Yee-Ying Lee · Teck-Kim Tang
Eng-Tong Phuah · Oi-Ming Lai *Editors*

Recent Advances in Edible Fats and Oils Technology

Processing, Health Implications, Economic and Environmental Impact

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Preface

Fats and oils are used extensively in food and non-food applications. Palm oil, soybean oil, rapeseed oil, and sunflower oil are few of the examples of vegetable oils that are widely traded as commodities. In 2020/2021, global production of fats and oils exceeded 200 million metric tons and is expected to grow at a rate of 4.45% per annum. World production and consumption of fats and oils are mainly dominated by Asia with Indonesia and Malaysia as the prominent countries. The processing method or analysis protocols adopted by the fats and oil industries have been well established in the past few decades. However, over time, discovery of some new findings further propels the fats and oils field. For instance, sustainability and renewable energy are the recent hot topics that have been greatly discussed in recent years, not to mention the occurrence of processing contaminants in edible oils as well as the green approaches utilized to modify the physical and chemical properties of fats and oils.

The book intends to capture a collection of up-to-date scientific advances in fats and oils technology over the past few years. It is contributed by esteemed researchers from academia and industries who are experts in their respective fields. The book covers the existing and recent advanced techniques adopted in the edible fats and oils research that touches on the processing and modification to the traceability and sustainability issues of fats and oils. For ease of reference, the book is structured into three different sections: (a) Chemistry and Processing, (b) Modification and Health Implications, (c) Renewal Energy, Safety, Adulteration, Sustainability, and Traceability. In the first section, the readers are introduced to the various types of edible fats and oils as well as their minor constituents. These chapters cover the sources and properties of vegetable oil, animal fat, seed oil, and minor components. Exotic oil which is a recent topic in the food industry is also revealed. This is then followed through with the modification approaches used to improve the functionality and nutritional value of fats and oils. These chapters touch on the traditional modification techniques like blending, hydrogenation, interesterification, fractionation as well as the current modification method such as oleogelation. A more advanced method using ionic liquid in fats and oils modification is also highlighted in one of the

chapters. Examples of the structured lipid synthesized from the aforementioned approaches and its food application such as diacylglycerols, medium and long-chain triacylglycerol, human milk fat substitute, and cocoa butter substitute are highlighted in the chapters. A chapter on the fats and oils that exist in the form of colloids in a nanosize known as nanoemulsion is also covered. The last part of the book wraps up the safety, adulteration, sustainability, and traceability of fats and oils. It deals with the techniques in the production of renewable energy biodiesel from sludge oil, formation, and detection of processing contaminants such as monochloropropane-diol (MCPD) and glycidyl ester (GE), recent techniques to detect adulteration of fats as well as sustainability and traceability of fats and oils.

To the scientists from academia or industries, we hope that the book will be useful to bring you new insights and keep you abreast with the latest updates of the oils and fats industry. For those who are embarking on their journey to pursue knowledge in the fats and oils area, we hope that the book can spark your interest in this field.

Last but not least, we thank all the authors for spending their precious time despite their busy schedules to contribute to the book chapters. Thank you for your patience given to us in completing the edited book, not to mention the editorial team at Springer Nature for their effort in coordinating the publication of the book.

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About the Editors

Yee-Ying Lee received her Ph.D. degree in Food Biotechnology and B.Sc. degree in Biotechnology from University Putra Malaysia. She is currently a lecturer with School of Science, Monash University Malaysia. She is a committee member of Monash-Industry Palm Oil Education and Research Platform. Her research interest is on lipid modification using lipase in which she has managed to co-author 7 book chapters and published 18 research articles. She has received numerous national and international professional awards for her Ph.D. work on structured lipid medium and long-chain triacylglycerol including 2016 Best Ph.D. award from the Institute of Bioscience, University Putra Malaysia, and 2015 Best Young Researcher Award from Asian Congress on Biotechnology. She and her team were awarded the second position in Developing Solutions for Developing Countries Competition organized by the Institute of Food Technologists in 2014.

Teck-Kim Tang earned his Master's Degree in Biotechnology from University Putra Malaysia in 2013, working on the production of diacylglycerol oil. He is currently pursuing his Ph.D. degree at Univeristi Putra Malaysia focusing on the synthesis of emulsion stabilized by microfibrillated cellulose. He has published 17 peer-reviewed articles and co-authored 3 book chapters in the area of lipid technology. He demonstrated a great passion in lipid science and technology research and has an immense knowledge in the area of structured lipid and emulsion technology. He and his team had won a second position in Developing Solutions for Developing Countries Competition organized by the Institute of Food Technologists in 2014.

Eng-Tong Phuah is a lecturer in Food Science and Technology, School of Applied Sciences and Mathematics at Universiti Tecknologi Brunei. He completed his Ph.D., specialized in bioprocess engineering at Universiti Putra Malaysia and his undergraduate studies at Universiti Sains Malaysia. His research interests mainly focus on the structural modification of edible fats and oils via enzymatic and chemical methods in order to alter and improve their physicochemical and nutritional

properties. Besides, he has been working actively in reaction kinetics and process simulation for improved process design. In addition, he has also investigated the potential use of structured lipids in a variety of food products, ranging from mayonnaise, margarine, shortening, and others, intended to provide and enhance their beneficial health effects. To date, he has published 16 peer-reviewed articles as well as several book chapters.

Oi-Ming Lai is a Professor in Enzyme Technology from the Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. She is the author or co-author of more than 150 referenced SCI publications and more than 10 book chapters. She has taught undergraduate and postgraduate courses in lipid technology, enzyme technology, food biotechnology, industrial biotechnology, and food biocatalysis. Oi-Ming's research program includes the development and improvement of structured lipids, upscaling and bioreactor designs and kinetics, and product diversification of various oilseed components and its by-products. She is also an Associate Editor of the *Journal of Food Science*.

Chapter 1
Vegetable Oils and Animal Fats: Sources, Properties and Recovery

Eng-Tong Phuah, Jeremy Wee-Lek Yap, Chei-Wei Lau, Yee-Ying Lee, and Teck-Kim Tang

Abstract Fats and oils can be found naturally in a wide range of animal and plant-based sources. They serve an important part of a balanced and healthy diet as they provide energy, support growth and development, provide the essential fatty acids and boost the immune system. Fats and oils are also high in fat-soluble vitamins especially vitamin E (tocopherols) which is well known for its antioxidant properties. Besides, these ingredients also help to enhance the sensory characteristics of various food products. Some fats and oils may even be used for medicinal purposes and biodiesel production. Obviously, fats and oils are essential components for both food applications and industrial uses. Based on the previous literatures, each fat and oil has its own unique fatty acid profile and physicochemical properties. Therefore, the present chapter reviews and focuses on the nutritional values and physicochemical properties of some common edible fats and oils extracted from both plant and animal sources.

Keywords Animal fats · Vegetable oils · Poultry fat · Lard · Tallow · Fish oil · Palm oil · Soybean oil · Sunflower oil · Coconut oil

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1.1 History, Production and Utilisation of Animal Fats

Animal fat has a long history of use in the Eastern region since Roman times for soap making. Tallow from cattle and sheep is utilised in candle making because of its high saturated fat content. Utilisation of animal fat further extended to food applications. In the 1940s, the consumption of lard among Americans was higher than the vegetable oil. Animal fat enhances the taste, flavour and texture of food. Lard and tallow blended with cottonseed oil provide an excellent source to be used as shortening for baking purposes. Animal fat has also been utilised widely as the frying medium for French fries attributed to their high thermal and oxidative stability. The industrial revolution switches the way animal fats being used in food applications. Public demand for vegetable oil increases over the concern of cholesterol-raising effect of animal fats in the 1990s.

Animal's fat is fat rendered from the fatty tissue or milk of animals such as beef (tallow), sheep (butter), swine (lard) and poultry (chicken, duck, goose fat) and others such as grease (yellow or white). The amount of fat rendered for the animal tissue or milk duct varies from 0 to 60% depending on the age, animal, sex and diet. It accounts for approximately 10% of the global fats and oils market and is valued at USD 227.9 billion in 2020. The major producers of tallow and lard are the United States of America and China, respectively. Table 1.1 shows the animal fats and oils production, consumption and stock in the United States in 2019. Poultry fat contributed to the majority of the animal fats market in the United States followed by tallow and lard. It should be noted that the animal fat is not produced for the edible purpose but is considered as by-products generated that is meant to produce meat, wool, dairy and skin. Today, the majority of the animal fat was utilised for biodiesel, animal feed, oleochemical and food applications. According to EFPRA, animal fat is

Table 1.1 United States: animal fats and oils production, consumption and stocks in 2019

	Jan 2019	Dec 2019	Total (Jan–Dec) ('000 pounds)
Lard			
(a) Production	27,411	30,755	384,396
(b) Removed for use in processing	26,208	31,377	345,524
(c) Stock on hand end of month	(D)	5938	
Poultry fats			
(a) Production	207,154	189,183	2,499,783
(b) Removed for use in processing	210,203	182,201	2,493,935
(c) Stock on hand end of month	23,720	31,499	
Tallow, edible			
(a) Production	67,184	87,347	996,693
(b) Removed for use in processing	68.894	81,301	1,030,983
(c) Stock on hand end of month	31,498	17,847	

Modified from USDA, National Agricultural Statistics Service. Fats and Oils: Oilseed Crushing, Production, Consumption and Stocks 2019 Summary (March 2020).
D: withhold to avoid disclosing data for individual operations

Table 1.2 Utilisation of animal fat in Europe

Table	Amount ('000 tonnes)
Edible fats	186
Oleochemicals	575
Animal fed and pet food	950
Solid fuel	1000

Modified from: EFPRA (Rendering in Numbers)

used mainly for biodiesel followed by animal feed and pet food, oleochemical and edible purposes in Europe. It was surprising to find that the demand for lard in Germany is growing due to its excellent properties to be used as a replacer for butter in the bakery sector. Lard-made pastries have a flakier texture. Goose fat and lard are also a popular spread used in Germany and France. Animal fat not only contributes to excellent solid like texture to bakery products but also it contains no unnatural trans fatty acid. Increasing demand for animal fat used for food application was believed to be due to the new regulation set up to ban the use of trans fatty acid in food products. The growth of the tallow market is also increasing because of the high demand for biodiesel (EMR Report) (Table 1.2).

1.2 Poultry Fat

Poultry fat is fat rendered from the fatty tissue of chicken, duck, goose and by-products of the poultry processing. For example, studies showed that chicken feather contains around 2–12% of the fat. Content of the fat increases with age. Young chicken has more fats depot than the hen. The predominant fatty acid composition of the poultry fat is oleic acid (43.3%), palmitic acid (21.3%) and linoleic acid (19.1%) followed by stearic acid (7.4%) (Table 1.3). However, the fatty acid composition of poultry fat can be manipulated by introducing different feeding diets as indicated in the previous literatures (Hargis and Van Elswyk 1993; Khatun et al. 2018). For example, Hargis and van Elswyk (1993) reported that the ω-3 fatty acids could be increased by the inclusion of marine oil in the diet. Under normal circumstances, poultry fat contains more unsaturated fatty acids than saturated fatty acids with a minute amount of vitamin D and vitamin E.

1.3 Lard Fat

Lard is the fresh clean and healthy fatty tissue from the skin rendered from swine where a certain amount is contributed by bones, ears and tails, internal organs. It appears to have a semi-solid like property and is whitish in colour. It was used as a substitute for butter in the nineteenth century but its usage started to decline because of its high saturated fat and cholesterol content. However, lard was reintroduced

Table 1.3 Fatty acid composition and nutrient composition in poultry fat

Fatty acid	Percentage (%)
C14	1
C16	21.3
C16:1	4.7
C18	7.4
C18:1	43.3
C18:2	19.1
C18:3	1.5
C18:4	1.1
C20	1.1
C20:1	0.4
Nutrient composition	
Vitamin D	1.91000 IU/kg
Vitamin E	27 mg/kg
Choline	1224 mg/kg

Modified from INRAE-CIRAD-AFZ Feed Tables, Thomas et al. (2000)

back following the trans fatty acid issue as a fat substitute to replace the partially hydrogenated fat. It is used widely as bakery and frying application. Table 1.4 shows the fatty acid composition of lard. The main fatty acid of lard is oleic acid (43.4%), palmitic acid (24.9%), stearic acid (15.5%) followed by linoleic acid (9.5%). Lard has a unique acylglycerol configuration whereby the *sn*-2 position is occupied with saturated fatty acid particularly palmitic. Lard is the only fat that are distinctly different from other edible fat and oils where most of the *sn*-2 position of vegetable oil is occupied by unsaturated fatty acid. The triacylglycerol composition in such configuration is similar to the human breast milk fat. The performance of lard for used as shortening in creaming and cake making can be improved through the interesterification process.

1.4 Tallow Fat

Tallow fat is the product rendered from the fatty tissue, muscles and bones of clean and sound bovine animals or sheep at the time of slaughter. Table 1.5 shows that tallow contains 38.5% oleic acid, 26.3% palmitic acid and 21.2% of stearic acid. Vitamin A, D, E, K are present in minute amounts in tallow. Fat derived from beef and sheep tend to have the saturated fatty acid located in the *sn*-1 and *sn*-3 position that resembles vegetable oil. Tallow contains natural trans fatty acid which is the conjugated linoleic acid that was produced through biohydrogenation process in the lumen of the ruminant species. The presence of natural trans fatty acid distinguishes it from other vegetable oil.

Table 1.4 Fatty acid composition, nutrient composition and physicochemical properties in lard fat

Fatty acid	Percentage (%)
C12	0.2
C14	1.7
C16	24.9
C16:1	2.8
C18	15.5
C18:1	43.4
C18:2	9.5
C18:3	0.8
C20	1.3
C20:1	0.7
C22:1	0.2
Nutrient composition	
Vitamin A	0.31000 IU/kg
Vitamin D	11,000 IU/kg
Vitamin E	5 mg/kg
Vitamin B_6 pyridoxine	0.2 mg/kg
Choline	497 mg/kg
Physicochemical properties	
Melting point	30–40 °C
Saponification value	193–202
Non saponifiable matter	0.1–1.0%

Modified from INRAE-CIRAD-AFZ Feed Tables, Thomas et al. (2000)

1.5 Fish Oil and Krill Oil

Fish oil is derived from the fatty tissue of fatty fish such as salmon, mackerel, anchovies and sardine. Fish oil is more valued than other animal fats. It has anti-inflammatory property that is essential to manage heart and brain health where it is sold as dietary supplements encapsulated in the form of pills or tablets. Fish oil differs from the animal fat derived from the land whereby the fatty acid derived from fish oil is made from the very long carbon atom and they are highly unsaturated. It is made predominantly from the ω-3 polyunsaturated fatty acid (PUFA) particularly C20, C22 and C24 known as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The fatty acid composition of the seawater fish is different from the freshwater fish where the former contains more C20 and C22 with a lesser oleic and linoleic content. Fish oil can also be obtained from many different organs. Cod, halibut and shark stored most of their fat in the liver. Unlike other fish oil, cod liver oil is valued for its vitamin A (15,000–50,000 IU/g) and vitamin D (40–200 IU/g). Sperm oil from whales is prized for their cosmeceutical application. Sperm oil is composed mainly of wax ester and a small amount of triacylglycerol mainly made from oleic acid. Recently, krill oil obtained from the tiny crustacean species that live in the Antarctic Ocean called the Antarctic krill is well sought after. It is also

Table 1.5 Fatty acid composition, vitamin and mineral content in tallow fat

Fatty acid	Percentage (%)
C12	0.2
C14	3.2
C16	26.3
C16:1	3.8
C18	21.2
C18:1	38.5
C18:2	2.8
C18:3	0.7
C20	1.1
C20:1	0.3
C22	0.1
Nutrient composition	
Vitamin A	18.31000 IU/kg
Vitamin D	0.31000 IU/kg
Vitamin E	27 mg/kg
Vitamin K	1.5 mg/kg
Choline	798 mg/kg
Physicochemical properties	
Melting point	44–55 °C
Saponification value	192–198
Non saponifiable matter	0.1–0.6%

Modified from INRAE-CIRAD-AFZ Feed Tables, Thomas et al. (2000)

composed mainly of phospholipid-derived fatty acid such as phosphatidylcholine of around 30–65% and the remaining is composed of triacylglycerol made from EPA and DHA. Contradictorily, fish oil is made mainly from triacylglycerol. The majority of the fatty acids attached to the phosphatidylcholine of krill oil are EPA and DHA which increases the bio-absorption of the essential fatty acid. Unlike other marine oil, krill oil contains an appreciable amount of astaxanthin which is a powerful antioxidant that is responsible for its red colour. Astaxanthin is a more potent antioxidant than Coenzyme Q10 and fish oil (Table 1.6).

1.6 History, Production and Utilisation of Vegetable Oils

Vegetable oils are a group of fats extracted from the plant sources such as seeds, nuts, cereal grains and fruits (Hammond 2003). The use of vegetable oils has been practised by the Ancients thousands of years ago. They collect the oil exuded from the plant by slowly heating up the oily plant products. For instance, archaeological evidence suggested the existence of olive tree dating back to 43,980 B.C. The applications of olive oil as cooking oil, perfume, anointment for the dead, soap and lights in ancient times are well described (Kiritsakis and Markakis 1988;

Table 1.6 Fatty acid composition and nutrient composition in fish oil and krill oil

Fatty acid (%)	Cod liver oil	Salmon oil	Sardine oil	Krill Oil
C12	n.d.	n.d.	0.1	n.d.
C14	3.7	3.7	7.6	15.1
C16	13	10.2	16.2	23.5
C16:1	8.9	8.7	9.2	5.73
C18	2.4	4.7	3.5	1.13
C18:1	24.3	18.6	11.4	19.7
C18:2	1.3	1.2	1.3	2.33
C18:3	0.5	0.6	0.9	0.12
C18:4	0.9	2.1	2	1.04
C20	n.d.	n.d.	0.4	n.d.
C20:1	11.3	8.4	3.2	0.06
C20:2	n.d.	n.d.	n.d.	0.06
C20:4	1.1	0.9	1.6	0.35
C20:5 (EPA)	11	12	16.9	19.92
C22:1	4.6	5.5	0.2	n.d.
C22:5 (DPA)	1.4	2.9	3.8	0.43
C22:6 (DHA)	10.8	13.8	2.5	10.6
Nutrient composition				
Vitamin A	10,001,000 IU/kg	n.d.	n.d.	n.d.
Vitamin D	1,001,000 IU/kg	n.d.	3.31000 IU/kg	n.d.
Vitamin E	219 mg/kg	219 mg/kg	219 mg/kg	n.d.
Vitamin K	1.5 mg/kg	1.5 mg/kg	1.5 mg/kg	n.d.

n.d. not detected. Modified from Ahn et al. (2018)

Clodoveo et al. 2014). Indeed, vegetable oils can be applied in various food, pharmaceutical and cosmetical products. Besides, vegetable oils also exhibit the potential to replace petroleum-based fuels in recent years because vegetable oil-based biofuels have renewable characteristics (Mekhilef et al. 2011; Issariyakul and Dalai 2014). In contrast to animal fats, vegetable oils are more preferable owing to its healthier fatty acid profiles. The vegetable oils contain predominantly unsaturated fatty acids such as oleic, linoleic and linolenic acids, depending on the oil sources. These unsaturated fatty acids are reported to exert beneficial health effects including suppressing coronary heart diseases and cardiovascular diseases as well as providing antioxidative effects (Orsavova et al. 2015). Even though more than 350 oil-bearing crops have been identified, soybean, palm, rapeseed, sunflower and coconut oils remain to be the major vegetable oils. Table 1.7 shows the global vegetable oil consumption (million metric tonnes). A steady increase in the global oil consumption with a compound annual growth rate (CAGR) of 2.2% is observed in tandem with the increasing world population. Worldwide consumption of palm oil is the highest, followed by soybean oil, rapeseed oil and sunflower oil. Others include palm kernel oil, coconut oil and etc. Palm oil offers several advantages such as being the most efficient and highest oil yielding vegetable crops, relatively inexpensive

Table 1.7 Global vegetable oil consumption from 2016/2017 to 2019/2020 (million metric tonnes)

Vegetable oils	2016/2017	2017/2018	2018/2019	2019/2020
Palm oil	61.6	66.99	73.06	71.48
Soybean oil	53.29	54.56	54.92	55.46
Sunflower oil	16.33	17.42	18.2	19.33
Palm kernel oil	7.42	8.09	8.65	8.56
Rapeseed oil	28.9	28.86	28.16	27.62
Coconut oil	3.09	3.4	3.54	3.65
Total	170.63	179.32	186.53	186.1

Modified from Statista (2020)

and it has a variety of uses from baking products and spreads to frying (Norhaizan et al. 2013; Lai et al. 2015). Palm oils are mostly exported to China, European Union, India, Pakistan and other countries. Soybean oil and rapeseed oil are also widely consumed throughout the world owing to their high PUFA content, low crystallisation temperature and reasonable cost.

1.7 Palm Oil

Palm oil is obtained from the mesocarp of the fruit of the oil palm tree, *Elaeis guineensis*. It is grown in several countries in the world where the five leading producing countries are Indonesia (43.5 million tonnes per year), Malaysia (19.3 million tonnes per year), Thailand (3.1 million tonnes per year), Colombia (1.7 million tonnes per year) and Nigeria (1.0 million tonnes per year). Oil palm is a perennial crop which has two crops per year. Two different types of oils can be produced from palm fruit which are crude palm oil from the mesocarp and palm kernel oil from the inside kernel. One hectare of land can produce around 4–5 tonnes of crude palm oil per year and an additional yield of about 1 tonne of palm kernel oil can be obtained from the same fruit bunch (MPOC 2020). Palm oil is considered as one of the highest oil yielding crops among other oil crops.

Palm oil exists in semi-solid form at ambient temperature. It is by far known as the most fractionated oil in the world. The semi-solid palm oil comprises of low- and high-melting triacylglycerol that can be physically refined and fractionated into a liquid fraction and a solid fraction namely palm olein and palm stearin, respectively (Omar et al. 2005). Palm olein is commonly used in cooking and frying oil whereas palm stearin is as solid fat in margarine and shortening blends (Law and Thiagarajan 1990).

Palm oil is well-known with its superior frying performance due to its good oxidative stability. Most importantly, there is a high percentage of symmetrical monounsaturated triacyl group present in palm oil with palmitic-oleic-palmitic (POP) predominating (Duns 1985). Because palm oil contains relatively high levels of carotene, which performs as pro-oxidant even in the presence of high tocopherol

Table 1.8 Fatty acid composition in palm oil

Fatty acid	Percentage (%)
C8	n.d.
C10	n.d.
C12	0.1–1.0
C14	0.9–1.5
C16	41.8–46.8
C16:1	0.1–0.3
C18	4.5–5.1
C18:1	37.3–40.8
C18:2	9.1–11.0
C18:3	0.4–0.6
C20	0.2–0.7
C20:1	n.d.
C20:5 (EPA)	n.d.
C22	n.d.

n.d. not detected. Modified from Koushki et al. (2015), Tan and Nehdi (2012), Noor Lida et al. (2002)

and tocotrienol concentrations, it has a high resistance to oxidation. In addition, it is not mandatory to undergo hydrogenation and the occurrence of trans-fat could be avoided owing to its semi-solid texture at ambient temperature (Koushki et al. 2015). This characteristic brings into the replacement of palm oil for hydrogenated oil in food application as it provides a healthy alternative to trans-fatty acids (May and Nesaretnam 2014). These distinctive physicochemical characteristics of palm oil and its derivatives have brought interest to this oil towards the confectionery and plastic fat products with health benefits (Saberi et al. 2010; Goh 2002).

Palm oil differs from other commodity oils as it consists of almost even proportion of saturated and unsaturated fatty acids. Generally, palm oil comprised of nearly 50% of saturated fatty acids, 40% of monounsaturated fatty acids (MUFAs) and 10% of PUFAs (Tan and Nehdi 2012). As shown in Table 1.8, palm oil is predominated by ~44% of palmitic acid, ~39% oleic acid, linoleic acid (~10%) and a small amount of myristic acid and stearic acid. Due to the high saturated fatty acid content, palm oil has a high oxidative stability which is excellent in frying.

The physicochemical properties of palm oil are tabulated in Table 1.9. The high level of palmitic acid (41.8–46.8%) contributes to the low iodine value (IV) in palm oil and its derivatives. Palm oil has a good oxidative and frying stability, not only due to the presence of the unique fatty acid composition, but also having a high smoke point and presence of phytonutrients. Crude palm oil has a reddish-orange colour due to its high carotene content and a characteristic "nutty" flavour. It is also rich in several phytonutrients such as tocopherols, tocotrienols and phytosterols. However, palm oil is always undergoing physical refining such as degumming, bleaching and deodorizing which almost reduces these natural antioxidants by half (Gee 2007).

Table 1.9 Physiochemical properties of palm oil

Characteristics	Range
Specific gravity (4 °C)	0.888–0.889
Iodine value (g I_2/100 g fat)	46.0–56.0
Saponification number (mg KOH/g oil)	190.0–202.0
Bioactive compounds (mg/kg oil)	
α-Tocopherol	129.0–215.0
α-Tocotrienol	44.0–73.0
β-Tocopherol	22.0–37.0
β-Tocotrienol	44.0–73.0
γ-Tocopherol	19.0–32.0

n.d. not detected. Almeida et al. (2019), Uddin et al. (2020), O′Brien(2009)

1.8 Soybean Oil

Modern soybean (*Glycine max (L.) Merr*) is an agricultural crop of significant economic importance. It belongs to the order of *Fabales* and the family of *Fabaceae*. The common belief is that modern soybean was first domesticated in China from its wild relative (G. soja) dated back to around 6000–9000 years ago (Carter et al. 2004). However, it was also suggested that modern soybean was domesticated from the complex of G. soja/G. max which diverged from a common ancestor of these two species of Glycine (Kim et al. 2010).

Soybean is currently one of the most important crops grown for its protein and oil. It has been incorporated into the human diet in many forms including tofu, soy sauce, soy milk, tempeh, texturized soy protein and so on. The by-products of soybean processing like soybean meal and okara can also be used as animal feed. Soybean can be grown worldwide on a large scale because a wide variety of soils, other than those which are very sandy, can support its growth with an optimum soil pH ranges from 6.0 to 6.5 (FAO 2020). Soybean is an excellent source of plant-based protein for both human and animal consumption, although its protein quality is limited by the low concentration of sulphur amino acids which can be improved through traditional breeding and genetic engineering (Krishnan 2005). Consumption of both protein and non-protein (e.g., isoflavones, lecithin, saponins and fibre) components of soybean have been associated with lower risk of cardiovascular disease (Ramdath et al. 2017).

Soybean oil is the second most consumed edible oil in the world after palm oil. It is also the most consumed vegetable oil in both China and the United States. In non-food application, soybean oil has also been used in the production of biodiesel which can probably perform better than the traditional gasoline (Demirbas 2007; Coppo et al. 2014; Vicente et al. 2010). Other than that, soybean oil can also be manufactured into inks, paints, varnishes, resins and plastics (Cahoon 2003). With

Table 1.10 Fatty acid composition in soybean oil

Fatty acid	Percentage (%)
C8	n.d.
C10	n.d.
C12	n.d.–0.1
C14	n.d.–0.5
C16	7.5–17.0
C16:1	n.d.–0.5
C18	1.6–5.5
C18:1	16.0–50.0
C18:2	35.0–60.0
C18:3	2.0–13.0
C20	n.d.–1.4
C20:1	n.d.–1.0
C20:5 (EPA)	n.d.–0.2
C22	n.d.–0.5

n.d. not detected. Modified from Zambiazi et al. (2007), Ivanov et al. (2011), Byun et al. (1996), Chowdhury et al. (2007), Dobarganes et al. (2002), List (2016), Medina-Juárez et al. (1998), Perkins (1995), Rafalowski et al. (2008), Sawada et al. (2014), Tuberoso et al. (2007), Noureddini et al. (1992a)

this, it is expected that soybean oil will remain as one of the most important vegetable oils in the future.

Soybean oil is rich in unsaturated fatty acids, a typical characteristic of vegetable oils. The five most abundant fatty acids in soybean oil are in the order of linoleic, oleic, palmitic, linolenic and stearic acids, as shown in Table 1.10. The wide range in the reported fatty acid compositions of the commercial soybean oil may be ascribed to the commercialisation of modified oils (Fehr 2007). The high content of PUFAs namely linolenic and linoleic acids reduces the oxidative stability of soybean oil which is undesirable for many food products and industrial applications. Conventionally, this can be overcome through chemical hydrogenation which also results in the formation of trans-fatty acids, a highly undesirable component in fats and oils which increases the risk of several health problems. As the alternatives, genetic approaches such as genetic modification and breeding program have been implemented to obtain improved oil yield, reduced linolenic acid as well as increased oleic acid and stearic acid for better oxidative stability of soybean oil, thereby reducing the reliance on hydrogenation (Clemente and Cahoon 2009). Table 1.11 gives some insights into the variation among the fatty acid compositions of the modified soybean oils.

Different temperatures during the seed development were found to affect the fatty acid composition of soybean oil. According to Rennie and Tanner (1989), the contents of stearic acid and oleic acid increased while linoleic acid and linolenic acid decreased when the day and night temperature decreased from 40 to 15 and 30 to 12 °C. This phenomenon could be ascribed to the effect of temperature on gene expression. On the other hand, exposure of soybeans to up to 5 kGy of γ -irradiation

Table 1.11 Fatty acid compositions in modified soybean oils

	Fatty acid composition (%)				
	C16	C18	C18:1	C18:2	C18:3
Unmodified	11.2	3.4	21.5	55.8	8
Low linolenic	10.1	5.3	41.1	41.2	2.2
Low palmitic	5.9	3.7	40.4	43.4	6.6
Low saturates	3	1	31	57	9
High palmitic	26.3	4.5	15	44.4	9.8
High stearic	8.6	28.7	16.2	41.6	4.9
High saturates	23.3	20	10.5	39.7	6.5
High oleic	6.4	3.3	85.6	1.6	2.2

Modified from Dijkstra (2016)

was reported to have no significant effect on the fatty acid composition of the resulting soybean oil (Byun et al. 1996). Still, the oxidative stability (induction period) was improved by more than 100% after γ-irradiation.

The conventional way to extract soybean oil is by solvent-extraction with hexane. However, leakage of hexane to the environment causes pollution while prolonged exposure to hexane residue in oil also results in several health issues (Kumar et al. 2017). Thus, the potential of other solvents such as ethanol and supercritical carbon dioxide (SCO_2) has been studied in the literatures. For ethanol, yield increased (up to 20%) by using a more concentrated ethanol with absolute ethanol showed the most consistent and best performance at a range of extraction temperature. Besides, the fatty acid composition of soybean oil obtained with ethanol extraction resembles that of typical soybean oil (Sawada et al. 2014). The composition of SCO_2-extracted soybean oil was reported to fulfil the FAO limit as well, although the yield appeared to be lower than that obtained with hexane (yield: 16.4–19.9% with SCO_2, 19.9–25.0% with hexane) (Dobarganes et al. 2002).

Consumption of fats and oils which are high in ω-6/ω-3 ratio can lead to low-grade inflammation, oxidative stress, endothelial dysfunction and atherosclerosis (DiNicolantonio and O'Keefe 2018). Soybean oil is naturally high in ω-6/ω-3 ratio with a typical reported range of 6–9. Notably, a very high value of this ratio (22.8) was reported by Rafalowski et al. (2008) in unrefined cold-pressed soybean oil.

Soybean oil is rich in tocopherols with γ-tocopherol as the major form which has been reported as high as 1559 mg/kg oil (Dijkstra 2016). Since it is of plant origin, cholesterol is absent in soybean oil. Nevertheless, soybean oil contains phytosterols with the highest total amount reported as 4050 mg/kg oil (Dijkstra 2016). Among them, β-sitosterol, campesterol and stigmasterol are the three dominant phytosterols being detected in soybean oil. Phytosterols possess therapeutic potential for obesity and diabetes by playing the role as nutritional modulators in regulating the immune response, oxidative stress, adipose tissue metabolism, hypercholesterolemia and gut dysbiosis (Vezza et al. 2020). Particularly, phytosterols can interfere with the solubilisation of cholesterol, thereby blocking its absorption in the small intestine (QuÍlez et al. 2003). Temperature during the development of soybean was reported

to affect the total phytosterols and tocopherols contents in soybean oil. As reported by Vlahakis and Hazebroek (2000), total phytosterols increased while total tocopherols decreased as the day/night temperature during the soybean development increased from 20/10 to 35/25 °C. This was accompanied by changes in the composition of the phytosterols with proportionally more campesterol at elevated temperature at the expense of stigmasterol and β-sitosterol.

Soybean oil was reported to be susceptible to oxidation, as reflected by its low induction period (4.84–5.24 h). The low oxidative stability of soybean oil is because of the high content of PUFA, particularly linoleic acid and linolenic acid. The shelf life of oil is estimated to be 37.8 weeks based on the accelerated shelf-life model (Alemayhu et al. 2019). As mentioned previously, oxidative stability of soybean oil can be improved when the soybean is subjected to γ-irradiation (Byun et al. 1996). Soybean oil, along with essential oils, has also been used in the making of antibacterial edible films to enhance the microbiological food safety (Zhang et al. 2015) (Table 1.12).

1.9 Rapeseed Oil

Rapeseed (*Brassica napus*), which contains more than 40% of oil, is an important crop grown for its oil which fulfils approximately 13% of world's demand for vegetable oil (Hajduch et al. 2006). It belongs to the order of *Brassicales* and the family of *Brassicaceae*. Rapeseed was domesticated around 400 years ago in Europe where its oil was used in lamps for lighting, soap making and as an edible oil (Gómez-Campo and Prakash 1999). It has been suggested that rapeseed ($2n = 38$) originated from the spontaneous hybridisation of *B. rapa* ($2n = 20$) and *B. oleracea* ($2n = 18$) (Hasan et al. 2008). Rapeseed has been the most important oilseed crop in the EU for more than a decade (Hasan et al. 2008; Krautgartner et al. 2018). Based on the latest data, Canada, EU, China and India are the top 4 countries and/or regions in the production of rapeseed and the world's production of rapeseed achieves 68.19 million metric tons in the year 2019/20.

Rapeseed can be grown on a wide variation of well-drained soils with optimum pH ranging between 5.5 and 8.3 (Ag Marketing Resource Center 2020). After the oil being extracted, rapeseed meal is produced as the by-product which can be used as animal feed. Typically, rapeseed meal contains around 40% of protein and 12% of crude fibre, but it is mostly used to feed ruminant because of its content of antinutrient glucosinolates (Rutkowski 1971). Glucosinolates are sulphur-containing compounds which can be harmful for the thyroid gland of monogastric animals. Through breeding programs, cultivars that are low in glucosinolates has been cultivated (Mailer 2016).

Rapeseed oil has a typical yellowish look and is the third most consumed edible oil in the world after palm and soybean oils. China, Germany, India, the United States are among the four largest rapeseed oil-consuming countries (Grain Central 2019). Other than food application in mayonnaise, salad dressing and others,

Table 1.12 Physicochemical properties of soybean oil

Characteristics	Range
Specific gravity (25 °C)	0.914–0.918
Iodine value (g I_2/100 g oil)	126–138
Saponification number (mg KOH/g oil)	189–195
Oxidative stability index (110 °C) (h)	4.84–5.24
Dynamic viscosity (cP)	48.1–57.1
Bioactive compounds (mg/kg oil)	
α-Tocopherol	44–282
β-Tocopherol	n.d.–69.6
γ-Tocopherol	332–1559
σ-Tocopherol	110–477
Phytosterol composition (mg/kg oil)	
β-Sitosterol	650–2360
Brassicasterol	n.d.–128
Campestanol	59.1
Campesterol	248–1310
Cycloartanol	27.2
Cycloartenol	47.1
Egrosterol	19.8
Stigmasterol	219–872
24-methylene-Cycloartanol	43.4
Δ5-Avenasterol	n.d.–135
Δ5-Stigmasterol	n.d.–150
Δ7-Avenasterol	n.d.–40
Squalene (mg/kg oil)	n.d.–181

n.d. not detected. Modified from Anwar et al. (2003), Bart et al. (2010), Byun et al. (1996), Dijkstra (2016), Dobarganes et al. (2002), Fang et al. (2016), Kania et al. (2004), List (2016), Medina-Juárez et al. (1998), Nergiz and Celikkale (2011), Noureddini et al. (1992a), Perkins (1995), Rafalowski et al. (2008), Sahasrabudhe et al. (2017), Sawada et al. (2014), Tran et al. (2018), Tuberoso et al. (2007), Yang et al. (2019), Vlahakis and Hazebroek (2000), Noureddini et al. (1992b), Siger et al. (2008)

rapeseed oil can also be utilized as the raw material to produce biodiesel and plastic (Mailer 2016; Encinar et al. 2018; Vicente et al. 2010).

Similar to most vegetable oils, rapeseed oil is rich in unsaturated fatty acid (>80%). This makes rapeseed oil to remain as clear liquid in the refrigerator and fractionation is usually not required to remove any solidified fat (List 2016). The five most abundant fatty acids in rapeseed oil are oleic, linoleic, linolenic, palmitic and stearic acids, as tabulated in Table 1.13. A huge range in the MUFA and PUFA contents, particularly those of oleic, linoleic and linolenic acids has been observed in the literatures. Rapeseed oil was originally rich in erucic acid which makes it suitable as lubricating oil in engines (Eskin 2016). However, long-term oral intake to erucic acid can induce myocardial lipidosis in both human and animals and a tolerable daily

Table 1.13 Fatty acid composition in rapeseed oil

Fatty acid	Percentage (%)
C8	n.d.
C10	n.d.
C12	n.d.
C14	n.d.–0.3
C16	2.5–10.5
C16:1	0.1–0.4
C18	0.9–6.9
C18:1	23.2–66.0
C18:2	15.2–30.0
C18:3	1.2–44.0
C20	n.d.–0.7
C20:1	0.6–9.1
C22	n.d.–0.4
C22:1	n.d.–1.5
C24	n.d.–0.3

n.d. not detected. Modified from Biljana et al. (2015), Dymińska et al. (2017), Kmiecik et al. (2009), List (2016), Matthaus et al. (2016), Noureddini et al. (1992a), Orsavova et al. (2015), Rafalowski et al. (2008), Sagan et al. (2019), Tuberoso et al. (2007), Zambiazi et al. (2007)

intake of erucic acid has been set at 7 mg/kg body weight (EFSA Panel on Contaminants in the Food Chain et al. 2016). Therefore, conventional breeding and genetic engineering have been applied for the past few decades to select and cultivate cultivars of rapeseed with altered fatty acid composition that suite certain functions which include those with low erucic acid content for human consumption (Sakhno 2010; Matthaus et al. 2016). Consequently, cultivars with relatively diverse MUFA and PUFA contents are available nowadays, as reported in the work of Biljana et al. (2015).

Rapeseed oil also contains a good balance of ω-6 and ω-3 fatty acids with ω-6/ω-3 ratio typically ranges between 1 and 3. A low ω-6/ω-3 ratio approaching the value of 1 which is believed to be the original value in human's history is associated with reduced risk of cardiovascular disease, cancer, and inflammatory and autoimmune diseases (Simopoulos 2002). However, an abnormally high ω-6/ω-3 ratio of 16.3 has been reported in a cold-pressed rapeseed oil by Orsavova et al. (2015) which can be attributed to the difference between cultivars. To further improve the ω-6/ω-3 ratio in rapeseed oil, Sagan et al. (2019) have suggested blending rapeseed oil with the ω-3-rich sage and cress oils in 70:10:20 ratio which was found to decrease the ω-6/ω-3 ratio by about 50%, but the oxidative stability was compromised due to the higher content of PUFA.

Oil is typically extracted from rapeseed by pressing followed by solvent extraction with hexane which could also cause harm to both the environment and human health (Kumar et al. 2017). As an alternative, extraction with hot pressurised ethanol has been proposed by Citeau et al. (2018). Briefly, the process consists of extraction

of rapeseed flakes with 95.6% ethanol at 95 °C and pressure of 340–360 kPa. The extraction mixture is then subjected to cooling separation at −20 to +13 °C which results in practically complete extraction of high-quality crude rapeseed oil that is free from free fatty acids, phospholipids and non-lipid components and the separated solvent can also reused for subsequent extraction without the need for complete solvent distillation.

Rapeseed oil contains good amounts of tocopherols. These tocopherols were mainly in γ- and α-forms whereas β- and σ-tocopherols were either not detected or detected in a very low percentage. Similar to other vegetable oils, rapeseed oil is free from cholesterol which is of animal origin. In contrast, diverse phytosterols have been detected in rapeseed oil. Among them, brassicasterol, campesterol and β-sitosterol are the three dominant phytosterols in rapeseed oil which have been detected in levels above 1000 mg/kg oil.

Rapeseed oil is also susceptible to oxidation, as reflected by the short induction period which ranged from 5.1 to 7.4 h as previously reported. Nevertheless, rapeseed oil is more oxidation-resistant than soybean oil due to the higher amount of MUFA particularly oleic acid in rapeseed oil (Table 1.14).

1.10 Sunflower Oil

Sunflower oil is the lipid extract obtained from the sunflower seed of the plant, *Helianthus annuus* L. Sunflower oil is another important edible oil besides palm, soybean and rapeseed oils because the sunflower seed contains approximately 44% of oil and 16% protein, making it an excellent edible oil source (Pilorgé 2020). Sunflower oil is comparable to soybean and corn oils due to their similar fatty acid profile. Sunflower oil is particularly high in unsaturated fatty acids such as linoleic and oleic acids, making it a valuable healthy oil. The sunflower genotype can be categorised according to its oleic content namely (1) regular (14–39%), (2) mid-oleic (43–72%) and (3) high-oleic (75–91%) (FAO 1999; Grompone 2005). The fatty acid composition of the sunflower oil may be affected by some environmental factors namely temperature, sunlight and precipitation that affects the growth of sunflowers, leading to different seed development (Akkaya et al. 2019). Table 1.15 shows the fatty acid composition of a typical sunflower oil.

The main triacylglycerols in sunflower oil are OLL, LLL, OLO and PLO (where L = linoleic; O = oleic and P = palmitic acids), which these triacylglycerols contribute to almost 80% of the total triacylglycerol composition (Noor Lida et al. 2002). Owing to the high degree of unsaturation, sunflower oil appears as light yellowish oil at ambient temperature. Previous study showed that sunflower oil is rich in α-tocopherol contents (403–935 mg/kg oil) (Schmidt and Pokorny 2018). Also, sunflower oil has a relatively high concentration of phytosterols (2850 mg/kg oil) which is higher than the recommended value of 1000 mg/kg to exert beneficial health effects (Yang et al. 2019). The main phytosterols are β-sitosterol, campesterol and stigmasterol. Besides, sunflower oil has a high oxidative stability. Therefore, it

Table 1.14 Physiochemical properties of rapeseed oil

Characteristics	Range
Specific gravity (25 °C)	0.903–0.907
Iodine value (g I_2/100 g oil)	109–113
Saponification number (mg KOH/g oil)	170–190
Oxidative stability index (110 °C) (h)	5.1–7.4
Dynamic viscosity (cP)	63.5–78.8
Bioactive compounds (mg/kg oil)	
α-Tocopherol	13–362
β-Tocopherol	n.d.–0.6
γ-Tocopherol	18–536
σ-Tocopherol	n.d.–6
Phytosterol composition (mg/kg oil)	
β-Sitosterol	2310–3941
Brassicasterol	530–1366
Campestanol	28.3
Campesterol	1500–3080
Cycloartanol	11
Cycloartenol	173
Egrosterol	25.4
Stigmasterol	n.d.–257
24-methylene-Cycloartanol	52.8
Δ5-Avenasterol	409
Squalene (mg/kg oil)	211.0–437.4

n.d. not detected. Modified from Anwar et al. (2003), Bart et al. (2010), Dymińska et al. (2017), Fang et al. (2016), Kmiecik et al. (2009), Matthaus et al. (2016), Nergiz and Celikkale (2011), Noureddini et al. (1992a, b), Rafalowski et al. (2008), Rudzińska et al. (2005), Sagan et al. (2019), Sahasrabudhe et al. (2017), Siger et al. (2008), Tran et al. (2018), Tuberoso et al. (2007), Vlahakis and Hazebroek (2000), Yang et al. (2019)

Table 1.15 Fatty acid composition in typical sunflower oil

Fatty acid	Percentage (%)
C12	n.d.
C14	0.1–0.2
C16	5.0–7.6
C16:1	0.1–0.3
C18	2.7–6.5
C18:1	14.0–39.4
C18:2	48.3–74.0
C18:3	0.1–0.3
C20:0	0.1–0.5
C20:1	n.d.–0.3
C22	n.d.–1.5

n.d. not detected. Modified from Panda et al. (2016), Rosa et al. (2009), CODEX (2001)

Table 1.16 Physiochemical properties of sunflower oil

Characteristics	Range
Specific gravity (25 °C)	0.915–0.919
Iodine value (g I_2/100 g oil)	120–140
Saponification number (mg KOH/g oil)	188–202
Oxidative stability index (98 °C) (h)	12.8–13.7
Dynamic viscosity (cP)	43–49
Bioactive compounds (mg/kg oil)	
α-Tocopherol	403–935
β-Tocopherol	6–41
γ-Tocopherol	1–27
σ-Tocopherol	n.d.
Phytosterol composition (mg/kg oil)	
β-Sitosterol	1400–2214
Brassicasterol	n.d.–18
Campestanol	3–48
Campesterol	272–296
Cycloartanol	n.d.–7
Cycloartenol	47–130
Egrosterol	n.d.–7
Stigmasterol	110–270
Sitostanol	40–45
Δ5-Avenasterol	70–178
24-methylene-cycloartanol	76–14.9
Squalene (mg/kg oil)	134–142

n.d. not detected. Modified from Vafakish et al. (2017), Chiplunkar and Pratap (2016), Crapiste et al. (1999), Tasan and Demirci (2005), Schmidt and Pokorny (2018), Hassanien (2013), CODEX (2001)

can be used for various applications including frying oil, salad oil and etc. (Grompone 2005) (Table 1.16).

1.11 Coconut Oil

Coconut oil is an edible oil extracted from the fresh and mature kernel of the coconut (*Cocos nucifera* L.). Coconut oil can be classified into either virgin coconut oil or refined coconut oil in which the former has not been exposed to any refining steps such as bleaching, degumming and deodorisation after extraction (Agarwal 2017; Soo et al. 2020; Satheesh and Prasad 2012). Therefore, virgin coconut oil was reported to retain most of the polyphenolic compounds. The fatty acid composition and nutritional content of the coconut oil are presented in Tables 1.17 and 1.18, respectively. Coconut oil consists predominantly of short and medium chain fatty acids particularly in lauric acid (46–48%) and followed by myristic acid (16–21%).

Table 1.17 Fatty acid composition in coconut oil

Fatty acid	Percentage (%)
C6	n.d.–0.7
C8	4.6–10.0
C10	4.5–8.0
C12	43.0–53.2
C14	16.0–21.0
C16	7.2–10.2
C16:1	n.d.
C18	2.0–4.0
C18:1	4.5–10.0
C18:2	1.0–2.5
C18:3	n.d.–0.2
C20:0	n.d.–0.2
C20:1	n.d.–0.2
C22	n.d.

n.d. not detected. Modified from Satheesh and Prasad (2012), Soo et al. (2020), Mansor et al. (2012), Prapun et al. (2016), Dayrit et al. (2007), APCC (2005), CODEX (2001), Bhatnagar et al. (2009)

Previous studies suggested that free lauric acids could exert potent antimicrobial activity on Gram-positive bacteria such as *Clostridium difficile*, *Staphylococcus aureus* and *Candida species* (Ogbolu et al. 2007; Abbas et al. 2017; Silalahi et al. 2014). Besides, a diet enriched with coconut oil could possibly suppress the low-density lipoprotein while improving the high-density lipoprotein which is associated with enhanced cardio-protective effect (Chinwong et al. 2017). An analysis on the triacylglycerol composition of coconut oil revealed 22–25% of LaLaLa, 14–16% of CCLa, 19–21% of CLaLa, 13–15% of LaLaM and 7–9% of LaMM (where La = lauric; C = capric and M = myristic acids) as the major triacylglycerol. Due to its substantial amount of medium-chain triacylglycerol, coconut oil has a relatively low melting temperature range (Marina et al. 2009). The physicochemical properties of coconut oil are similar to palm kernel oil in that they exhibit sharp melting points and excellent oxidative stability in addition to high lauric and myristic fatty acids. Coconut oil is also found to contain trace amount of α-tocopherol (17–60 mg/kg), depending on the extraction technique (Prapun et al. 2016; Desai et al. 1988; Arlee et al. 2013). Fermentation technique tends to produce coconut oil with high phenolic compounds. Qualitative analysis of the phenolic compositions of coconut oil revealed the presence of caffeic, catechin, ferulic and p-coumaric acids as the predominant phenolic compounds (Seneviratne and Sudarshana 2008).

Table 1.18 Physicochemical properties of coconut oil

Characteristics	Range
Specific gravity (25 °C)	0.915–0.920
Iodine value (g I_2/100 g oil)	4.1–11.0
Saponification number (mg KOH/g oil)	248–280
Oxidative stability index (110 °C) (h)	11.3
Dynamic viscosity (cP)	40
Bioactive compounds (mg/kg oil)	
α-Tocopherol	17–60
β-Tocopherol	n.d.
γ-Tocopherol	n.d.
σ-Tocopherol	n.d.
Phytosterol composition (mg/kg oil)	
β-Sitosterol	27.1
Brassicasterol	n.d.
Campestanol	n.d.
Campesterol	4.1
Cycloartanol	n.d.
Cycloartenol	n.d.
Egrosterol	n.d.
Stigmasterol	8.5
Sitostanol	n.d.
Δ5-Avenasterol	n.d.
Squalene (mg/kg oil)	20

n.d. not detected. Modified from CODEX (2001), APCC (2005), Dayrit et al. (2007), Soo et al. (2020), Prapun et al. (2016), Arlee et al. (2013), Rajan et al. (2010), Bhatnagar et al. (2009), Koh and Long (2012), Pazzoti et al. (2018), Gopakumar and Thankappan (1986), Tan et al. (2002)

1.12 Conclusion

Dietary lipids can be derived from both animal and vegetable sources. They are essential nutrients required to regulate and maintain healthy body functions. In contrast to animal fats, vegetable oils are composed primarily of unsaturated fatty acids (oleic and linoleic acids) with some exceptions. On the other hand, animal fats are high in saturated fatty acids (palmitic and stearic acids) with a considerable amount of MUFAs. However, the fatty acid profile of animal fats can be modified through feeding diet to meet the recommended nutrient requirement. Vegetable oils are also rich in tocopherols and phytosterols which are powerful antioxidants and capable of reducing low-density lipoprotein, thereby lowering the risk of coronary heart diseases. Animal fats also contain some important nutrients including vitamin D and K. In short, both animal fats and vegetable oils can be used for various food applications, depending on their distinctive physicochemical properties.

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Chapter 2
Exotic Oil: Sources, Properties and Recovery

Eng-Tong Phuah, Li-Choo Chong, Chee-Hao Kuan, and Ali Yassoralipour

Abstract Due to the increasing demand for agricultural products accompanied by the declining availability of agricultural land, insects have therefore provided a very promising alternative source to replace the conventional food crops and animal-based food in near future. Studies clearly demonstrated that insects have high nutritional values with a large quantity of high-quality proteins and fats, making up to 80% dry weight of insect. Besides, insects are also rich in minerals, vitamins and bioactive compounds including tocol and sterol compounds. Recently, insect-based lipid has received tremendous attention owing to its high level of unsaturated fatty acids particularly linoleic and linolenic acid. Diets enriched with polyunsaturated fatty acid tend to reduce the incidence of cardiovascular disease and other related complications. Besides, insect-based lipid may contain a considerable amount of antioxidative compounds such as α-, β-, γ-, σ- tocopherol or tocotrienol, carotenoid and other lipid-soluble antioxidants. In addition, the physicochemical properties (iodine value, melting and crystallization properties, etc) of the insect-based lipids are comparable to vegetable oils and animal fats. These characteristics indicate the potential application of this fat system in food, pharmaceutical and cosmetic industry.

Keywords Insect lipid · Mealworm · Black soldier fly · Silkworm · Cricket · Melon bug · Sorghum bug · Dubia cockroach · Locust · Grasshopper

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2.1 Introduction

The consumption of insects had been practiced by many cultures globally over the centuries (Govorushko 2019; Ramos-Elorduy 2009; Zielińska et al. 2018). More than 2000 species are reported as edible insects, of which five orders are with at least 100 records (Govorushko 2019; Tang et al. 2019). In recent years, the concept of 'entomophagy', the introduction of edible insect in human diet has gained increasing interest with more than two billion people consuming insects nowadays (Orkusz et al. 2020). Consumer curiosity is the major driving factor for the acceptance of insects as food, arousing their interest in trying insect-based food products such as cricket flour pasta or pancake, cricket chips and other insect-based products available in the market (Cicatiello et al. 2016; Yen 2009). Besides, increasing familiarity and awareness about the nutritional values of edible insects also positively affects the consumer behaviours towards insect consumption (Cicatiello et al. 2016; Orkusz et al. 2020; Tan et al. 2015). Nevertheless, taste and appearance of the insect-based products hinder the diffusion of entomophagy concept in some countries. The prejudice against insect as food could be reduced when the insects are not served or presented in its real biological form, thereby making them less disgusting at the first sight (Cicatiello et al. 2016; Orkusz et al. 2020; Schösler et al. 2012). In fact, insects are rich in nutritional values with high protein and fat content, ranging from 40–75% and 20–40% on dry weight (DW), respectively (Tang et al. 2019). Interestingly, the amount and quality of insect-based protein is comparable to the animal-based protein with analogous amino acid composition. The protein has a high digestibility (76–96%) and can satisfactorily provide almost all the essential amino acids in an adequate level for adults as recommended by World Health Organization (WHO) (Payne et al. 2016; Tang et al. 2019; WHO 2007). Also, the insects are superior than cereal- and legume-based protein because the insect contains a high amount of lysine, threonine and methionine (Spranghers et al. 2017; van Huis et al. 2013). Depending on the insect species, the amino acid content may vary, either higher or lower than the recommended dietary intake level. For instance, the amount of threonine in the protein extract of striped sand grasshopper (*Melanoplus foedus*) (103 mg/g protein) is almost four times higher than the amount suggested by WHO (Table 2.1) (Oibiokpa et al. 2018). On the other hand, some insects reported very low amount of tryptophan (<4 mg/g protein) including mealworm (*Tenebrio molitor*) (Finke 2002; Hussain et al. 2017) and cricket (*Acheta domesticus*) (Tang et al. 2019).

Adapted from Swaminathan et al. (2012), WHO (2007).

Lipid is another important biological compound found in the insect bodies. The insect lipid provides energy, protects the insects from harmful pathogens, helps in communication among the population individuals *via* pheromones, builds up the cell membranes and provides and other physiological functions (Alnajim et al. 2019; Beenakkers et al. 1985; Tzompa-Sosa and Fogliano 2017). Insects are generally high in fats. The fat content and fatty acid composition of insect lipids depends on a variety of factors namely feed composition, cultivation conditions, species, sex, developmental stage as well as geographical locations (Dreassi et al. 2017;

Table 2.1 Dietary indispensable amino acid requirements of adult humans

Indispensable amino acids	mg/g protein
Isoleucine	30
Leucine	59
Valine	39
Lysine	45
Methionine + cysteine	22
Phenylalanine + tyrosine	38
Threonine	23
Tryptophan	6
Histidine	15
Total	277

Spranghers et al. 2017; Tang et al. 2019; Tzompa-Sosa and Fogliano 2017). These parameters greatly affect the fatty acid profile of insects. For example, mealworm (*Tenebrio molitor*) oil is high in unsaturated fatty acids (USFAs) while the contrary is observed for black soldier fly (*Hermetia illucens*) oil where the lipid extract is dominated by saturated fatty acid (SFA). Besides, studies also indicated the wild caught insects are higher in ω-3 fatty acids than those commercial insects (Tzompa-Sosa and Fogliano 2017). Triacylglycerol (TAG) is the major form of insect lipid while other minor components including partial acylglycerols, cholesterol, free fatty acids, phospholipids, tocols and sterols compounds are also present and can be drawn out from the insect during the lipid extraction process (Tzompa-Sosa and Fogliano 2017; Tzompa-Sosa et al. 2019, 2014). The present chapter will review and provide a complete analysis of the lipid extract from different insect species.

Edible insects are also excellent source of vitamins and micronutrients. This can be illustrated by caterpillars with high B1, B2 and B6 nutrients (Rumpold and Schlüter 2013), bee brood (pupae) with significant levels of vitamin A and D (Finke 2005), red palm weevil with high vitamin E content (Bukkens and Paoletti 2005) and adult cricket with sufficient vitamin B12 (Finke 2002). Insect meal also provides a complete range of mineral contents such as phosphorus, magnesium, sodium, potassium, chloride, selenium, manganese and zinc except calcium (<100 mg/g DW) (Finke 2002; Rumpold and Schlüter 2013; Tang et al. 2019). However, some insects such as house fly larvae and melon bugs are typically rich in calcium (Tang et al. 2019). All insect species are also rich in fibre and energy content (Finke 2002; Tang et al. 2019). Most importantly, insect meals are considered to be safe for consumption and the anti-nutrients such as oxalate, phytate, tannin and hydrocyanide are reported to be far below the toxicity levels (Ekop et al. 2010; Omotoso 2006; Rumpold and Schlüter 2015). Even so, the consumption of some insects may result in allergic reaction due to the presence of tropomyosin and arginine-kinase (well-known allergens) (Rumpold and Schlüter 2015).

Another reason that leads to the gradual shift from agricultural products to insect-based diet is because of the fast-growing world population coupled with the slow or unsustainable growing pace of the production of agricultural products. The increased competition for land, water and energy in addition to reduce the negative impacts of food production on the environment (e.g. deforestation; climate change, etc.) will

eventually lead to critical food shortage (Godfray et al. 2010; Payne et al. 2016; van Huis 2013; Yen 2009). The phenomenon urges for the search for alternative food source. As mentioned earlier, insects are readily available food source with excellent amount of protein, lipids, energy, certain vitamins and minerals. Above all, the mass production of edible insects has the advantages as follows: (1) less land and water are required; (2) lower greenhouse gas emission; (3) insects have high feed conversion ratio; (4) insects are safe to be consumed by human and can act as animal feed; (5) low capital investment, technology and skills required; (6) insects have rapid breeding cycles and (7) fast and high returns on investments (Dickie et al. 2019; Govorushko 2019; Madau et al. 2020; Makkar et al. 2014; Oonincx et al. 2011; Reverberi 2020; van Huis and Oonincx 2017). In response, insect farming can ensure sustainable foods for animals and humans.

2.2 Mealworm Oil

Mealworm can be categorised into yellow mealworm (*Tenebrio molitor*), giant mealworm (*Zophobas morio*) and lesser mealworm (*Alphitobius diaperinus*) where the former is always the species of interest. Yellow mealworms are the larval form of the mealworm beetle, a species of darkling beetle. The insect belongs to the order of Coleoptera and family of Tenebrionidae (Selaledi et al. 2019). They are indigenous to Europe and now distributed throughout the world. Their presence can be detected in a variety of staple grains such as oat, wheat, rice and flour, infesting the food grains in the storage facilities. A complete life cycle of mealworm beetle involves egg, larva, pupa and adult. The duration of egg stage is around 4–34 days, depending on the room temperature (Siemianowska et al. 2013). The larval stage is the most variable stage in the life cycle with duration ranges from 57 to 629 days in ambient conditions. The pupal stage may last for 6–20 days before emerging into an adult (Aguilar-Miranda et al. 2002; Ribeiro et al. 2018). Young larvae are white, darkening with age whereas adults are shiny, dark-brown or black (Heidari-Parsa et al. 2018).

Mealworms are easy to culture and mass production of this species is possible due to its high environmental tolerances as well as minimal physical space is required, making them an important alternative and cost-effective protein and oil source as compared to some common agricultural products such as soybean (Li et al. 2013a; Makkar et al. 2014; Selaledi et al. 2019). Previous studies revealed that mealworm is relatively high in nutritional profiles with significant amount of protein (45–70% DW) and fats (30–40% DW) with significant energy contribution ranging from 379–573 kcal/100 g, depending on the growing stage (Bovera et al. 2015; Finke 2002, 2015; Zhao et al. 2016). A detailed examination of the amino acid composition showed that the mealworm protein contains most of the essential amino acids at adequate levels except sulphur amino acids (methionine + cysteine) (Finke 2002, 2015; Son et al. 2020; Zhao et al. 2016). Mealworm also appears to be a good source of mineral content including phosphorus, magnesium, sodium, zinc and iron (Finke

2002, 2015; Igor et al. 2019; Zhao et al. 2016). However, the nutritional values of the mealworms are well correlated with the environmental (temperature and relative humidity) and dietary conditions which greatly govern their development and survival rate (Alves et al. 2016; Liu et al. 2020; Ribeiro et al. 2018; Rumbos et al. 2020).

Mealworm oil has attracted a considerable attention due to its similar physicochemical properties as compared to vegetable oils (Son et al. 2020). After all, mealworm farming has low environmental impact with minimal land, water, energy input, toxic fertilizer and pesticide required than that of vegetable oil combined with available mass production technology, making mealworm farming a potential solution to current massive palm oil deforestation issue (Liu et al. 2020).

2.2.1 Fatty Acid Composition of Mealworm Oil

Mealworm oil is high in USFAs with oleic acid (~45%) being the most abundant, followed by essential linoleic acid (~20%) as shown in Table 2.2. Other major fatty acids in mealworm oil include myristic, palmitic, steric, palmitoleic and linolenic acids even if the data reported in the literature is highly variable. Mealworm oil appears as a light-yellow liquid at ambient temperature. It shows a distinct fatty acid profile as compared to animal-based lipid where the polyunsaturated fatty acids (PUFA) in mealworm oil is 2–10 times higher than the animal fat (Enser et al. 1996). Besides, mealworm oil has a comparable and high ω-6/ω-3 fatty acids ratio which is a pronounced characteristic of vegetable oils (Son et al. 2020). These ω-6 and ω-3 PUFAs can only be integrated from the diet as the human body does not synthesise them. The PUFAs play an important physiological role in immune regulation and inflammation (Glick and Fischer 2013; Simopoulos 2002, 2008).

Previous studies reported that the quality of mealworm oil is associated with feed composition, rearing conditions (Francardi et al. 2017; van Broekhoven et al. 2015) as well as phenological and physiological parameters namely pupal sex, development stage and generation (Dreassi et al. 2017). For instance, Dreassi et al. (2017) found that different feeding diets altered the fatty acid composition of mealworm oil, specifically in oleic and linoleic acid content. Similarly, van Broekhoven et al. (2015) pointed out that high consumption of diet high in ω-6/ω-3 ratio led to mealworm oil with high ω-6/ω-3 ratio. The author reported a direct correlation between feed diet and fatty acid composition of mealworm oil. Another study demonstrated that mealworm fed with diet enriched with 10-wt% linseed showed an increase of linolenic acid (ω-3) and a significant decrease in SFAs in mealworm oil (Francardi et al. 2017). On the contrary, Ravzanaadii et al. (2012) reported no obvious correlation between the fatty acid profile of mealworm oil and the feeding substrates, indicating a distinct physiological regulation of lipids in insects.

Recently, Otero et al. (2020) evaluated the possibility of applying advanced lipid extraction methods (ultrasound-assisted and pressurised-liquid extraction methods) to improve the nutritional properties of mealworm oil. Surprisingly, a significant

Table 2.2 Fatty acid composition of mealworm oil

Characteristics	Range
Oil yield (% DW)	28.5–40.5
Fatty acid composition, %	
C-8:0 Caprylic	0.1
C-10:0 Capric	n.d.
C-12:0 Lauric	0.1–0.6
C-14:0 Myristic	2.9–4.5
C-16:0 Palmitic	9.5–21.3
C-16:1 Palmitoleic	1.7–3.1
C-18:0 Stearic	1.6–8.0
C-18:1 Oleic	35.5–53.1
C-18:2 Linoleic	10.6–29.2
C-18:3 Linolenic	0.2–1.4
C-20:0 Arachidic	0.1–0.9
C-20:1 Gadoleic	0.1
C-20:5 Eicosapentaenoic	0.3–0.4
C-20:4 Arachidonic	0.4–0.5
C-22:4 Docosatetraenoic	0.5–0.6
Total SFA	21.5–36.5
Total USFA	63.5–78.5
Total ω-3	0.3–0.9
Total ω-6	12.7–28.7
Total MUFA	44.6–52.5
Total PUFA	18.9–26.0
Ratio ω-6 / ω-3	24.4–51.6

Data are reported based on the previous studies; n.d. = not detected. Adapted from Alves et al. (2016), Heidari-Parsa et al. (2018), Jeon et al. (2016), Morales-Ramos et al. (2015), Otero et al. (2020), Paul et al. (2017), Purschke et al. (2017), Sipponen et al. (2018), Son et al. (2020), Tzompa-Sosa and Fogliano (2017), Tzompa-Sosa et al. (2019), Tzompa-Sosa et al. (2014), Uğur (2019), Ugur et al. (2020), Zhao et al. (2016)

reduction in total SFA and an increase in total PUFA was observed for the ultrasound-assisted extraction technique using ethanol-water as the extraction solvent. They reported an increase in linoleic acid by 30% as compared to the conventional solvent extraction approach (Otero et al. 2020). Similarly, Sipponen et al. (2018) also successfully altered and improved the lipid profile of the mealworm oil *via* supercritical carbon dioxide extraction method where mealworm oil with a higher degree of unsaturation (an increase from 1.17 to 1.34) could be attained. Besides, high hydrostatic pressure-assisted extraction method can also be employed to increase PUFAs composition in mealworm oil as reported by Ugur et al. (2020).

Attention has been heavily focused on the lipid extract of mealworm larvae to date. In fact, the nutritional value of the mealworm pupae should not be overlooked. The mealworm pupal oil may contain similar or even superior nutritional quality as

Table 2.3 Fatty acid composition of mealworm pupal oil

Characteristics	Range
Oil yield (% DW)	32.0–44.7
Fatty acid composition, %	
C-8:0 Caprylic	n.d.
C-10:0 Capric	0.1
C-12:0 Lauric	0.1–1.0
C-14:0 Myristic	2.3–8.2
C-16:0 Palmitic	17.3–21.4
C-16:1 Palmitoleic	0.1–2.4
C-17:0 Heptadecanoic	0.1–0.3
C-18:0 Stearic	3.0–5.0
C-18:1 Oleic	36.0–45.9
C-18:2 Linoleic	17.2–32.4
C-18:3 Linolenic	0.2–1.9
C-20:0 Arachidic	0.6–0.8
Total SFA	23.5–36.8
Total USFA	63.2–76.5
Total ω-3	0.2–1.9
Total ω-6	17.2–32.4
Total MUFA	28.9–59.1
Total PUFA	17.4–34.3
Ratio ω-6 / ω-3	2.0–12.9

Data are reported based on the previous studies; n.d. = not detected Adapted from Adámková et al. (2017), Dreassi et al. (2017), Morales-Ramos et al. (2015)

compared to its larvae oil. However, limited study was reported as today for mealworm pupal oil. Previous study indicated that a similar fat content was obtained in both mealworm larvae and pupae with approximately 35% of oil extracted (Adámková et al. 2017; Dreassi et al. 2017; Morales-Ramos et al. 2015). The fatty acid composition of mealworm pupal oil is similar to that larvae oil as shown in Table 2.3. Morales-Ramos et al. (2015) demonstrated that oleic and stearic acids were significantly higher in pupae than in larvae whereas the other major fatty acids (palmitic and linoleic acid) did not differ much between larvae and pupae. Nevertheless, the lipid extract from mealworm pupae is not well studied and further investigation may be required.

2.2.2 Physicochemical Properties of Mealworm Oil

Tocopherol (Vitamin E) is an important natural antioxidant commonly found in lipids. Its capability of preventing free radical and hydroperoxyl radical oxidation of lipids is well documented (Seppanen et al. 2010; Yamauchi 1997). Mealworm oil consists a reasonable amount of tocopherol with γ-tocopherol being the primary

tocopherol, accounting for almost 90% of the total tocopherol (Jeon et al. 2016; Son et al. 2020). The total phenolic content of mealworm oil is only 10–20% to that in olive oil and grape seed oil (Son et al. 2020). However, the total phenolic content was increased by 10 times (400–500 mg GAE/kg of oil) when the high hydrostatic pressure-assisted extraction method was employed to extract the mealworm oil (Ugur et al. 2020). Besides, mealworm oil contains minor amount of cholesterol which is the building block required for the biosynthesis of various steroid hormones, vitamin D and bile salts (Son et al. 2020).

Mealworm oil was reported to exhibit a low induction time of 36.0 h, indicating that they are easily oxidised. The shelf life of oil is estimated to be 305 d based on the accelerated shelf-life model. However, mealworm oil showed an improved oxidative stability when the mealworms were roasted at 200 °C for 5–15 mins as the preliminary step. This was attributed to the deactivation of enzymes and formation of Maillard reaction products which can terminate the lipid oxidation reactions (Jeon et al. 2016). Other than that, Dabbou et al. (2020) also reported that mealworm oil was capable of delaying the bacterial growth of Gram-positive and Gram-negative pathogenic bacteria (Dabbou et al. 2020).

The TAG profile of mealworm oil was found within Equivalent Carbon Number (ECN) 50–54 with a minute amount of TAG with ECN 36–38 which is distinct than that of vegetable oil and animal fat (Tzompa-Sosa et al. 2014). When analysing the crystallisation and melting properties of mealworm oil, it was found that mealworm oil showed four clear separated crystallisation peaks and three melting peaks in the thermograms (Crystallisation - Peak 1 = −45.4 °C, Peak 2 = −19.9 °C, Peak 3 = -5 °C and Peak 4 = 3.6 °C) (Melting = Peak 1 = −24.7 °C, Peak 2 = −17.4 °C and Peak 3 = 16.7 °C) (Tzompa-Sosa et al. 2016). This is attributed to the large differences in fatty acid composition within the TAG molecules, therefore causing the TAG to crystallise independently (Tzompa-Sosa and Fogliano 2017). A large separation between the crystallisation peaks indicates that the solid and the liquid part coexist at certain temperature (Tzompa-Sosa et al. 2019). Therefore, separation of the mealworm oil into both olein and stearin fractions *via* dry fractionation technique is therefore possible. Tzompa-Sosa et al. (2016) successfully fractionated the two fractions at operating temperatures of 2 °C and 4 °C. The olein fraction obtained has its highest crystallisation points of -5 °C and 3.6 °C being removed while its last melting point being reduced from 16.7 °C to 1.7 °C and 3.2 °C, respectively (Tzompa-Sosa and Fogliano 2017). These reports clearly demonstrate the potential applications of mealworm oil as a food ingredient due to its desired physicochemical characteristics, excellent nutritional value with a significant amount of PUFAs, high antioxidant capacity and anti-inflammation activity.

The typical characteristics and physicochemical properties of mealworm oil are presented in Table 2.4.

Table 2.4 Physicochemical properties of mealworm oil

Characteristics	Range
Specific gravity (15 °C)	0.85–0.89
Iodine value (g I_2/100 g oil)	72.5–91.6
Saponification number (mg KOH/g oil)	224.0–227.6
Cholesterol, %	0.1–0.6
Oxidative stability index (98 °C) (h)	35.3–36.7
Viscosity (cP)	235–330
Tocols compounds (mg/kg oil)	
α-Tocopherol	6.2–6.4
β-Tocopherol	7.9–9.1
γ-Tocopherol	120.8–126.2
σ-Tocopherol	5.8–6.4
Squalene (mg/kg oil)	16.1–26.1
Total polyphenol (mg GAE/g oil)	3.7–19.3
Colour	L (lightness): 38.9 a (redness): −1.9 b (yellowness): 7.5

Data are reported based on the previous studies. Adapted from Alves et al. (2016). Heidari-Parsa et al. (2018), Jeon et al. (2016), Morales-Ramos et al. (2015), Otero et al. (2020), Paul et al. (2017); Purschke et al. (2017), Sipponen et al. (2018), Son et al. (2020), Tzompa-Sosa and Fogliano (2017), Tzompa-Sosa et al. (2019), Tzompa-Sosa et al. (2014), Ugur et al. (2020),Zhao et al. (2016)

2.3 Black Soldier Fly Larvae Oil

Hermetia illucens or commonly known black soldier fly (BSF) belongs to the family Stratiomyidae of the order Diptera. BSF is one of the important insect species due to its ability to compost organic waste, making BSF an environmental-friendly and cost-effective way of minimising the global food wastes. Most importantly, adult flies do not bite or eat, therefore avoiding the transmission of diseases to humans (da Silva and Hesselberg 2020). The BSF larvae can be grown on a wide range of low-value organic wastes such as rotting fruits and vegetables, food waste, decaying organic solid waste material or palm kernel meal. The larvae then convert these biomass nutrients into their own valuable biomass (proteins and lipids). This characteristic reduces the financial and environmental costs associated with composting (Popa and Green 2012). The larvae can be used as animal feed or the oil can be extracted for biodiesel production (Li et al. 2015). BSFs are commonly found in areas with compostable materials exposed where they attract the flies to lay eggs. Eggs normally take about 5 days to hatch. The larvae will then feed on the biowaste to grow for approximately 12 days before finding a suitable place to pupate (Shelomi 2020). BSF can be raised up easily with low land and water requirements, similar to that of the mealworm, making mass production of this insect species feasible (Matthäus et al. 2019).

Studies showed that BSF larvae is a good sustainable source of protein and fat, ranging from 17.5 to 63% and 11.2 to 46.7%, respectively. They contain all essential

amino acids and the predominant amino acids are lysine, valine, arginine, aspartic acid, glutamic acid and proline (De Marco et al. 2015; Finke 2013; Liland et al. 2017; Spranghers et al. 2017). Besides, the BSF protein has a high digestibility value with apparent protein digestibility score of 76.0, comparable to 77.2 for soybean meal (Shelomi 2020). Furthermore, commercially produced BSF larvae showed 0.77 mg/100 g thiamine, 1.62 mg/100 g riboflavin, 3.85 mg/100 g pantothenic acid, 7.1 mg/100 g niacin, 0.6 mg/100 g pyridoxine, 0.27 mg/100 g folic acid, 0.035 mg/100 g biotin, 5.58 mg/100 g vitamin B12, 110 mg/100 g choline, 8.38 mg/100 g carnitine and 0.62 mg/100 g vitamin E, indicating its outstanding nutritional profile (Finke 2013). With regard to mineral content, calcium is the most abundant macromineral (20–60 g/kg DW) found in BSF larvae which appears to be the limiting nutrient for other insect species. Other minerals include phosphorus (3–15 g/kg DW), potassium (6–16 g/kg DW), magnesium (3–5 g/kg DW), sodium (0.6–1.5 g/kg DW) and some microminerals such as zinc (0.2–0.6 g/kg DW) and iron (0.2–2 g/kg DW) (Barragan-Fonseca et al. 2017; Chia et al. 2020; Liu et al. 2017; Spranghers et al. 2017). The BSF also contains around 9% of chitin, a fibrous aminopoly-saccharide polymer which can be applied in various applications such as food, cosmetic, pharmaceuticals and textile industries (Waśko et al. 2016). However, the nutritional composition of BSF larvae varies, depending on the feeding diets and development stages (Liland et al. 2017; Liu et al. 2017). A detailed analysis on the nutrient contents of BSF throughout the entire life cycle (egg, larva, pupa and adult stages) had been examined and reported by Liu et al. (2017).

2.3.1 Fatty Acid Composition of Black Soldier Fly Larvae Oil

The lipid extracts from BSF mainly consist of medium-chain fatty acids in which lauric acid (C12:0) is the predominant fatty acid (Table 2.5). The high level of medium-chain SFA (C10–14) in BSF larvae oil (40–80% SFAs) is not common as animal fats are often characterised by similar levels of unsaturated and saturated fatty acids. The presence of lauric acid has the ability of disrupting the lipid membranes in microorganism, thereby making BSF larvae oil a useful antibacterial ingredient in pharmaceutical products (Khoramnia et al. 2013). Its antibacterial properties are further supported by a recent study conducted by Dabbou et al. (2020). The authors found that the BSF oil suppressed the growth of both Gram-positive and Gram-negative bacteria. In-vitro experiments also found that lauric acid exhibited potent antibacterial activity against *Propionibacterium acnes* (Nakatsuji et al. 2009). In addition, the administration of lauric acid could considerably reduce the total serum cholesterol and enhance the synthesis of high-density lipoprotein (HDL) cholesterol (Sheela et al. 2016). A low total serum cholesterol to HDL cholesterol is associated with lower atherosclerotic risk (Malaspina et al. 1981).

The fatty acid composition of BSF larvae oil is almost similar to palm kernel fat and coconut fat, the traditionally used lauric-based fats (Matthäus et al. 2019), but differ significantly from other insect lipids. Therefore, the BSF larvae-based fat may

Table 2.5 Fatty acid composition of black soldier fly larvae oil

Characteristics	Range
Oil yield (% DW)	11.2–46.7
Fatty acid composition, %	
C-8:0 Caprylic	n.d.
C-10:0 Capric	0.8–3.1
C-12:0 Lauric	28.8–60.9
C-14:0 Myristic	3.9–11.5
C-16:0 Palmitic	8.2–21.9
C-16:1 Palmitoleic	2.3–8.0
C-18:0 Stearic	1.5–5.3
C-18:1 Oleic	9.5–23.4
C-18:2 Linoleic	1.4–13.0
C-18:3 Linolenic	0.1–3.6
C-20:0 Arachidic	0.1
C-20:1 Gadoleic	n.d.
C-20:5 Eicosapentaenoic	0.1
C-20:4 Arachidonic	0.1–1.3
C-22:4 Docosatetraenoic	n.d.
Total SFA	45.3–82.8
Total USFA	15.1–35.2
Total ω-3	0.9–2.3
Total ω-6	4.6–11.6
Total MUFA	9.5–19.1
Total PUFA	6.8–16.6
Ratio ω-6 / ω-3	2.0–12.9

Data are reported based on the previous studies; n.d. = not detected. Adapted from Barragan-Fonseca et al. (2017), Caligiani et al. (2018), Caligiani et al. (2019); Ewald et al. (2020), Liland et al. (2017), Liu et al. (2017), Mai et al. (2019), Matthäus et al. (2019), Ramos-Bueno et al. (2016), Ravi et al. (2019), Spranghers et al. (2017)

replace palm kernel fat or coconut fat in various food applications, particularly in the development of cocoa butter substitutes with a steep melting profile. The BSF larvae oil also contains moderate amount of oleic acid (16%) and linoleic acid (7%) in which their composition may vary depending on the feeding media lipid profile (Liland et al. 2017; Matthäus et al. 2019; Ramos-Bueno et al. 2016). For instance, linolenic acid or eicosapentaenoic acid (ω-3 fatty acids) was present in the BSF larvae oil when these fatty acids occurred in their diet (Sealey et al. 2011).

2.3.2 Physicochemical Properties of Black Soldier Fly Larvae Oil

BSF larvae oil has a relatively low cholesterol content ranging from 19–248 mg/kg oil, which is 10 to 20 times lower than the animal fats such as lard with an average cholesterol concentration of 1000 mg/kg fat (Liland et al. 2017; Matthäus et al. 2019). In comparison to vegetable oils, BSF larvae fat contains low concentration of vitamin E active substances (53–85 mg/kg) with α-tocopherol (27–30 mg/kg) as the predominant tocols member while γ-tocopherol, σ-tocopherol and α-tocotrienol were only found in small amount (< 3 mg/kg) (Liland et al. 2017; Matthäus et al. 2019). Interestingly, the sterol composition of BSF larvae fat is comparable to seed oils with β-sitosterol as main representative, contributing to more than 50% of this sterol groups. The low cholesterol level coupled with high concentration of phytosterol makes BSF fat a healthier choice of dietary lipids because phytosterols could potentially reduce low density lipoprotein (LDL) cholesterol (Caligiani et al. 2019). BSF larvae fat also contains significant amount of Δ5-avenasterol compound (122.1 mg/kg), comparable to the amount detected in some seed oils such as sunflower seed oil (170 mg/kg), black cumin seed oil (202 mg/kg), Origanum seed oil (170 mg/kg), Pistachio oil (72.6–170 mg/kg) and others (Cheikhyoussef et al. 2020; Kiralan et al. 2014; Matthäus et al. 2018; Yahyavi et al. 2020). Previous study indicated that Δ5-avenasterol is a potent antioxidant and can act as an anti-polymerisation at elevated or frying temperature due to the structural element of an ethylidene group in the side chain (Kochhar 2000). As a consequence, BSF larvae fat showed a high oxidative stability of 50.5 h induction period as compared to some seed oils such as black cumin seed oil with an induction time of 19.6 h (Kiralan et al. 2014), pistachio oil with an induction time of 31.3 h (Rabadán et al. 2018), grape seed oil with an induction period of 10 h (Bjelica et al. 2019), and chia seed oil with an induction period of 2.3 h (Ixtaina et al. 2012).

The crystallisation and melting profile of fats are closely related to the TAG composition. BSF larvae fat is reported to be dominated by saturated TAGs such as LaLaLa, LaLaM, LaMM, contributing to approximately 60% of the total TAG content (Matthäus et al. 2019). The TAG composition of BSF larvae fat is comparable to that of coconut oils and palm kernel fats. However, both coconut oils and palm kernel fats also contain significant amount of TAGs with fatty acids less than 10 carbon atoms namely CCLa and CLaLa (Chen et al. 2007; Soo et al. 2020). During the crystallisation of BSF larvae fat, it shows two closely separated exothermic peaks at 8.98 °C and 3.57 °C in the thermogram. The former peak is due to the presence of high melting TAG fractions while the latter is caused by the TAG fractions with USFAs (MOM, LaPO and MPL) (Matthäus et al. 2019). The two crystallisation peaks are very close together with a narrow temperature range. Similar observation was reported for comercial coconut oil where two exothermic peaks appeared at 8.29 °C and 2.08 °C, respectively (Soo et al. 2020). The same crystallisation pattern was observed for palm kernel oil (Liu et al. 2019; Norizzah et al. 2018). On the other hand, the melting curve of BSF larvae fat demonstrates a

broad melting temperature range where the onset melting temperature starts at 8 °C and ends up at 30 °C. The fat exhibits one significant melting peak at 27.2 °C with two small and contiguous shoulder peaks. Norizzah et al. (2018) also reported one single broad endothermic peak with melting temperature ranges from 10 °C to 31 °C and a peak temperature appears at around 25 °C for palm kernel oil.

Therefore, it can be concluded that the physicochemical properties of the BSF are very similar to coconut fats and palm kernel fats, opening up the potential applications of BSF-based fats in replacing these lauric-based fats in various food applications as well as developing new products. The typical characteristics and physicochemical properties for BSF larvae fat are shown in Table 2.6.

2.4 Silkworm Pupal Oil

Silkworms are well known as the producer of silk thread. Among the various species of silkworms, mulberry silkworms (*Bombyx mori*), oak silkworm (*Antheraea pernyi*) and the eri silkworm (*Samia cynthia ricini*) are the common species used in sericulture and the former is the main insect species model for research (Zhou and Han 2006). When a silkworm enters the pupa phase, it creates a protection cocoon made of raw silk with high mechanical strength. Just before the moth emerges, the pupa is killed by boiling, drying or soaking in NaOH. After that, the silk will be extracted. Silkworm pupae are the main by-products produced after extracting the silk threads, and they contribute to approximately 60% of the cocoon weight (Altomare et al. 2020; Hu et al. 2017). These pupae are considered as industrial waste and they are normally used as animal feed or fertilizer (Altomare et al. 2020). The consumption of silkworm pupae in Asia countries particularly in China, Thailand and India to provide the nutrition benefits is well documented (Mishra et al. 2003; Yang et al. 2009; Zhou and Han 2006).

Previous study indicates that silkworm pupae are high in protein (55.6%) and fat content (32.2%) (Tomotake et al. 2010; Yang et al. 2009). The pupae exhibit excellent amino acid profile with significant amount of essential amino acids namely valine (47–66 mg/g), methionine (15–46 mg/g) and phenylalanine (46–81 mg/g) (Tomotake et al. 2010). These essential amino acids satisfy the nutrient requirement recommended by WHO (WHO 2007; Zhou and Han 2006). Besides, the silkworm pupae protein hydrolysate shows angiotensin I-converting enzyme inhibitory activity (Wang et al. 2008). Therefore, the silkworm protein hydrolysate form can act as a potential source to treat cardiovascular disease. In addition, the antimicrobial, anti-inflammatory and antioxidative activity of silkworm pupae peptide were also reported (Altomare et al. 2020; Cheng et al. 2006). Furthermore, Tomotake et al. (2010) pointed out that the silkworm pupae contain 1-deoxynojirimycin (DNJ) which can act as potent α-glucosidase inhibitor, retarding the digestion and glucose absorption. As a result, it lowers the blood glucose levels (Li et al. 2013b; Ma et al. 2019). The mineral analysis indicates a high concentration of potassium (34.0 mg/g)

Table 2.6 Physicochemical properties of black soldier fly larvae oil

Characteristics	Range
Specific gravity (4 °C)	0.908–0.914
Iodine value (g I_2/100 g fat)	19.3–75.6
Saponification number (mg KOH/g oil)	213–252
Cholesterol, %	0.002
Oxidative stability index (120 °C) (h)	49.2–52.0
Viscosity (cP)	96–101
Tocols compounds (mg/kg fat)	
α-Tocopherol	30.2–30.4
α-Tocotrienol	2.7
β-Tocopherol	9.6–9.8
β-Tocotrienol	19.7–19.9
γ-Tocopherol	22.2
Sterol compounds (mg/kg fat)	
Cholesterol	17.5–20.5
Brassicasterol	16.5–21.7
24-Methylencholesterol	15.9–18.1
Campesterol	868.9–910.5
Campestanol	142–153.2
Stigmasterol	67.5–72.7
7-Campesterol	46.9–50.5
5,23-Stigmastadienol	27.4–30.2
Chlerosterol	8.9–11.1
β-Sitosterol	1823.8–1908.2
Sitostanol	184.7–190.3
Δ5-avenasterol	120.2–124
5,24-stigmastadienol	23.5–23.7
Δ7-stigmastenol	85.2–86.6
Δ7-avenasterol	5.4–39
Total polyphenol (mg GAE/g oil)	6.9–20.1
Colour	L (lightness): 65.24 C (chroma): 21.52 H (hue angle): 110.4

Data are reported based on the previous studies; n.d. = not detected. Adapted from Barragan-Fonseca et al. 2017; Caligiani et al. 2018; Caligiani et al. 2019; Ewald et al. 2020; Liland et al. 2017; Liu et al. 2017; Mai et al. 2019; Matthäus et al. 2019; Ramos-Bueno et al. 2016; Ravi et al. 2019; Spranghers et al. 2017)

in silkworm pupae with a significant amount of essential trace elements (copper, zinc, iron and selenium) required for physiological functions (Zhou and Han 2006).

2.4.1 *Fatty Acid Composition of Silkworm Pupal Oil*

Silkworm pupal oil contains about 18–32% DW of lipid extract, depending on the extraction process. For example, a research report indicated that only less than 7% of the silkworm pupal oil was obtained using the maceration extraction method with petroleum ether for different varieties of Thai silkworm (Winitchai et al. 2008). The USFA accounts for more than 70% of the total fatty acid composition in silkworm pupal oil in which linolenic acid (45%) and oleic acid (25.0%) are the principal fatty acids (Hu et al. 2017; Pan et al. 2012; Tomotake et al. 2010). However, some studies revealed that the fatty acid composition might vary, depending on the species origin, feeding diet, season and geographical regions (Shanker et al. 2006). Shanker et al. (2006) reported that the Eri silkworm pupal oil fed with tapioca-based diet has 35% higher in linolenic acid as compared to castor-based diet. Also, Eri silkworm pupal oil is observed to contain 50% higher in PUFAs as compared to mulberry silkworm pupal oil (Hu et al. 2017; Shanker et al. 2006). Similarly, Pan et al. (2012) also pointed out that the variation in fatty acid composition in silkworm pupal oil is related to the type of species. The concentration of linolenic acid in oak silkworm pupal oil is 10% lower than the mulberry silkworm pupal oil (Pan et al. 2012).

Based on the fatty acid composition as shown in Table 2.7, the silkworm pupal oil can be considered as an excellent source of edible oil. The pupal oil is exceptionally high in PUFAs especially ω-3 fatty acids. These ω-3 fatty acids have anti-inflammatory, antithrombotic, antiarrhythmic, hypolipidemic and vasodilatory properties. They can prevent and tackle chronic diseases such as cardiovascular disease, hypertension, cancer and others (Simopoulos 1999, 2002, 2008). It is worth-mentioning that the ω-6/ω-3 ratio in silkworm pupal oil is as low as 0.2 which is comparable to chia seed oil and flaxseed oil (Ciftci et al. 2012; Kulczyński et al. 2019; Nitrayová et al. 2014). Simopoulos (2002) reviewed and emphasised on the importance of low ratio of ω-6/ω-3 essential fatty acid. A low ω-6/ω-3 ratio reduces the inflammation by reducing the leukotrienes and prostaglandin generated from arachidonic acid. Consequently, it can exert a protective effect against the development and growth of cancers and provide other beneficial health effects (Simopoulos 1999, 2002, 2004, 2008; Simopoulos and Cleland 2003). The statement was further supported by a study conducted by Mentang et al. (2011). The authors reported that the administration of silkworm pupal oil suppressed the formation of arachidonic acid in adult Wistar rats. Arachidonic acid is required for the formation of ω-6 PUFA-derived eicosanoids. Overexpression of these eicosanoids will cause cell proliferation, arthritis and inflammatory disorders (Gerster 1998). In the meanwhile, the study also showed that the silkworm pupal oil reduced the plasma TAG and glucose level in rat subject, thereby preventing the body fat accumulation (Mentang et al. 2011).

Table 2.7 Fatty acid composition of silkworm pupal oil

Characteristics	Range
Oil yield (% DW)	18.0–32.2
Fatty acid composition, %	
C-8:0 Caprylic	n.d.
C-10:0 Capric	n.d.
C-12:0 Lauric	n.d.
C-14:0 Myristic	0.1–0.7
C-16:0 Palmitic	15.8–30.3
C-16:1 Palmitoleic	0.6–4.8
C-17:0 Heptadecanoic	0.2–0.6
C-18:0 Stearic	2.0–7.1
C-18:1 Oleic	9.1–39.4
C-18:2 Linoleic	3.7–7.3
C-18:3 Linolenic	28.0–60.4
C-20:0 Arachidic	0.2–0.7
C-20:3 Eicosatrienoic	0.2
C-20:5 Eicosapentaenoic	n.d.
C-20:4 Arachidonic	n.d.
C-22:4 Docosatetraenoic	n.d.
Total SFA	28.2–28.8
Total USFA	71.3–71.6
Total ω-3	28.0–60.4
Total ω-6	3.7–7.3
Total MUFA	27.7–35.7
Total PUFA	41.6–43.6
Ratio ω-6 / ω-3	0.1–0.3

Data are reported based on the previous studies; n.d. = not detected. Adapted from (Hu et al. 2017; Kotake-Nara et al. 2002; Pan et al. 2012; Shanker et al. 2006; Tomotake et al. 2010; Wei et al. 2009)

2.4.2 Physicochemical Properties of Silkworm Pupal Oil

The physicochemical properties of silkworm pupal oil are presented as in Table 2.8. Past study showed that silkworm pupal oil consists high concentration of tocopherols (125–224 mg/kg) as compared to vegetable oils such as soybean oil (265 mg/kg) and linseed oil (244.2 mg/kg) (Kotake-Nara et al. 2002). Besides, silkworm pupae were reported to contain significant level of carotenoids such as lutein and neoxanthin. When evaluating the oxidative stability of silkworm pupal oil by measuring the oxygen consumption in the headspace of gas chromatography at 50 °C, Kotake-Nara et al. (2002) observed a low reduction of oxygen concentration even after incubating the silkworm pupal oil for 800 h. On the other hand, a drastic drop in oxygen concentration was reported for both linseed oil and soybean oil after incubation periods of 180 h and 300 h, respectively. The results obtained clearly

Table 2.8 Physicochemical properties of silkworm pupal oil

Characteristics	Range
Specific gravity (25 °C)	0.91–0.93
Iodine value (g I_2/100 g oil)	119–124
Saponification number (mg KOH/g oil)	105–140
Oxidative stability index (110 °C) (h)	26.7–64.6
Refractive index (25 °C)	1.44–1.47
Tocols compounds (mg/kg oil)	
α-Tocopherol	73.3–131
α-Tocotrienol	n.d.
β-Tocopherol	8.5–15.0
β-Tocotrienol	n.d.
γ-Tocopherol	23.1–42.1
σ-Tocopherol	20.3–36.0
Carotenoid compounds (mg/kg oil) *	
Neoxanthin	12.8
Violaxanthin	4.6
Lutein	74.2
Sterol compounds (mg/kg oil)	
Cholesterol	586.3–895.6
Campesterol	47.0–68.6
Stigmasterol	43.0–47.6
β-Sitosterol	240.0–366.0
Total polyphenol (mg GAE/kg oil)	42.0–79.6

Data are reported based on the previous studies; n.d. = not detected, *Carotenoid profile is calculated based on the assumption that carotenoid compounds are concentrated in the lipid fraction of silkworm pupae. Adapted from Hu et al. (2017), Kotake-Nara et al. (2002), Pan et al. (2012), Shanker et al. (2006), Tomotake et al. (2010), Wei et al. (2009)

indicated the high stability of pupal oil. The high oxidative stability of the pupal oil might be attributed to the high levels of carotenoids and tocopherols.

Among the sterols, cholesterol is the major sterol compound found in silkworm pupal oil. Nevertheless, the cholesterol level is still comparatively lower than other animal fats (600–1000 mg/kg) (Orczewska-Dudek et al. 2012). Most of the TAG molecular species of silkworm pupal oil lie in ECN 40 which is identified to be LLLn, LnLnP or LnLnO. This group of TAG species contributes to almost 40% of the total TAG compositions, followed by TAG molecules with ECN 44 (LnOO, OLL, SLLn, PLL or PLnP) (23%). More importantly, Shanker et al. (2006) pointed out that the *sn*-2 position of TAG backbone is dominated by USFAs such as linoleic and linolenic acid. The predominance of USFAs at *sn*-2 position is essential to enhance the bioavailability of these fatty acids. Thereafter, the biosynthesis of their higher homologues such as arachidonic, eicosapentaenoic and docosahexaenoic acids and their subsequent conversion to eicosanoids is made possible (Shanker et al. 2006).

2.5 Cricket Oil

Cricket belongs to the family Gryllidae of the order Orthoptera. There are two major categories of cricket namely European house cricket (*Acheta domesticus*) and Jamaican field cricket (*Gryllus assimilis*). Field cricket is an interesting cricket species because it shows resistance to Acheta domesticus densovirus (AdDNV) which threatens the house cricket and hinders the large-scale breeding of the house cricket (Liu et al. 2011). Cricket has cylindrical bodies with 16–21 mm in length, round heads and long antennae as well as light yellowish-brown in colour. Crickets are distributed throughout the world, but they are once thought to be native to Southwestern Asia (Mariod and Mirghani 2017). Crickets are normally consumed as food or snack in Southeast Asia (Oonincx et al. 2015). The life cycle of cricket involves egg, nymph and adult. The cycle is usually completed within 2 to 3 months under standard rearing conditions (Uğur 2019; von Hackewitz 2018). A single female may lay around 728 eggs. The eggs hatch after 11–15 days and start to grow and become an adult after around 50 days (von Hackewitz 2018). These crickets can be grown in large colonies in a controlled environment and cricket farming has now become popular in Southeast Asian particularly Thailand (Starčević et al. 2017; van Huis 2013).

Crickets are high in protein and fat contents, ranging from 65–71% and 10–23% DW, respectively. The protein content of cricket is higher than some other edible insects such as melon bugs (27%) and sorghum bug (28%) (Mariod et al. 2011c). The cricket protein has a complete set of essential amino acids at sufficient amount for adults as recommended by WHO (2007). The total essential amino acid is reported to be around 400 mg/g protein with phenylalanine + tyrosine being the highest (88 mg/g), followed by leucine (80 mg/g), lysine (58 mg/g), valine (53 mg/g), isoleucine (41 mg/g), threonine (40 mg/g), methionine + cysteine (23 mg/g), histidine (22 mg/g) and tryptophan (6 mg/g) on average (Finke 2002; Mariod and Mirghani 2017; Rumpold and Schlüter 2013; Tang et al. 2019). These properties make cricket a suitable food source to replace conventional animal or plant-based protein. A study conducted to evaluate the protein quality of cricket meals on weanling rats suggested that the cricket protein is comparable or even superior than soy protein (Finke et al. 1989). Crickets also contains high level of vitamin A (24.3 μg), vitamin B12 (7.1 μg), vitamin B complex (85 mg), vitamin C (9.74 mg), vitamin E (72 IU/kg). Besides, cricket meal also contains approximately 7% of chitin (Adámková et al. 2017). The mineral analysis of the cricket meal also revealed a high amount of calcium, potassium, magnesium, sodium, iron, zinc, manganese, copper and selenium with mean concentration of 200, 1200, 100, 500, 12, 20, 3.5, 1.5 and 0.05 mg/100 g insect, respectively (Mariod and Mirghani 2017; Rumpold and Schlüter 2013). The sodium-to-potassium ratio is important as it is associated with the risk of high blood pressure, stroke and cardiovascular disease (Iwahori et al. 2017). Based on the previous data, the ratio of sodium-to-potassium ratio is calculated to be 2.5:1 which is close the ideal ratio of 3:1, indicating that the cricket meal is a healthy food.

2.5.1 Fatty Acid Composition of Cricket Oil

Cricket contains a lower amount of oil ranging from 10–23% DW. The lipid extract of cricket is composed of USFAs relative to SFAs in the ratio of 2:1 (Table 2.9). Linoleic acid is the major fatty acid found in cricket oil, followed by oleic acid and palmitic acid. These three fatty acids contribute to almost 85% of the total fatty acid content. Therefore, cricket oil can be regarded as ω-6 and ω-9 rich insect lipid. Previous study showed that cricket oil has a unique feature in which it consists of vaccenic acid (C18:1 trans11) which makes this fatty acid specific for this insect species (data is not shown in Table 2.9). Its presence might be due to the microbial biohydrogenation of fatty acid from unsaturated C18 to C18 trans isomers in ruminal gut (Tzompa-Sosa et al. 2014). The beneficial health effect of vaccenic acid is scarce and remains to be a subject of debate even though some studies reported its

Table 2.9 Fatty acid composition of cricket oil

Characteristics	Range
Oil yield (% DW)	9.8–22.8
Fatty acid composition, %	
C-8:0 Caprylic	n.d.
C-10:0 Capric	n.d.
C-12:0 Lauric	0.2–0.3
C-14:0 Myristic	0.4–1.8
C-16:0 Palmitic	17.2–29.9
C-16:1 Palmitoleic	1.0–2.8
C-17:0 Heptadecanoic	0.1–0.3
C-18:0 Stearic	4.6–10.6
C-18:1 Oleic	19.5–32.3
C-18:2 Linoleic	26.9–41.7
C-18:3 Linolenic	0.7–10.9
C-20:0 Arachidic	0.1–0.6
C-20:3 Eicosatrienoic	0.2–0.5
C-20:5 Eicosapentaenoic	0.5–1.5
C-20:4 Arachidonic	0.1
C-22:6 Docosahexaenoic	0.1–0.2
Total SFA	31.3–39.5
Total USFA	54.8–68.3
Total ω-3	2.0–12.4
Total ω-6	29.1–34.4
Total MUFA	23.5–34.0
Total PUFA	31.3–36.5
Ratio ω-6 / ω-3	12.8–37.0

Data are reported based on the previous studies; n.d. = not detected. Adapted from Adámková et al. (2017), Laroche et al. (2019), Otero et al. (2020), Paul et al. (2017), Ramos-Bueno et al., (2016), Starčević et al. (2017), Tzompa-Sosa et al. (2019), Tzompa-Sosa et al. (2014), Uğur (2019), Ugur et al. (2020)

beneficial effect in reducing tumour growth and coronary heart disease (Field et al. 2009). Besides, eicosapentaenoic acid was also detected in cricket oil at trace amount (~1.0%) (Laroche et al. 2019; Tzompa-Sosa et al. 2014). These findings are not common as most of the terrestrial and aquatic insects show minute (< 0.3%) or absence of these eicosapentaenoic and docosahexaenoic acids in the insect lipids (Fontaneto et al. 2011). Surprisingly, a study reported that the lipid extract from field cricket showed an elevated eicosapentaenoic acid content when linolenic acid-enriched diet was provided (Komprda et al. 2013). The observation could be explained by the capability of cricket in synthesizing linolenic acid or higher homologues from oleic or linoleic acid due to the presence of Δ12-desaturase that uses oleoyl-CoA as a substrate (Cripps et al. 1986). The ω-6/ω-3 ratio for cricket oil is 17:1 which is higher than the recommended value of 10:1 (FAO 2010). Therefore, attention should be given when consuming cricket oil. Besides, cricket oil can also be taken together with other edible oils rich in ω-3 fatty acids such as linseed or chia seed oil in order to achieve a balanced ω-6/ω-3 ratio.

Different extraction techniques can be used to alter or modify the fatty acid content of cricket oil to meet the desired composition (Laroche et al. 2019; Otero et al. 2020; Sipponen et al. 2018; Tzompa-Sosa et al. 2019; Ugur et al. 2020). Enrichment of linoleic acid from 39.3% to 57.2% in cricket oil was achieved using the supercritical CO_2 extraction method as compared to the conventional solvent extraction method (Sipponen et al. 2018). In another study, Otero et al. (2020) successfully improved the PUFA/SFA ratio from 1.5 to 3.0, an increase of 100% *via* ultrasound-assisted extraction method using ethanol-water as the solvent mixture. This makes the insect lipid extract to have an almost similar PUFA/SFA profile as soybean oil (~4.0). In addition, the fatty acid composition of cricket oil is positively correlated with the fatty acid profile of the feeding diet (Oonincx et al. 2020; Starčević et al. 2017). For example, diet-enriched with linseed oil (high linolenic acid) significantly increased the amount of linolenic acid in cricket tissues. The study reported that the supplementation of 5% linseed oil increased the linolenic acid in cricket by 11.3% (Starčević et al. 2017). Similar observation was also reported by Oonincx et al. (2020) in which linolenic acid in cricket oil would increase by 3% for every 1% flaxseed oil (high in linolenic acid) added into the feeding diet. Moreover, another study also revealed that fish oil-fed crickets exhibited high amount of higher ω-3 homologues with eicosapentaenoic acid and docosahexaenoic acid ranges from 0.78–1.35% and 0.16–0.29%, respectively (Starčević et al. 2017).

2.5.2 Physicochemical Properties of Cricket Oil

The physicochemical properties of cricket oil are presented in Table 2.10. Cricket oils are mainly comprised of TAG species with ECN 50–54, in which ECN 52 contributing to approximately 35% of the total TAG composition, followed by ECN 54 (25%) and ECN 50 (20%). Low concentration of glyceride with ECN 36–38

Table 2.10 Physicochemical properties of cricket oil

Characteristics	Range
Iodine value (g I_2/100 g oil)	99
Cholesterol, %	0.7–3.6
Total polyphenol (mg GAE/g oil)	0.4–0.8
Colour	L (lightness): 60.2 a (redness): 8.9 b (yellowness): 63.8

Data are reported based on the previous studies; n.d. = not detected. Adapted from Adámková et al. (2017), Laroche et al. (2019), Otero et al. (2020), Paul et al. (2017), Ramos-Bueno et al. (2016), Starčević et al. (2017), Tzompa-Sosa et al. (2019), Tzompa-Sosa et al. (2014), Uğur (2019), Ugur et al. (2020)

were also spotted, suggesting that the phospholipid and diacylglycerol (DAG) with two fatty acid chains might also present in the crude oil extract (Tzompa-Sosa et al. 2014). Cricket oil showed four clear separated peaks in the crystallisation thermogram. The peaks appear at −48.7 °C, −33.1 °C, −11.2 °C and 7.6 °C, respectively. The obvious separation among these crystallisation points indicates the presence of high-melting and low-melting TAG fractions in cricket oil. To broaden the applications of cricket oil, separation of these two fractions can be performed using fractionation techniques. On the other hand, the melting profile of cricket oil covers a wide melting temperature range with onset and endset melting temperatures of −30.7 °C and 22.7 °C, respectively. Four well-separated melting peaks are observed (Peak 1 = −30.7 °C, Peak 2 = -13 °C, Peak 3 = 1.2 °C and Peak 4 = 22.7 °C) (Tzompa-Sosa et al. 2014). Therefore, cricket oil tends to appear in liquid form at room temperature due to its predominance of low-melting fractions.

The physicochemical properties of cricket oil are not well explored or investigated to date. Therefore, more studies can be conducted to reduce the current research gap and to enhance our understanding on cricket oil.

2.6 Melon Bug Oil

The melon bug *Coridius viduatus* (Heteroptera: Dinidoridae; formerly *Aspongopus Viduatus*) is known locally as Um-buga in Sudan. The insects are widely distributed in the Kordofan and Darfur regions of Sudan (Mariod 2020c; Tarla et al. 2013). The insect is oval in shape, flat and relatively large (20 mm long and 10 mm broad). They have very distinctive front wings, called hemelytra with one half is leathery and the apical half is membranous. The bugs are considered as the main pest for watermelon. The adult nymphs pierce leaves, stems and young fruits and suck the sap, resulting in wilting, fruit drop and death of the plant (Mariod et al. 2004; Mariod 2020c). The oil extracted from melon bug are used for cooking (during famine and shortage of food) and some medical applications including skin lesion remedy. This has been the traditional practice in Sudan for decades (Mariod et al. 2004, 2011c).

The proximate analysis of the melon bug shows that the insects contains about 8.3% moisture, 27.0% crude protein, 54.2% fat, 3.5% ash and 7.0% carbohydrate, indicating that melon bug is rich in fat content. The melon bug contains 16 known amino acids, including all the essential amino acids. The total amount of essential amino acid was reported to be 208.5 mg/kg protein, which is slightly lower than the recommended amount of 277 mg/kg protein (WHO 2007). The melon bug protein has a moderate amino acid score in which lysine, leucine and phenylalanine + tyrosine are the limiting amino acids of the melon bug protein (Mariod et al. 2011c; Tang et al. 2019). Besides, melon bug was also found to contain considerable amount of sulphur-containing amino acids (methionine + cysteine) (57.1 mg/g protein) which are important precursors for the synthesis of glutathione, a low molecular thiol-tripeptide in maintaining the intracellular redox balance (Clemente-Plaza et al. 2018). Previous study reported that gelatine could also be extracted from the melon bug and a maximum of 3% gelatine was obtained using hot water extraction method (Mariod et al. 2011d). Gelatine is a useful food ingredient that can be applied to improve elasticity, consistency and stability of the food system. An analysis of the melon bug-based gelatine suggested that it has a fine structure network with very small voids and shows comparable properties to that of the commercial gelatine. In a subsequent study, the melon bug-based gelatine was used as a stabiliser in ice-cream making (Mariod and Fadul 2015). It is worth mentioning that the melon bug insect is typically abundant in calcium content (1021 mg/100 g) as this is not common in insect meal. Other mineral contents include magnesium (301 mg/100 g), potassium (200 mg/100 g), phosphorus (1234 mg/100 g) and sodium (401 mg/100 g). Both magnesium and phosphorus contents are far beyond the recommended daily intake of 240 mg/day and 700 mg/day, respectively.

2.6.1 Fatty Acid Composition of Melon Bug Oil

The melon bug oil appears to be dark light red in colour. Melon bug contains approximately 50% of oil which is predominated by 46.3% oleic and 31.1% palmitic with minute amount of linoleic acid (4.3%). Therefore, melon bug oil is considered as ω-9 rich edible oil. A closer look on the data summarised in Table 2.11 showed that the fatty acid composition of the melon bug oil is comparable to that of palm oil in which the palm oil contains significant amount of palmitic acid and oleic acid in the ratio of approximately 1:1. These two fatty acids contribute to almost 80% of the fatty acid content in palm oil (Mancini et al. 2015; Montoya et al. 2014). Hence, it could be hypothesised that melon bug oil may be suitable for deep-drying purpose due to its high level of SFA and monounsaturated fatty acid (MUFA), rendering it being oxidised at elevated frying temperature. Previous studies indicated that both SFA and MUFA are resistant to oxidation while PUFAs are the predominant factor in the oxidation of vegetable oils (Cao et al. 2015). Furthermore, the melon bug oil is also rich in palmitoleic acid (~10%), an important fatty acid in exerting beneficial

Table 2.11 Fatty acid composition of melon bug oil

Characteristics	Range
Oil yield (% DW)	45.0–55.0
Fatty acid composition, %	
C-8:0 Caprylic	n.d.
C-10:0 Capric	n.d.
C-12:0 Lauric	n.d.
C-14:0 Myristic	0.3–0.4
C-16:0 Palmitic	30.5–31.6
C-16:1 Palmitoleic	10.4–10.9
C-17:0 Heptadecanoic	2.3–2.5
C-18:0 Stearic	3.2–3.8
C-18:1 Oleic	45.2–47.4
C-18:2 Linoleic	3.6–4.9
C-18:3 Linolenic	0.1–0.5
C-20:0 Arachidic	0.2–0.3
C-20:1 Gadoleic	0.2
C-20:5 Eicosapentaenoic	n.d.
C-20:4 Arachidonic	n.d.
C-22:4 Docosatetraenoic	n.d.
Total SFA	37.3–38.5
Total USFA	60.3–62.7
Total ω-3	0.4–0.5
Total ω-6	3.6–4.9
Total MUFA	56.4–57.5
Total PUFA	3.9–5.5
Ratio ω-6 / ω-3	7.2–12.3

Data are reported based on the previous studies; n.d. = not detected. Adapted from Alnadif et al. (2007), Mariod (2011), Mariod et al. (2004), Mariod et al. (2011a), Mariod et al. (2005), Mariod et al. (2006a, 2008), Mariod et al. (2011b), Mariod (2013), Mariod et al. (2011c), Matthäus et al. (2015)

health effects. Previous study demonstrated that palmitoleic acid is related to the increased insulin sensitivity, thereby reducing the lipid accumulation in the liver (Frigolet and Gutiérrez-Aguilar 2017). Besides, its potent anti-inflammatory properties of palmitoleic acid as compared to oleic and palmitic acid is also well explored (de Souza et al. 2018). Another interesting part about the melon bug oil is that the fatty acid profile of the lipid is predominated with MUFA (~60%) which is not observed in other insect lipids. A diet high in MUFA enhances β-oxidation and shows its positive effects on weight control in a number of animal and human studies (Bes-Rastrollo et al. 2006; Clifton et al. 2004; Liao et al. 2010; Schwingshackl and Hoffmann 2012).

2.6.2 *Physicochemical Properties of Melon Bug Oil*

The typical characteristics and physicochemical properties for melon bug oil are presented in Table 2.12. Previous studies showed that melon bug oil is low in tocopherol (3 mg/kg oil) but a high oxidative stability was observed (Alnadif et al. 2007; Mariod et al. 2004). This observation is not expected as the concentration of the tocopherol has a direct correlation with the oxidative stability of oil. The antioxidant behaviour of tocopherol in prohibiting the formation of hydroperoxide is well reported (Gizachew 2020; Kamal-Eldin 2006; Yamauchi 1997).

Melon bug oil was reported to exhibit high total phenolic compounds (206.6 mg/kg oil) as compared to some conventional oils including groundnut oil, coconut oil, rice bran oil, mustard oil, sunflower oil and sesame oil with total phenolic content of 30.9 mg/kg, 18 mg/kg, 8.9 mg/kg, 5.6 mg/kg, 4.9 mg/kg and 3.3 mg/kg, respectively (Janu et al. 2014; Matthäus et al. 2015). In another study, Matthäus et al. (2015) identified the phenolic compounds and flavanoids in melon bug oil to be t-cinnamic acid and syringic acid, quercetin and pelargonin, respectively. However, quantification of these compounds was not conducted in their study. The total amount of sterol in melon bug oil is roughly 1200 mg/kg oil with β-sitosterol being the major sterol

Table 2.12 Physicochemical properties of melon bug oil

Characteristics	Range
Oxidative stability index (120 °C) (h)	36.5–46.0
Kinematic viscosity (40 °C) (mm^2/s)	34.9–35.1
Tocols compounds (mg/kg oil)	
α-Tocopherol	0.7–3.8
α-Tocotrienol	n.d.
β-Tocopherol	n.d.
β-Tocotrienol	n.d.
γ-Tocopherol	0.4–2.4
σ-Tocopherol	n.d.
Sterol compounds (mg/kg oil)	
Cholesterol	14.0–40.0
Campesterol	18.0–171.0
Stigmasterol	8.0–40.0
β-Sitosterol	106.0–696.0
Δ5-avenasterol	5.0–25.0
Δ7-stigmastenol	9.0–45.0
Δ7-avenasterol	n.d.
Others^{+}	175.0–1063.0
Total polyphenol (mg GAE/g oil)	206.3–206.9

Data are reported based on the previous studies; n.d. = not detected, + Others include 24-methylcholesterol, campestanol, chlerosterol, sitostanol and 5,24-stigmastadienol Adapted from Alnadif et al. (2007), Mariod (2011), Mariod et al. (2004), Mariod et al. (2011a), Mariod et al. (2005), Mariod et al. (2006a, 2008), Mariod et al. (2011b), Mariod 2013), Matthäus et al. (2015)

species (Alnadif et al. 2007; Mariod et al. 2006a). High variation in the sterol contents was observed in the previous experiments. Mariod et al. (2006) pointed out that the amount of tocopherols and sterols extracted from melon bug depends on the extraction solvent. The author reported that organic-based solvent tends to improve the tocopherols and sterols content in melon bug oil by 23% and 406%, respectively as compared to water-extracted melon bug oil.

Previous studies indicated that the oxidative stability of oil is associated with the presence of phenolic compounds, tocopherols and other parameters (phopholipids, trace metals and fatty acid composition of oil) (Mariod et al. 2006a, 2006b). Due to its high concentration of sterol content coupled with low degree of unsaturation as indicated earlier, melon bug oil showed a remarkable high induction period of 36.5–46.0 h. In a subsequent study, Mariod et al. (2008) also reported that melon bug oil has high storage stability. The fatty acid compositions of melon bug oil remained unchanged after a 24-month of storage period at 30 °C (Mariod et al. 2008). Another study also revealed that the oxidative stability of the sunflower oil can be improved by blending the sunflower oil with melon bug oil. A positive correlation between the induction period of the sunflower oil and the percentage of melon bug oil in the blends was observed (Mariod et al. 2005). These results clearly point out the suitability of melon bug oil to be used for various food applications especially as cooking oil.

The melon bug oil also exhibited excellent antibacterial activities in which the crude oil and phenolic compounds-free oil could inhibit the growth of several food-related pathogenic bacteria isolates particularly *Staphylococcus aureus*, *Salmonella enterica* serovar Paratyphi, *Escherichia coli* and *Bacillus cereus* (Mustafa et al. 2008). Therefore, the melon bug oil can be applied on the meat products as a natural preservative to suppress the growth of Gram-positive bacteria. However, further investigation may be required to confirm its appropriateness in food applications.

2.7 Sorghum Bug Oil

Sorghum bug with scientific name of *Agonoscelis versicoloratus* (Heteroptera: Pentatomidae; formerly *Agonoscelis pubescens*) is also known as Sudan millet bug or cluster bug sometimes (Mariod et al. 2011d; Mariod and Fadul 2015). They are widely distributed in both rain-fed and irrigated areas. In Sudan, they are locally called as Dura andat. The adult sorghum bug is shield-shaped, about 11–13 mm long and 6–7 mm wide. Both the upper- and undersides of its body are covered with fine silvery pubescence (Mariod 2011, 2020b). The bugs have piercing-sucking mouth-parts throughout the life cycle. They are considered as insect pests as they suck the plant juice and injure the host plants. The bug has a characteristic musty flavour which is detectable when a large number of the bugs are present or when the bugs are crushed (Mariod et al. 2006a, 2008; Mariod 2020b). The sorghum bug can attack a number of crops including lucerne (Medicago sativa), sorghum, sunflower, wheat and sesame, leading to the huge losses of grains. The adults infest sorghum during

the milky-maturity stage of the plant. The bug feed on the developing grains, which become atrophied. The adults shelter during the dry season in clusters for about 9 months on the stems and branches of trees and bushes (Mariod 2011, 2020b).

The consumption of sorghum bug is common in some areas where the adult insects are usually collected and eaten after frying. The oil can also be extracted from the sorghum bug and used for cooking and medicinal purposes (Mariod 2011, 2020b). To improve the consumer acceptance level of sorghum bug, there was also research study that incorporated the sorghum-insect meal into biscuit, making it into a value-added baked product (Awobusuyi et al. 2020). To our surprise, biscuits supplemented with sorghum insect-based ingredients were more acceptable to panellists than the control. Besides, the tar obtained from highly heated bugs can also be used to treat camels with dermatological infections, indicating its potential antibacterial activity (Mariod et al. 2006a).

Sorghum bug has moderate amount of protein but high fat content which contributes to 28% and 57%, respectively of the total dry mass (Mariod et al. 2011c). The total essential amino acids in sorghum bug were found to be relatively low (119.3 mg/g protein) which is almost 60% below the recommended dietary intake (WHO 2007). Besides, most of the essential amino acids have a low amino acid score except histidine that is barely close to the recommended value. These characteristics obviously indicate the poor quality of protein extracted from sorghum bug. However, the situation can be improved by consuming sorghum bug meal together with other higher quality protein meal to compensate for the low essential amino acid content. Additionally, a nutritive protein component, gelatine, was detected and could be extracted from the sorghum bug at a reasonable amount (~3%) (Mariod et al. 2011d; Mariod and Fadul 2015). Mariod and Fadul (2015) later reported that the functional properties of sorghum bug-based gelatin are comparable to that of the commercial gelatin. The ice-cream developed using melon bug and sorghum bug-based gelatin has comparable sensory characteristics with regard to their taste and texture when compared with ice-cream prepared using commercial gelatin (Mariod and Fadul 2015). The major mineral content in Sorghum bug includes magnesium (309 mg/100 g), calcium (760 mg/100 g), potassium (413 mg/100 g), phosphorus (923 mg/100 g) and sodium (304 mg/100 g) (Mariod et al. 2011c; Rumpold and Schlüter 2013).

2.7.1 Fatty Acid Composition of Sorghum Bug Oil

The sorghum bug oil is yellowish in colour. Sorghum bug has high fat content (~60%) as compared to other insects including mealworm, black soldier fly and silkworm abovementioned. The sorghum bug oil is composed of 40.9% oleic acid, 34.5% linoleic acid and 12.1% palmitic acid as the major fatty acid groups. The fatty acid profile of sorghum bug oil is almost similar to that of the sesame oil (oleic acid – 41%, linoleic acid – 41%, palmitic acid – 10%) (Frančáková et al. 2015) and rice bran (oleic acid – 43%, linoleic acid – 33%, palmitic acid – 20%) (Frančáková et al.

2015; Orsavova et al. 2015). The USFA contents of these edible oils are as high as 80%. This is an important characteristic as studies comparing the disease rates in different countries have suggested an inverse association between USFA intake and the occurrence of cardiovascular disease (Lunn and Theobald 2006). The beneficial health impact of sorghum bug oil is limited and scarce to date. However, its potential health effect is hypothesised to represent the health benefits exerted by rice bran oil owing to the similarity in fatty acid profile. Previous literatures indicated that the rice bran oil reduces the LDL-cholesterol while improves the HDL-cholesterol (Chanita et al. 2020; Zavoshy et al. 2012). The PUFA-to-SFA ratio is an important indicator of lipid composition in a healthy diet. A diet with PUFA-to-SFA ratio equals to 1 is normally recommended. A diet with high PUFA-to-SFA ratio (> 3) would promote tumour whereas a low PUFA-to-SFA ratio (< 0.33) in the diet would induce atherosclerosis (Liao et al. 2010; Paul et al. 2017). The sorghum bug oil has a PUFA-to-SFA ratio of 1.8, implying its excellent nutritional value (Table 2.13).

2.7.2 Physicochemical Properties of Sorghum Bug Oil

Sorghum bug oil contains approximately 340 mg/kg of tocopherol compounds in which γ-tocopherol contributes the highest proportion of tocopherol groups, followed by α-tocopherol (Mariod et al. 2004). Conventional hexane extraction method is normally employed to extract the fats or oils from various sources. Previous study reported that a relatively low concentration of α-tocopherol content (9 mg/kg oil) was obtained using the conventional method. α-tocopherol is an important compound that can act as antioxidant or radical scavenger, slowing down the oxidation mechanism (Gizachew 2020; Kamal-Eldin 2006; Yamauchi 1997). Mariod et al. (2010) later reported that the use of supercritical fluid (CO_2) extraction approach could enhance the α-tocopherol content, carotenoid and chlorophyll pigments in sorghum bug oil as compared to conventional solvent extraction method. A significant increase in the amount of α-tocopherol from 9 mg/kg to 40 mg/kg was observed using the supercritical fluid extraction method under the operating conditions of 60 °C and 300 bar. Besides, a much higher DPPH radical-scavenging activity in sorghum bug oil was observed (Mariod et al. 2010).

Sorghum bug oil contains a moderate amount of total phenolic compounds (9.5 mg GAE/kg), comparable to rice bran oil (8.9 mg GAE/kg), mustard oil (5.6 mg GAE/kg) and sunflower oil (4.9 mg GAE/kg) (Janu et al. 2014). A qualitative analysis of the phenolic compounds in sorghum bug oil reported vanillic acid, callistephin, sinapic acid, t-cinnamic, epicatechin and luteolin to be the phenolic species present in the oil (Matthäus et al. 2015). In addition, sorghum bug oil shows a substantial amount of sterol compounds (4499 mg/kg oil) with β-sitosterol being the most significant followed by stigmasterol. These two sterol compounds account for 65% of the total sterol components (Alnadif et al. 2007). Due to its high concentration of tocopherols and sterols compounds, sorghum bug oil should therefore exhibit high storage and frying stability. However, a contrary result was

Table 2.13 Fatty acid composition of sorghum bug oi

Characteristics	Range
Oil yield (% DW)	56.5–60.0
Fatty acid composition, %	
C-8:0 Caprylic	n.d.
C-10:0 Capric	n.d.
C-12:0 Lauric	0.1
C-14:0 Myristic	0.2
C-16:0 Palmitic	11.3–12.6
C-16:1 Palmitoleic	0.6–1.4
C-17:0 Heptadecanoic	0.1–0.2
C-18:0 Stearic	5.3–9.3
C-18:1 Oleic	40.4–41.4
C-18:2 Linoleic	34.0–35.4
C-18:3 Linolenic	0.9–1.4
C-20:0 Arachidic	0.6–0.8
C-20:1 Gadoleic	0.1–0.2
C-20:5 Eicosapentaenoic	n.d.
C-20:4 Arachidonic	n.d.
C-22:4 Docosatetraenoic	n.d.
Total SFA	20.0–21.0
Total USFA	78.9–80.2
Total ω-3	0.9–1.4
Total ω-6	34.0–35.4
Total MUFA	42.7–43.4
Total PUFA	36.2–36.8
Ratio ω-6 / ω-3	24.3–39.3

Data are reported based on the previous studies; n.d. = not detected. Adapted from Alnadif et al. (2007), Mariod, (2011), Mariod et al. (2004), Mariod et al. (2006a, 2006b, 2008), Mariod (2013, 2020b); Mariod et al. (2011c); Mariod et al. (2010); Matthäus et al. (2015)

observed when sorghum bug oil was used for deep-frying at 175 °C. The oil became unsuitable for human consumption after 6 to 12 h, considering its low sensory scores, drastic increase in the amount of polar compounds, oligomer TAG and free fatty acids (Mariod et al. 2006b). The result could be explained by the presence of phospholipid, trace metal and other factors that might play a role in reducing the oxidative stability of sorghum bug oil. The typical characteristics and physicochemical properties for sorghum bug oil are presented in Table 2.14.

Table 2.14 Physicochemical properties of sorghum bug oil

Characteristics	Range
Oxidative stability index (120 °C)	4.7–5.5 h
Kinematic viscosity (40 °C) (mm^2/s)	26.9–27.1
Tocols compounds (mg/kg oil)	
α-Tocopherol	6.8–10.8
α-Tocotrienol	n.d.
β-Tocopherol	n.d.
β-Tocotrienol	n.d.
γ-Tocopherol	317.6–327.6
σ-Tocopherol	4.8–10.3
Plasto-chromanol-8	1.0–3.0
Sterol compounds (mg/kg oil)	
Cholesterol	22.0
Campesterol	116–958
Stigmasterol	218–254
β-Sitosterol	2323–2688
Δ5-avenasterol	132–163
Δ7-stigmastenol	23–28
Δ7-avenasterol	16
Others$^+$	1212
Total polyphenol (mg GAE/g oil)	9.4–9.6

Data are reported based on the previous studies; n.d. = not detected, + Others include 24-methylcholesterol, campestanol, chlerosterol, sitostanol and 5,24-stigmastadienol. Adapted from Alnadif et al. (2007); Mariod (2011); Mariod et al. (2004); Mariod et al. (2006a, 2006b, 2008); Mariod (2013, 2020b), Mariod et al. (2011c), Mariod et al. (2010), Matthäus et al. (2015)

2.8 Dubia Cockroach Oil

Dubia cockroach (*Blaptica dubia*) is also known as South American Dubia cockroach and Orange-spotted cockroach. The insects belong to the order of Blattodea and family of Blaberidae. Other cockroach species that receives much research attention includes American cockroach (*Periplaneta americana*) and Australian cockroach (*Periplaneta australasiae*). Dubia cockroach is large in size and can grow up to 4.0–4.5 cm. They are sexually dimorphic blaberid cockroach. The wings are fully developed in the male adults but they rarely fly. The insects are not able to climb on smooth surfaces owing to the poor development of arolium between their claws in both nymph and adult stages. Dubia cockroach is an ovoviviparous species and gives birth to live young (Wu 2013; Wu et al. 2013). This reproduction system is typical of the family Blaberidae of Blattodea (Roth 2003). The insects are normally used as animal feed for the reptiles and amphibians. Because of its low nurturing technique required, inexpensive and low maintenance cost with minimum smell as compared to other cockroach species, Dubia cockroach farming has become popular in some areas. Besides, this insect is considered as

organic waste decomposer that is capable of converting the rotten fruits, vegetables and grains into its own body biomass, thereby making the insect a natural way of eliminating the global food waste burden (van Huis et al. 2013).

Dubia cockroach consists of higher protein content (~50%) as compared to other insect sources such as melon bug (27%), sorghum bug (28%), termites (35%), grasshopper (37%) and others (Rumpold and Schlüter 2013; Tang et al. 2019; Yee et al. 2018). Its protein composition may even be superior than some of the conventional meat products including lean red meat sources of beef (23.2%), veal (24.8%) and mutton (21.5%) (Kinyuru et al. 2009). Dubia cockroach also shows high protein digestibility (> 80%), an important marker to determine the overall digestibility such as the utilisation and absorption rate of protein in the human body. Most importantly, Dubia cockroach protein can provide 361 mg/g essential amino acids which far exceed the recommended dietary intake of 277 mg/g protein. Besides, all essential amino acids are present in a quantity that is necessary for humans. Amino acids phenylalanine + tyrosine are found to be two times higher than the amount suggested by WHO. These two essential amino acids play a critical role for the production of dopamine, a hormone and neurotransmitter in the human body.

Besides, the dubia cockroach also consists significant amount of fat ranging from 32–43% DW. An analysis on the mineral content of Dubia cockroach also indicates that the cockroach is a poor source of calcium (~30 mg/100 g DW), similar to other insects. Relatively low vitamin A and E content in cockroach is also reported (Oonincx and Dierenfeld 2012). The Dubia cockroach contains around 5% chitin, providing a cheap alternative source for extraction. Also, the food safety evaluation has confirmed that cockroach is not poisonous to animals and human (Yee et al. 2018). The dubia cockroach can therefore act as healthy food and feed materials due to its high nutritional values.

2.8.1 Fatty Acid Composition of Dubia Cockroach Oil

Previous literatures show that approximately 40% of the fat can be extracted from Dubia cockroach which is comparable to that of mealworm and melon bug insects. Table 2.15 presents the fatty acid composition determined in Dubia cockroach oil. The most abundant USFA in dubia cockroach oil is oleic acid (~50%) while the most abundant SFA is palmitic acid (~19%). The MUFA remains to be a major fatty acid group in Dubia cockroach oil. This MUFA group is linked to the reduced risk of cardiovascular disease and diabetes (Schwingshackl and Hoffmann 2012). Schwingshackl and Hoffmann (2012) reviewed the beneficial health effects of MUFAs and they pointed out that MUFA-enriched diet resulted in an increase of HDL-cholesterol and a corresponding decrease in serum TAG. In addition, MUFA-enriched diet was also found to exert a hypoglycemic effect and reduce glycosylated hemoglobin in type-2 diabetes subject. Nevertheless, the beneficial health effect of MUFA is not conclusive.

Table 2.15 Fatty acid composition of Dubia cockroach oil

Characteristics	Range
Oil yield (% DW)	31.8–43.3
Fatty acid composition, %	
C-8:0 Caprylic	n.d.
C-10:0 Capric	n.d.
C-12:0 Lauric	0.2
C-14:0 Myristic	1.1–1.2
C-16:0 Palmitic	18.1–20.2
C-16:1 Palmitoleic	5.0–5.5
C-17:0 Heptadecanoic	0.1–0.2
C-18:0 Stearic	3.7–4.3
C-18:1 Oleic	49.5–51.5
C-18:2 Linoleic	15.7–17.6
C-18:3 Linolenic	1.1–1.3
C-20:0 Arachidic	0.1
C-20:1 Gadoleic	0.1
C-20:5 Eicosapentaenoic	n.d.
C-20:4 Arachidonic	n.d.
C-22:4 Docosatetraenoic	n.d.
Total SFA	23.3–25.8
Total USFA	74.1–76.3
Total ω-3	1.1–1.3
Total ω-6	15.7–17.6
Total MUFA	56.1–57.6
Total PUFA	16.9–19.1
Ratio ω-6 / ω-3	13.8–15.0

Data are reported based on the previous studies; n.d. = not detected. Adapted from Tzompa-Sosa et al. (2019), Tzompa-Sosa et al. (2014); Yee et al. (2018), Yi et al. (2013)

Previous study conducted by Tzompa-Sosa et al. (2014) indicated that the fatty acid profile of Dubia cockroach oil could be modified *via* different extraction methods. The authors demonstrated that a higher ω-6/ω-3 fatty acid ratio was obtained using the Folch method whereas the aqueous extraction approach tends to increase the amount of ω-3 fatty acid (lower ω-6/ω-3 fatty acid ratio) in Dubia cockroach oil.

2.8.2 *Physicochemical Properties of Dubia Cockroach Oil*

Table 2.16 illustrates the physicochemical properties of Dubia cockroach oil. It could be seen that the physicochemical properties of cockroach oil particularly in the compositions of tocopherol and sterol compounds are still limited to date.

Table 2.16 Physicochemical properties of Dubia cockroach oil

Characteristics	Range
Cholesterol, %	0.2–0.9
Colour	L (lightness): 45.9 a (redness): 6.9 b (yellowness): 67.5

Data are reported based on the previous studies; n.d. = not detected. Adapted from Tzompa-Sosa et al. (2019), Tzompa-Sosa et al. (2014), Yee et al. (2018)

Previous study showed that the lipid extract from Dubia cockroach has glycerides highly concentrated in ECN 50–54 coupled with low concentration of glycerides that fall within ECN 36–38. The ECN pattern differs from animal fats with ECN 48–50 and vegetable oil with ECN 44–48. The result suggested that the crude fat extract has a significant amount of phospholipids and DAGs with two fatty acid chains (Tzompa-Sosa et al. 2014). In the subsequent study, Tzompa-Sosa et al. (2019) examined the crystallisation and melting profile of Dubia cockroach oil. They found that Dubia cockroach oil showed three clear separated peaks at −11.2 °C, −34 °C and − 53.7 °C with crystallisation onset at around -10 °C in their thermogram during crystallisation. The relatively low crystallisation temperature of Dubia cockroach oil could have been related to its high concentration of triunsaturated (UUU) or diunsaturated (SUU, USU or UUS) TAGs. On the other hand, the melting profile of Dubia cockroach oil has melting temperatures ranged from −25.2 °C to 6.6 °C with one main melting peak at −2.8 °C and three shoulder peaks overlapping with the main peak. Overlapping of peaks is due to the re-crystallisation of TAGs. Re-crystallisation is a process in which the crystallised TAGs transform to a more stable or become part of another crystal with a higher melting temperature (ten Grotenhuis et al. 1999).

The supercooling (temperature difference between the first crystallisation peak and the last melting peak) was large (17.8 °C) for Dubia cockroach oil. This difference is explained by crystallisation kinetics since fats need to be undercooled by at least 5 – 10 °C to form crystal nuclei, which will further grow and form crystals (Marangoni and Wesdorp 2012). The thermograms showed large separation among crystallisation and melting peaks in cockroach oil (Tzompa-Sosa et al. 2019). Therefore, fractionation of oil can be performed to widen its range of applications and increase the value of its fractions.

2.9 Locust and Grasshopper Oil

With regard to locusts, desert locust (*Schistocerca gregaria*) and migratory locust (*Locusta migratoria*) are the two main locust species that receive much of the research attention. They belong to the order of Orthoptera and family of Acrididae. The presence of desert locust can be detected in Africa, the Middle East and Asia decades ago while the migratory locust is the most widespread locust species which

can be found throughout the world. Various subspecies of migratory locust have been identified such as *Locusta migratoria manilensis*, *Locusta migratoria capito*, *Locusta migratoria tibetensis* Chen etc. because they occupy a huge geographical area with different ecological zones (Mariod et al. 2017). The locust has a high reproduction capacity where one female can lay up to 140 eggs per deposition. The eggs would hatch after 10 days under optimal rearing conditions. The high fecundity coupled with high acceptability by animals are the main reasons this species has gained considerable attention and locust-farming is wide practised globally.

Locust is always mixed up with grasshopper as the two insects are the same in appearance. Locusts can appear in two different phenotypic forms namely solitary and gregarious phases, whereas most grasshoppers do not show this kind of changes. At low population density, locusts behave as individuals and avoid each other, much like grasshoppers. When the population density reaches a critical level, the individuals would undergo physiological and behavioural changes, known as phase polyphenism. They aggregate and enter a gregarious phase, resulting in bands of nymph and migratory swarms of adults. In addition to behavioural changes, changes in body shape and colour, metabolism, development and morphology are also observed during the gregarious phase (Chen et al. 2011). Hence, the increasing scale of population and the migratory behaviour distinguish locust species from grasshoppers.

The locust swarm is one the most devastating threats to agricultural crops such as maize, sorghum, barley, rice and etc. A locust adult can consume almost its own weight every day, targeting food crops and forage (FAO 2020). Locust treatment can be very challenging due to their flying ability. Besides, the locusts have very high mobility and they can travel up to 150 km per day, enhanced by the direction of wind. A locust swarm contains almost 10 billion insects, weighing up to 30,000 tonnes, making it a considerable source of biomass. In Africa, the desert locust, the migratory locust, the red locust and the brown locust are commonly eaten (Kinyuru 2020a; Kinyuru and Ndung'u 2020; van Huis et al. 2013).

In recent years, the chemical composition of locusts has received attentions as the locusts are potentially rich in protein, fibre and essential fatty acids. Proximate analysis of locust meal revealed an average protein of 55%, average crude fat of 29%, while carbohydrates shows an average value of 12% based on the previous literatures (Mariod 2020a; Mariod et al. 2017; Oonincx and van der Poel 2011; Rumpold and Schlüter 2013). The amount of protein in locust is far higher than the plant-based protein including beans (23.5%), lentils (26.7%) or soybean (41.1%) (Blásquez et al. 2012). However, the protein content can vary greatly within the range of 7.5% to 91% DW (Bukkens and Paoletti 2005; Oonincx and van der Poel 2011; Rumpold and Schlüter 2013). These large differences are attributed to the variation in feeding diet, development stage, geographical region as well as gender (Mariod 2020a). For instance, commercially reared desert locusts have high protein content with significant levels of essential amino acids and fatty acids (Zielińska et al. 2015). Therefore, the diet can be altered or adjusted to produce locust meals that meet the nutritional requirement for adults. Depending on the species, locust protein can potentially provide almost all the essential amino acids and leucine is the

most abundant essential amino acid detected (Rumpold and Schlüter 2013; Zielińska et al. 2015). Leucine is essential for regulating blood-sugar level, tissue regeneration and metabolism (Pedroso et al. 2015). Previous studies also showed that insect locust is a good source of iron, copper, manganese and zinc (Kinyuru 2020b; Mohamed 2015; Oonincx and van der Poel 2011; Rumpold and Schlüter 2013; Zielińska et al. 2015). In addition, the locust insect is also considered to be an energy-dense diet with an average energy of 450 kcal/100 g (Kinyuru 2020b; Ochiai et al. 2020). Most importantly, acute and sub-chronic analysis of locust powder indicates no sign of toxicity in rats (Ochiai et al. 2020).

On the other hand, grasshopper is a non-destructive insect and it does not damage the crops and vegetation. The grasshopper can be grouped under three families (Tettigoniidae, Acrididae and Pyrgomorphidae). Most of the research studies focus on longhorn grasshopper (*Ruspolia differens*) and cone-headed grasshopper (*Ruspolia nitidula*) as they are commonly eaten by the Africans. They are categorised under the family Tettigoniidae of the order Orthoptera. The insect has high cultural and economic value in Africa where they are normally harvested from the wild during the two annual swarming seasons (November – December and March – May) (Sorjonen et al. 2020; van Huis et al. 2013). Recently, mass rearing programs are started and developed for local communities.

Grasshopper is very nutritious with 36–40% protein, 41–49% fat, 10–13% dietary fibre, 900–2300 μg/100 g carotenoids and 4–6 mg/kg potassium and phosphorus as reported by Ssepuuya et al. (2016). The total essential amino acid in grasshopper is determined to be 259 g/100 g protein which is slightly lower than the recommended value suggested by WHO. Lysine (57.4 mg/100 g) is the major essential amino acid detected, followed by histidine (44.1 mg/100 g) and threonine (28.6 mg/100 g) (Siulapwa et al. 2014). Despite the high amount of protein content recorded for grasshopper, information on the protein digestibility remains unknown. These markers can indicate the quality of dietary protein and more research works are therefore required. It is worth mentioning that the grasshopper is an excellent source of dietary fibre. Its fibre content is much higher than those plant-based food such as peas (4.7%), sesame (7.9%), mango (2.4%), guava (3.7%) and avocado (3.4%) (Ssepuuya et al. 2016). Grasshopper also shows remarkable carotenoids content which is not common in conventional meat products. However, the amount of carotenoid in grasshopper is linked to the season of harvesting where higher amount of carotenoid was observed during the spring season. Grasshopper also contains reasonable amount of mineral content in which calcium remains to be the limiting mineral, similar to the other insect sources (Kinyuru et al. 2009; Siulapwa et al. 2014). Consumption of 100 g of grasshopper meal could contribute 2.5% calcium, 14% magnesium, 10% potassium, 21% sodium, 67% iron, 16% phosphorus, 114% zinc based on recommended nutrients intake (CODEX 1991).

Table 2.17 Fatty acid composition of locust oil

Characteristics	Range
Oil yield (% DW)	18.9–38.3
Fatty acid composition, %	
C-6:0 Caproic	0.4
C-8:0 Caprylic	n.d.
C-10:0 Capric	0.1
C-12:0 Lauric	0.1–1.0
C-14:0 Myristic	1.6–3.6
C-16:0 Palmitic	22.6–37.7
C-16:1 Palmitoleic	1.0–1.9
C-17:0 Heptadecanoic	0.2–0.6
C-18:0 Stearic	2.4–10.0
C-18:1 Oleic	30.4–38.0
C-18:2 Linoleic	8.4–24.9
C-18:3 Linolenic	3.0–18.3
C-20:0 Arachidic	0.4–0.5
C-20:0 Behenic	0.1
C-20:3 Eicosatrienoic	0.3
C-20:4 Arachidonic	n.d.
C-22:6 Docosahexaenoic	0.1
Total SFA	33.6–41.0
Total USFA	59.0–66.5
Total ω-3	11.3–12.1
Total ω-6	5.6–14.6
Total MUFA	31.7–41.2
Total PUFA	17.8–34.8
Ratio ω-6 / ω-3	0.6–2.4

Data are reported based on the previous studies; n.d. = not detected. Adapted from Clarkson et al. (2018), Kinyuru (2020b), Mohamed (2015), Osimani et al. (2017), Ramos-Bueno et al. (2016), Zielińska et al. (2015)

2.9.1 Fatty Acid Composition of Locust Oil

Based on the data summarised in Table 2.17, the locust contains a significant amount of fat content (~30%) which is higher than the average of orthopteran species (~14%) (Rumpold and Schlüter 2013). Previous study indicated that percentage of lipid extract from locust depends on (1) the feeding diet in which Oonincx and van der Poel (2011) showed higher fat composition for locust fed with diet enriched with wheat bran, an increase from 18.2% to 23.1% was observed as compared to control; (2) geographical location in which Osimani et al. (2017) reported a much lower amount of fat in migratory locust sourced from Netherlands, and other factors as mentioned earlier.

A detailed analysis on the fatty acid composition of locust oil revealed that the insect is rich in USFA with oleic acid (34%), linoleic acid (17%) and linolenic acid (11%) being the dominant fatty acid species. Similar findings were also reported by Mohamed (2015) and Ramos-Bueno et al. (2016). However, Osimani et al. (2017) reported a much lower amount of USFA, particularly linolenic acid, in locust obtained from the Netherlands, indicating the strong correlation between the nutritional profile of the locust and its geographical origin. The locust oil can be regarded as a healthy lipid owing to its high linolenic acid (ω-3) content and ideal ω-6/ω-3 ratio (~1.5) which is much lower than the maximum threshold level of 10:1 (FAO 2010; Simopoulos 2002). Furthermore, the PUFA-to-SFA ratio of locust oil is found to be 0.7, which is close to the ideal value of 1. All these characteristics clearly indicate that locust is a potential healthy oil source.

2.9.2 Physicochemical Properties of Locust Oil

Table 2.18 shows the physicochemical properties and the bioactive compounds of locust oil. Locust oil has a high degree of USFA as indicated by the high iodine value (IV) of 75, which is comparable to some conventional vegetable oils such as olive oil (IV 80) but lower than some insect-based oils including silkworm pupal oil (IV 122) and cricket oil (IV 99). The locust meal itself is high in α-tocopherol (267.5 μg/g DW), therefore it can be assumed that the locust oil may contain high proportion of α-tocopherol, taking into account that all lipid-soluble tocols compounds are extracted together with the insect lipid. Retinol, lutein, β-carotene are present in high concentrations in adult locusts (0.2 mg/kg DW, 2.0 mg/kg DW and 5 mg/kg DW, respectively) that fed with wheat bran and carrots as part of the diet, thereby translating into possible higher amount of these nutrients in locust oil even though it is not reported elsewhere (Oonincx and van der Poel 2011).

With regards to sterols composition, previous study indicated that most insects including locust, are not able to synthesise sterols *de novo* from the isoprenoid precursors (Blásquez et al. 2012; Jing et al. 2013). Therefore, the insects can only acquire these nutrients from the dietary sources. Phytosterols play an important role in our human body by exerting cardiovascular protective effects, modulating endothelial function and acting as potent antioxidants (Patel and Thompson 2006; Yoshida and Niki 2003). For example, 7-dehydrocholesterol is the main precursor of vitamin D3, a key modulator of keratinocyte differentiation and proliferation in the skin (Glossmann 2010). Besides, fucosterol is another sterol compound that exhibits antioxidant, anti-inflammatory and antidiabetic properties (Abdul et al. 2016).

The presence of phytosterols in vegetable oils is common but some insect-based oil also showed a substantial amount of sterol compounds including mealworm oil, black soldier fly oil and others as discussed earlier. Dessert locust fed *ad libitum* on wheat seedlings also contains high sterol content as reported by Cheseto et al. (2015). Surprisingly, the authors detected five unique sterols compounds namely

Table 2.18 Physicochemical properties of locust oil

Characteristics	Range
Specific gravity (4 °C)	0.93–0.94
Iodine value (g I_2/100 g oil)	74.3–75.7
Saponification number (mg KOH/g oil)	170–172
Refractive index (25 °C)	1.44–1.48
Tocols compounds (mg/kg oil) *	
α-Tocopherol	827
Carotenoid compounds (mg/kg oil) *	
Retinol	0.2–1.7
Lutein	1.3–3.0
Zeaxanthin	0.1–0.3
β-Cryptoxanthin	0.2–0.5
α-Carotene	0.1
Cis-β-carotene	1.0–2.3
Trans-β-carotene	4.2–9.0
Lycopene	n.d.
Sterol compounds (ng/g oil) +	
Cholesterol	1880.5
7-Dehydrocholesterol	921.4
Lathosterol	n.d.
Desmosterol	232.3
Campesterol	n.d.
Stigmasterol	n.d.
(3β,5α) Cholesta-,14,24-trien-3-ol,4,4-dimethyl	102.9
Cholesterol, 7-oxo	1251.9
(3β,20R) Cholesta-5,24-dien-3,20-ol	59.0
β-Sitosterol	436.6
Fucosterol	235.2

Data are reported based on the previous studies; n.d. = not detected, *Tocopherol and Carotenoid profile is calculated based on the assumption that tocopherol and carotenoid compounds are concentrated in the lipid frac-tion of locust after extraction. + Data are reported based on the fat body of locust. Adapted from Cheseto et al. (2015), Kinyuru (2020b), Oonincx and van der Poel (2011)

7-dehydrocholesterol, desmosterol, fucosterol, (3β,5α) cholesta-,14,24-trien-3-ol,4,4-dimethyl and (3β,20R) cholesta-5,24-dien-3,20-ol which are not found in the feeding diet. This implies that the desert locust could amplify the phytosterols retrieved from the feeding diet and metabolises them into different derivatives with potential health benefits.

Table 2.19 Fatty acid composition of grasshopper oil

Characteristics	Range
Oil yield (% DW)	41.0–49.1
Fatty acid composition, %	
C-8:0 Caprylic	n.d.
C-10:0 Capric	0.1–0.5
C-12:0 Lauric	0.2
C-14:0 Myristic	0.3–1.5
C-16:0 Palmitic	27.2–32.3
C-16:1 Palmitoleic	1.3–3.0
C-17:0 Heptadecanoic	0.1–0.2
C-18:0 Stearic	5.3–8.7
C-18:1 Oleic	23.1–44.4
C-18:2 Linoleic	13.8–31.5
C-18:3 Linolenic	1.4–4.7
C-20:0 Arachidic	n.d.
C-20:0 Behenic	n.d.
C-20:3 Eicosatrienoic	n.d.
C-20:4 Arachidonic	0.6–0.7
C-20:5 Eicosapentaenoic	0.1–0.3
Total SFA	37.9–39.3
Total USFA	59.6–62.3
Total ω-3	3.0–4.7
Total ω-6	29.3–31.5
Total MUFA	23.1–46.1
Total PUFA	16.2–34.6
Ratio ω-6 / ω-3	8.8–9.1

Data are reported based on the previous studies; n.d. = not detected. Adapted from Fombong et al. (2017), Kinyuru et al. (2009), Opio (2015), Rutaro et al. (2018a), Rutaro et al. (2018b), Rutaro et al. (2018c), Siulapwa et al. (2014), Ssepuuya et al. (2016)

2.9.3 Fatty Acid Composition of Grasshopper Oil

The grasshopper can provide almost 45% DW of the fat content (Table 2.19). The grasshopper oil is predominated by palmitic acid (30%), followed by linoleic acid (30%) and oleic acid (25%). The amount of USFA and SFA in grasshopper oil are found to be 60% and 40%, respectively. A high USFA content is desired as it reduces the risk of cardiovascular disease (Lunn and Theobald 2006). A close observation on the fatty acid profile of the grasshopper can notice the high similarity in the fatty acid composition between the grasshopper oil and locust oil. However, a detailed examination on the physiochemical properties especially melting and crystallisation behaviour and the TAG composition, is required to show their differences.

A great variation in the fatty acid content was detected in the previous literatures because there is a close connection between the fatty acid profile of the insect and the diet (Lehtovaara et al. 2017; Rutaro et al. 2018a,b,c). Therefore, numerous studies have explored the possibility of manipulating the fatty acid profile of the grasshopper lipid extract to achieve desired nutrient requirement. Lehtovaara et al. (2017) reported that diet-enriched with USFAs (linoleic, linolenic, eicosapentaenoic or docosahexaenoic acid) could increase these fatty acid contents in the grasshopper. Interestingly, the author observed a drastic increase of linolenic acid in the grasshopper oil from ~7% to ~42%, an increase of almost 600% when the grasshopper was treated with linolenic-enriched diet as compared to mixed diet. However, there is an insignificant change in PUFAs with higher homologues even though the grasshopper oil is high in linoleic and linolenic acid, suggesting that the grasshopper is lack of desaturase and elongase enzyme to modify these fatty acids. Nevertheless, diet high in lipid creates stresses on the development of grasshopper and leads to slower biomass gain with low final weight (Lehtovaara et al. 2017). Another study also supported that the feeding diet could potentially affect the final fatty acid composition of the grasshopper lipid (Rutaro et al. 2018b). The authors reported that a mixed diet (mixtures of two and above natural plant inflorescences) reduced the proportion of essential fatty acids (PUFAs) in the grasshopper oil. Similar result was reported by Rutaro et al. (2018a).

2.10 Conclusion

In conclusion, the rapid growing population has been coupled with the growing needs of nutrients globally. The unparalleled growing pace between the population and the food supply has pushed forward food diversification to prevent nutritional crisis. Therefore, entomophagy is the key solution to ensure food security. From a nutritional point of view, insect-based food can provide high amount of proteins, fats, vitamins and minerals that meet the recommended dietary allowance. Besides, insect-farming has great environmental and economic advantages due to lower emission of greenhouse gases, less space and water needed, low capital investment as well as high return on investment. Thus, edible insects can act as potential sustainable food alternatives. The present chapter reviews and provides a complete analysis of the lipid content obtained from various edible insects. Obviously, each insect lipid has its own unique fatty acid profile and physicochemical properties, for instance, mealworm oil is high in USFAs, black soldier fly larvae oil is high in SFAs particularly in lauric acid, silkworm pupal is rich in essential ω-3 fatty acid, linolenic acid, melon bug oil is predominated by oleic acid and etc. Hence, these insect oils can be applied in various food applications, depending on their characteristics. However, more research works are required before it becomes feasible. Furthermore, insect oil is rich source of PUFAs, tocopherols, carotenoids and sterols compounds, implying the potential health benefits of insect oil for tackling certain coronary heart diseases, diabetes, and other health problems.

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Chapter 3
Seed Oil: Sources, Properties and Recovery

Chee Chin Chu and Kar Lin Nyam

Abstract Seed oils are excellent and valuable lipid sources attributed to their health-promoting fatty acid contents and lipid-soluble phytochemicals. The nutritional benefits and functional properties of the fatty acids and bioactive components (tocopherols, phytosterols and phenolic compounds) from the seed oils are well documented and explored. Previous literatures indicated that the extraction methods such as the conventional and innovative approaches as well as the processing techniques including degumming, neutralization, bleaching, dewaxing and deodorization could affect the macro- and micro-constituents in the seed oils. In addition, different post-processing treatments (heating and storage) of seed oils may alert or degrade important bioactive constituents. This chapter aims to describe the sources, properties and recovery of seed oils mainly focusing on their chemical composition, physicochemical characteristics, nutritional quality and health-promoting properties. Seed oils provide many benefits to health such as the improvement of general health being, prevention of hyperlipidemia, hypercholesterolemia, Type-2 diabetes, inflammatory properties and cancers. The major and minor components in the oils have made them as highly healthful food and novel therapeutical source for food applications.

Keywords Seed oil · Solvent extraction · Mechanical expression · Supercritical fluid extraction · Microwave-assisted extraction · Ultrasonic-assisted extraction · Refining

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3.1 Introduction

The United Nations projected that the world population is expected to exceed eight billion by 2030 (United Nations 2019). In 2040, this number will grow to more than nine billion. In 2055, the number will rise to over 10 billion from the current 7.7 billion in 2019. The growing of world population then leads to the increase in food demand. In the future perspective, oil crops consumption is expected to rise more rapidly than that of cereals (FAO 2015). According to the Food and Agricultural Organization (FAO) of the United Nations, vegetable oil showed the highest trade share with 40% of the production of all agricultural commodities (FAO 2016). Most of the vegetable oil is extracted from seeds. Seed oil is a vegetable oil that obtained from plant seeds since antiquity. It is widely used in cooking and as functional food. The oilcrops vary from numerous species of plants and the seed oil produced not only can be used as functional oil but also as raw materials for oleochemical industries. Vegetable oil not only can be applied in dietary consumption but also could serve as renewable alternatives to petroleum-based chemicals and biodiesel. Therefore, there is high demand in vegetable oil due to their numerous applications, increasing in population size, improving living standards and changing in dietary habits. From Gunstone et al. (2007), the application of vegetable oil has recorded a steady growth rate of approximately 5% per annum for the past few decades and the consumption is forecasted to double over the next 15 years (Li et al. 2017).

3.2 Sources

From the market assessment published by FAO (2016), the world production of major oilcrops consisted of oil palm, soybean, rapeseed, cottonseed, groundnut, sunflower seed, palm kernel and coconuts (Table 3.1). Four oilcrops (oil palm, soybean, rapeseed and sunflower seed) account for 83% of the world production (Alexandratos and Bruinsma 2012). Indonesia and Malaysia are the world's two major exporters for palm oil. Both countries will continue to dominate vegetable oil

Table 3.1 World production of major vegetable oils in 2018/2019

Oil	Amount (in million metric tons)
Palm	73.49
Soybean	56.97
Rapeseed	27.96
Sunflower	19.45
Palm kernel	8.57
Groundnut	5.57
Cottonseed	5.20
Coconut	3.63
Olive	3.10

trade and palm kernel oil is produced alongside palm oil. Whereas, soybeans are produced and exported by the United States and Brazil. For rapeseeds, they are produced by China and the European Union together with groundnuts and sunflower seeds. Besides, coconuts are mainly produced in the Philippines, Indonesia and Oceanic islands. The production of palm kernel oil and coconut oil have important industrial uses. However, the dominance has shifted towards palm kernel oil in tandem with the growing production of palm oil. While for the cottonseed oil, it is a by-product derived from cotton and mainly produced in India, the United States, Pakistan and China. In general, vegetable oil production is anticipated to increase globally at a higher rate than most agricultural commodities

Seed oil can be categorised into edible and non-edible seed oils. The existing seed oils that are widely consumed by human are palm oil, sunflower oil, canola oil, coconut oil, soybean oil, grapeseed oil and olive oil. In recent years their consumption has been increasing because of their nutraceutical properties in lowering the bad cholesterols, protecting against cardiovascular diseases and so forth (Cicero et al. 2017). In the United States, soybean oil is the most popular and consumed edible oil followed by canola oil and palm oil. However, the global vegetable oil market is largely dominated by palm oil followed by soybean oil, canola oil and sunflower oil and palm oil market contributes more than one-third of the total vegetable oil consumption (Research and Markets 2019). While for the non-edible seed oil including castor and jatropha oils. They are widely used in cosmetic and pharmaceutical industries

Seed oil is an essential component in the human daily diet and the agricultural industry is seeking ways to maximise plants' yield while reducing environmental effects of crop cultivation, especially land use. The enhancement in the production of seed oil in a sustainable and cost-effective way are crucial to fulfil the market's need for seed oil and high-quality seedcrops. The seedcrops can be cultivated via conventional breeding and genetic engineering. The increased yield in seed oil will benefit the society with adequate edible oil supply meanwhile contribute to the production of biofuel. In recent years, biofuel is being used in various applications, including powering machines to reduce fossil fuel contamination and fuelling long-distant transportation by automobiles, ships and aeroplanes. Seedcrops can be modified by advancement in plant technology and the advent of metabolic engineering. The transgenic seedcrops have novel biosynthetic genes which were taken from non-commercial plants that provide good fatty acids. Most of the genetically modified crops have been engineered for resistance to insects, tolerance to herbicides or both.

3.3 Fatty Acid Composition

Triacylglycerols (TAGs), proteins and carbohydrates represent the major constituents of plant seeds (Li et al. 2017). In terms of composition, TAGs are the major components in vegetable oil account for more than 95% (Liu and Lu 2018). TAGs

are made by esterification of three fatty acids on the glycerol skeleton. Fatty acid is a hydrocarbon molecule with various degrees of unsaturation, terminating with a carboxylic group. The fatty acid was classified according to the number of double bonds as saturated (without double bond), monounsaturated (with one double bond) and polyunsaturated (with two or more double bonds) fatty acids (Orsavova et al. 2015).

Saturated fatty acid (SFA) exhibits a completely saturated hydrocarbon chain, while monounsaturated fatty acid (MUFA) possesses one double bond and polyunsaturated fatty acid (PUFA) has more than one double bonds. Besides, cis or trans fatty acid depends on the structural configuration of the double bond whereas n-3 or n-6 polyunsaturated fatty acid is classified based on the position of the first double bond, counting from the methyl end (Orsavova et al. 2015). Cis-fatty acids are naturally occurring form of fatty acids while trans-fatty acids may be produced by hydrogenation of vegetable oil (Orsavova et al. 2015). Cis-fatty acids play important roles in cell signalling to regulate lipid metabolism and control the synthesis and secretion of inflammatory mediators (Bozza and Viola 2010). Whereas, an excessive intake of trans-fatty acids has been associated with adverse effects, such as the increased risk of coronary heart disease, diabetes mellitus and increased markers for systematic inflammation (Duffy et al. 2006). Unlike animal fats that are made up of mainly SFA, vegetable oils contain mainly of MUFA or PUFA, except coconut (Orsavova et al. 2015) and palm kernel oils that are predominantly made up of SFA.

The physicochemical properties of TAGs are significantly correlated with their constituent fatty acids and the fatty acid composition of oilcrops varies widely (Orsavova et al. 2015). As shown in Table 3.2, the fatty acid composition of some examples of seed oils are compiled from Dubois et al. (2007) and Chew et al. (2018). Besides that, the oil content of seedcrops varies from 10–15% to over 50% while carbohydrates, mainly polysaccharides, range from 15 to 30% in the seeds. Most of the seedcrops showed low protein around 15–25%, except soybean oil. Soybean oil consists of a very high content of protein, at up to 40% and it is a good source of protein (Clemente and Cahoon 2009)

3.4 Physicochemical Properties

The physicochemical parameters of seed oils are very important to characterize the seed oils. Those common parameters include iodine value, saponification value, peroxide value, free fatty acid, acid value, moisture content, refractive index and viscosity. The iodine value determines the degree of unsaturation in oil in which a high iodine number indicates a greater amount of unsaturated double bonds present in the lipid source. On the other hand, the saponification value represents the average molecular weight of the total fatty acid chains present in the oil sample. It is analysed by the amount in milligrams of potassium hydroxide required to saponify 1 g of oil sample. Also, the peroxide value determines the state of oxidation by quantifying the oxidised substances, particularly hydroperoxides that displace iodine from the

Table 3.2 Fatty acid composition of seed oils

	Oil (%)							
Fatty acid	Soybean	Rapeseed	Sunflower seed	Groundnut	Cotton seed	Palm kernel	Coconut	Kenaf seed
C8:0	–	–	–	–	–	4.1	7.6	–
C10:0	–	–	–	–	–	3.7	6.5	–
C12:0	–	–	0.5	–	–	46.0	48.2	–
C14:0	0.1	0.1	0.1	0.1	0.8	17.8	18.5	0.1
C16:0	10.8	5.1	6.4	10.4	24.2	8.4	8.7	19.3
C16:1	0.2	0.2	0.1	0.2	0.7	–	–	0.5
C18:0	3.9	1.7	4.5	3.0	2.3	1.6	2.7	4.3
C18:1	23.9	60.1	22.1	47.9	17.4	16.4	6.0	36.1
C18:2	52.1	21.5	65.6	30.3	53.2	3.1	1.8	36.2
C18:3	7.8	9.9	0.5	0.4	0.2	–	0.1	1.3
C20:0	0.3	0.6	0.3	1.2	0.2	–	0.1	0.7
C22:0	0.2	0.3	0.8	2.3	0.1	–	–	0.3
C24:0	0.3	0.2	0.2	1.4	0.1	–	–	0.2
C24:1	–	0.3	–	–	–	–	–	0.7
SFA	15.6	8.0	12.8	18.3	27.8	81.9	92.6	24.9
MUFA	24.1	60.6	22.3	49.6	18.2	16.4	6.1	37.5
PUFA	59.9	31.4	66.1	30.8	53.4	3.1	1.9	37.5

Source: Compiled from Dubois et al. (2007) and Chew et al. (2018). *SFA* saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid, – no data available

potassium iodide compound. Hydroperoxides are normally produced during oxidation of fatty acids. As vegetable oils contain mainly of the MUFAs and PUFAs and their double bonds are very reactive. The abstraction of a proton from neighbouring un-saturated fatty acids produces lipid hydroperoxides and regeneration of a carbon-centred lipid radicals, thereby propagating the radical reactions. Besides that, the presence of free fatty acids is related to high acid value and the quality of oil. Whereas, refractive index of oil exhibits a positive direct correlation with the degree of unsaturation and the fatty acid chain length. Table 3.3 shows the physicochemical properties of seed oil compiled from Abitogun et al. (2008), Ariharan et al. (2013), Bahadi et al. (2019), Chew et al. (2018), Dilsat et al. (2015, 2019), Long et al. (2008), Nyam et al. (2009) and Rosa et al. (2009)

3.5 Properties of Seed Oils

3.5.1 Antioxidant Activity

Antioxidant compounds are important in reducing cellular oxidative stress. It is closely related to antiaging, anti-inflammation and many diseases and cancers prevention. Antioxidants can scavenge and destroy aggressive oxidising agents and free radicals. For instance, antioxidants like vitamin C, vitamin E, flavonoids, and phenolic acids play an important role in fighting against free radicals and negative skin changes (Korac and Khambholia 2011). A free radical is a molecular species capable of independent existence that contains an unpaired electron in its atomic orbital which is unstable and highly reactive (Lobo et al. 2010). They can either donate an electron to or accept an electron from other molecules likes oxidants or reductants. On the other hand, antioxidants delay or inhibit cellular damage through their free radical scavenging property. As a stable molecule, it can enable the donation of an electron to an unstable free radical and neutralize it, thus reducing the damaging effect of the free radical (Lobo et al. 2010). Common edible oils such as olive and sesame oils have been reported to have antioxidative activities

Human body naturally contains antioxidants such as vitamin E and enzymatic antioxidants like glutathione peroxidase and superoxide dismutase (Vinardell and Mitjans 2015). During aging or exposure to sunlight, it reduces antioxidants in the skin that protect skin from sun damage and reduce the incidence of sunburn (Zhao et al. 2016). Vegetable oil is a good source of antioxidant such as phenolic compounds that can neutralise the free radicals formed in the body (Kozlowska et al. 2016). Thus, there is high demand in the consumption of seed oil through cooking oil and salad oil for general health being. Besides that, the presence of antioxidant activities in seed oil has raised great interest in the cosmetic field for skin health.

Tocopherols and phytosterols are components commonly present in seed oils. Vitamin E comprises of a family of eight substances with tocopherols being the most abundant form of vitamin E, followed by tocotrienols. Both class of compounds can exist in four isomeric forms (α-, β-, γ- and δ-isomers). Among the tocopherols,

Table 3.3 Physicochemical properties of seed oils

	Oil (%)							
	Soybean	Rapeseed	Sunflower seed	Groundnut	Cotton seed	Palm kernel	Coconut	Kenaf seed
% oil	21.00	41.00	36.14	41.00	18.2	10.00	42.00	20.80
RI	0.15	1.47	1.47	1.46	–	1.41	1.45	1.47
AV (mg KOH/g)	2.72	0.07	0.80	1.88	0.96	2.70	0.40	1.60
PV (meq O^2/kg)	21.38	5.73	1.33	8.39	5.60	1.43	0.20	2.64
SV (mg KOH/g)	199.63	184.10	193.90	175.78	217.51	244.04	258.80	171.00
IV (g I^2/100g)	119.21	116.10	132.30	111.19	105.30	20.8	5.50	89.56
FFA (%)	1.36	0.65	0.37	1.36	2.2	1.35	0.20	0.72

Source: Compiled from Abitogun et al. (2008), Ariharan et al. (2013), Bahadi et al. (2019), Chew et al. (2018), Dilsat et al. (2015, 2019), Long et al. (2008), Nyam et al. (2009) and Rosa et al. (2009). *RI* refractive index, *AV* acid value, *SV* saponification value, *IV* iodine value, *PV* peroxide value, *FFA* free fatty acid; –, no data available

α-tocopherol is known to be the most biologically active isoform (Montenegro 2014). α-tocopherol is the primary form of vitamin E found in the body due to its specific transport protein (Michels 2012). The significant quantity of vitamin E in seed oil could improve their oxidative stability. The presence of antioxidant compounds in seed oil prevent oxidative stress of the human cells, protect against oxidation and thus contributed to anti-carcinogenic properties.

3.5.2 Skin Repair

According to Mansur et al. (2016), the antioxidant compounds from plant that capture reactive oxygen species can minimise erythema and enhance SPF value of the cosmetic formulation. For instance, topical application of soybean oil, peanut oil and sesame oil protects against UV radiation and UV-induced cutaneous erythema (Lin et al. 2018). In addition, some studies have shown that antioxidants reduce the signs of aging by minimising wrinkles and preserving the skin texture (Mansur et al. 2016; Lin et al. 2018). Seed oils from sunflower seed, sesame seed and safflower seed have been recommended as good choices in promoting skin barrier homeostasis. Those bioactive compounds present can inactivate reactive oxygen species and restore skin homeostasis thus preventing erythema and premature skin aging (Mansur et al. 2016). Nowadays, the consumption of healthy seed oil and the use of plant derived natural antioxidants in cosmetics is preferred over synthetic antioxidants. Natural antioxidants derived from the plant could exert synergistic effects, thereby showing better effects and less toxicity

3.5.3 Anti-hyperlipidemia and Anti-hypercholesterolemia

Phytosterols are evidenced to be capable of reducing the blood cholesterol, thereby reducing the risk of cardiovascular diseases (Yang et al. 2019). Sterols constitute the major fraction of the unsaponifiable matter in many oils. From more than 40 phytosterols identified, β-sitosterol, campesterol and stigmasterol account for more than 95% of total phytosterols dietary intake. These phytosterols are commonly found in vegetable oils, nuts, seeds and legumes as integral natural components of the plant cell membranes (Cicero et al. 2017). The concentration and compositions of the phytosterols varies depending on the sources of edible oils in which higher contents of phytosterols are reported in rice bran oil, corn oil and rapeseed oil as compared to other vegetable oils (Yang et al. 2019). They are of interest due to their antioxidant activity and beneficial impact on human health. Phytosterols are reported to exhibit a wide spectrum of beneficial biological effects on human health such as anti-inflammatory, antioxidative, anticarcinogenic activities and cholesterol-lowering capability (Yang et al. 2019). Phytosterols have similar structure to cholesterol, but they are different in the side chain at C24 that presents a methyl or ethyl group (campesterol

and β-sitosterol) or an extra double bond in C22 (stigmasterol). Studies showed that phytosterols inhibit the intestinal absorption of exogenous cholesterols by competing with them for the formation of solubilized micelles. Thus, they are found to be able to lower blood cholesterol and low-density lipoprotein levels (Cicero et al. 2017)

In recent years, human dietary lipid intake has shifted towards polyunsaturated fatty acids (PUFAs) for their hypocholesterolemic effects (Yang et al. 2017). Linoleic and linolenic acids are synthesized in large quantities in plants, while they are not produced in humans, so they are taken from external sources. Cooking oils from canola, corn, olive, peanut, sunflower and soybean were suggested by the American Heart Association. Besides, the association also suggested some speciality oils, like avocado, grapeseed, rice bran and sesame, can be healthy choices for consumption. Intake of seed oils with a high amount of unsaturated fatty acids was found to be able to promote good health and reduce the risk of heart diseases. In addition, the hypolipidemic activity of seed oils used as a dietary supplement has been reported in the regulation of plasma lipid concentration and cholesterol metabolism.

The seed oils with high PUFAs was proven to be able to inhibit lipogenesis and upregulation of fatty acid β-oxidation in the liver. Omega-3 fatty acids are PUFAs which contain a double bond in position 3 at the end of the carbon chain. Omega-3 can be found in animal (fatty fish) and plant (flaxseed, walnut, edible seeds) sources. The intake of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) has the ability to maintain normal blood TAG levels (Yang et al. 2017; Cicero et al. 2017). In recent years, the American Heart Association has recognized n-3 fatty acids as preventive nutraceuticals for cardiovascular diseases. Apart from that, the hypolipidemic effect of seed oils is closely related to their antioxidant capacity. This is because high fat diet was found to induce oxidative stress in a variety of tissues and liver injury or hepatotoxicity is one of the main relative consequences of hyperlipidemia (Yang et al. 2017). Thus, the antioxidant activity of seed oil can help in preventing oxidative damage caused by high fat diet.

α-Linolenic acid (ALA), γ-linolenic acid (GLA) and stearidonic acid (SDA) are naturally produced in oilseeds such as flaxseed, rapeseed, soybean, borage, primrose seed, hemp seed and black currant seed (Abedi and Sahari 2014). ALA is an essential n-3 fatty acid with three double bonds located at carbon-9, 12 and 15. The fatty acid cannot be synthesized by the human body and can only be obtained from the plant-based diet. ALA is a fatty acid precursor to the production of other beneficial n-3 fatty acids such as EPA and DHA with the aids of elongase and desaturase enzymes. These fatty acids could contribute to normal brain development, normal vision and decreased risk of heart diseases (Cicero et al. 2017). Rapeseed is a member of the Cruciferae family. Canola oil is one of the good sources of ALA and contains a low amount of saturated fatty acid. In addition, GLA is also an important unsaturated fatty acid which is required for the biosynthesis of prostaglandin. Recently, GLA has been found to be capable of preventing and treating cardiovascular disorders. GLA was found in the oil of evening primrose and borage with 8–10% (w/w) GLA and 24–25% (w/w) GLA, respectively (Abedi and Sahari 2014). Besides that, SDA also plays a vital role in human health although the fatty acid is uncommon in plants. It is

an intermediate in the bioconversion of ALA to EPA and DHA. SDA is found in the oil obtained from borage contains 9–16% SDA, while hemp seed contains 2–3%, and blackcurrant seed has about 2% of the fatty acid (Abedi and Sahari 2014)

3.5.4 Type-2 Diabetes Prevention

According to literatures, the intake of MUFAs from plant sources, such as olive oil and pumpkin seed oil as a specific dietary compound has beneficial effects on reducing the risk to develop Type-2 diabetes and increasing glucose metabolism (Schwingshackl et al. 2017; Nishimura et al. 2014). Seed oils because of its favourable composition of bioactive compounds showed the potential role in the prevention and management of Type-2 diabetes. MUFAs play crucial roles in glycemic control through the reduction of glycemic load especially when replacing carbohydrates with MUFAs and the consecutive attenuation in insulin secretion and sensitivity (Schwingshackl et al. 2017). Besides that, the polyphenols in seed oils might also inhibit carbohydrate digestion and absorption which affect glucose metabolism that benefits Type-2 diabetes as well (Schwingshackl et al. 2017)

3.5.5 Anti-inflammatory Properties

Inflammation is a critical factor in the pathogenesis of many inflammatory disease states including cardiovascular disease, cancer, diabetes, rheumatoid arthritis and neurodegenerative diseases (Lucas et al. 2011). Many types of seed oil were found to have anti-inflammatory properties due to the presence of phenolic compounds (Lin et al. 2018). A traditional Mediterranean diet with high intake of olive oil was found can protect against the pathology of chronic diseases through the attenuation of pro-inflammatory mediators (Lucas et al. 2011; Santangelo et al. 2018). Virgin olive oil or extra virgin olive oil contains high amount of phenolic compounds that exert potent anti-inflammatory actions. Oleocanthal which present in virgin olive oil possesses similar anti-inflammatory properties to ibuprofen (Lucas et al. 2011). Apart from that, kenaf seed oil and roselle seed oil contain significant amount of phytochemicals were proven to have anti-inflammatory effect in edema-induced rats (Nyam et al. 2015)

3.6 Methods of Seed Oil Extraction

Extraction is an important step to separate, identify and recover valuable compounds from different plants. The nature of the targeted compounds will determine the choice of extraction technique in order to achieve the highest yield and purity. Oil

is extracted from seeds, nuts and fruits for the application in cooking, functional foods, biodiesel production and oleochemical industry, such as soap making, cosmetics and detergents. Extraction of seed oil can be done by using different methods and it is the first step in the oil refining process. The common methods which are widely applied are mechanical pressing and solvent extraction (Kumar et al. 2017). For instance, cold-pressing is applied to extract the virgin olive oil from the fruits through physical means by mechanical pressing. For most of the oils, however, the process is more complex with the combination of pressing, cooking and solvent extraction. Basically, before the oil extraction, the seeds will be cleaned and dried. Seeds with a high oil content (rapeseed and sunflower seed oil) will normally go through the preheat treatment in an indirectly heated conditioner prior to mechanical expeller pressing. The pressure exerted in the process squeezes out the oil. In addition, solvent extraction is commonly applied for oil extraction as well. The pre-processed seeds will be treated in a multistage counter-current process with solvent to reduce the remaining oil content to the lowest possible level. The solvent will then be separated from the mixture through distillation. The solvent is normally recycled to reduce the operating cost and minimise the toxic waste generation. The crude oil obtained will be stored prior to further processing (refining). The combination of mechanical pressing and solvent extraction is employed in large-scale operations for more complete extraction of seed oil.

3.6.1 Traditional Extraction Method

The traditional method of seed oil extraction can be carried out by kneading and heating the paste (Nkouam et al. 2017). The seeds will be thermally treated, crushed and ground to form the paste. After that, mixing the paste with water or boiling to extract the oil. Water plays a vital role in hydrolysing the paste, which displaces oil from hydrophilic surfaces in the paste. The paste is suspended in boiling water and boiled with liberated oil floating on the surface. Further quantities of water are added after boiling to replace the lost water that occurred during evaporation and to facilitate the floatation of the oil to the surface. The oil is carefully scooped from the surface of the water. The last process for the traditional extraction method will be the boiling of oil obtained in order to evaporate water (Nkouam et al. 2017). The traditional method is described as labour intensive, time-consuming, inefficient and low in yield and quality.

3.6.2 Conventional Methods

The conventional methods are the well-known and widely practised methods of oil extraction namely, mechanical expression and solvent extraction (Gaber et al. 2018). Many seed oils are extracted by either of the two methods or a combination of the

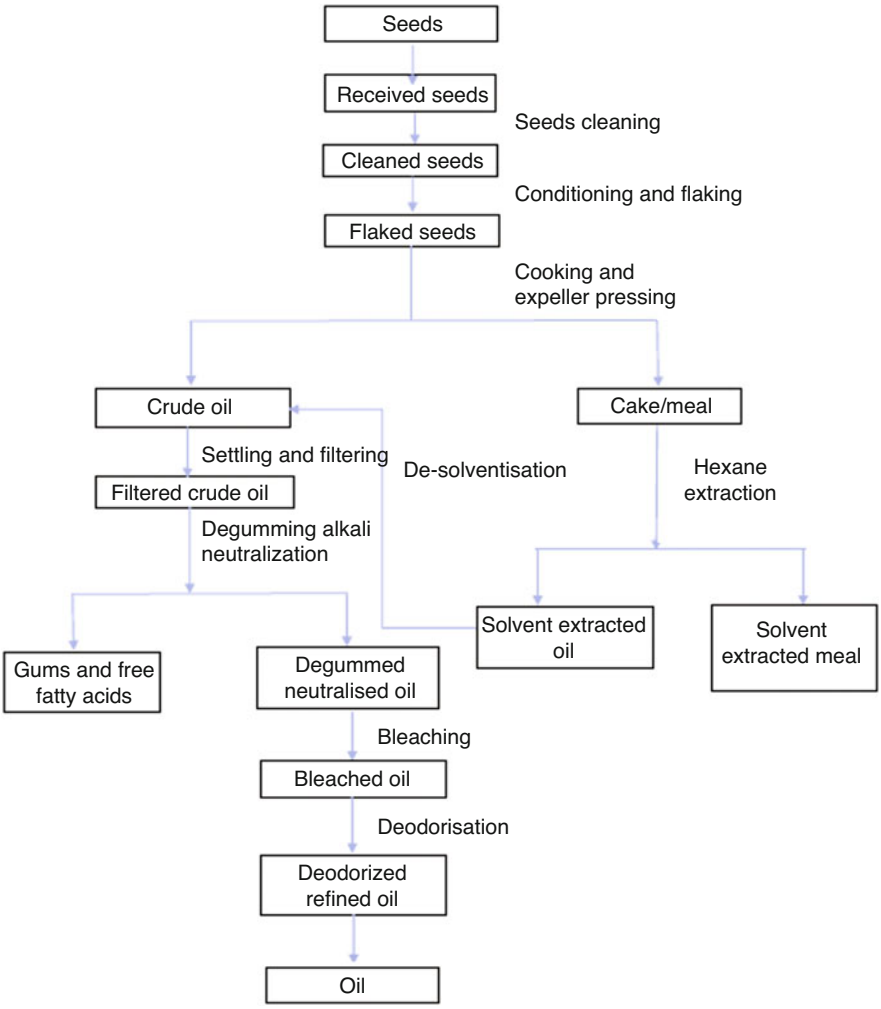

Fig. 3.1 Conventional seed oil extraction process: pressing, solvent extraction and oil refining steps [Adapted from Gaber et al. 2018]

two (Fig. 3.1). Pressing is one of the most common oil extraction method from oilseeds in which the method can be subdivided into both cold-pressing and hot pressing. Cold-pressed method is preferable as there is no heat involved

Oil extraction by mechanical pressing can be done by using a manual ram press or an engine-driven screw press. Ram press can extract around 60–65% of oil while an engine-driven screw press can extract around 68–80% of the available oil from seeds. Prior to the oil expression, the oilseeds will be pre-treated by cooking, size reduction by grinding or mechanical sieving by dehulling (Savoire et al. 2012). Pre-treatment of the seeds can increase the oil yield. Cooking can change the

moisture content of seeds, reduce the viscosity of the oil, increase plasticity and break the cell wall of seeds, provide sterilization, deactive the thermosensitive enzymes and destruct the thermolabile toxic components of the seeds (Savoire et al. 2012). Besides, reducing the size of seeds increase surface area thus increasing the efficiency of oil extraction and ensure high yield extraction. While dehulling is used to separate the oil-bearing nut from hulls.

After the pre-treatment process, they will then subject to the application of pressure using hydraulic or screw presses. Hydraulic presses can be found in antiquity while screw presses were invented in the early twentieth century (Savoire et al. 2012). Some researchers found that screw presses offer greater advantages over hydraulic presses due to its slightly higher yields and their continuous mode of operation. The mechanical expression is simple and safer compared to solvent extraction of vegetable oils in lab scale. However, on the industrial scale, mechanical expression is often used to extract seed with high oil content, such as the seed with more than 20% of oil. Generally, this method has the advantage of low operation cost (Savoire et al. 2012; Danlami et al. 2014) and can produce high quality oil with low concentration of undesired by-products such as free fatty acids (FFAs) (Danlami et al. 2014). Unfortunately, the processing technique suffers several drawbacks including lower oil yield with a large portion of oil left in the cake or meal after extraction. For example, mechanical pressing for castor oil only able to remove about 45% of the oil, while the remaining oil will be extracted using the solvent extraction method

3.6.2.1 Cold-Pressed Extraction

Cold-pressed technique involves the application of screw device that is tightened against the cake to extract the oils without applying heat. Compression pressure is therefore the sole operating parameter affecting the oil yield. Despite the lower oil yields for this method, the cold-pressed oil demonstrates a better quality with superior native properties compared to hot-pressed and solvent extracted oil. Besides, cold-pressed oil is free from chemical involved in the refining steps.

According to Chew (2019), cold-pressing comprises of three major stages namely (1) seed defatting, (2) seed fragmentation and (3) seed conditioning. Seed defatting reduces chlorophylls and other trace elements such as metals and pesticides, producing oil with brighter colour and enhanced oxidative resistance. Nevertheless, the method is not widely applied in the industry due to several technical difficulties (Chew 2019). After that, the seeds will be subjected to seed fragmentation. The seeds will be broken up by grinding, flaking or rolling. During seed conditioning, the seeds will be pre-treated in an oven condition the seed humidity before subjected to mechanical pressing to extract the oil. This pre-treatment process can enhance the oil extraction yield. In some instances, oil is produced using a variation of a high-pressure system. This is known as the expeller pressed method. It may generate some heat energy owing to intense friction forces and added pressures involved even though no supplemental heat is supplied to the system. Having said that, oil

produced using expeller method is still considered cold-pressed oil. Cold-pressed oil is regulated differently in different countries. For example, oil extraction temperature should not exceed 100 °F during the extraction process in order to be certified as "cold-pressed oil" in Europe. On the other hand, the United States does not regulate the oil making processes, certain analytical tests will be conducted to determine if the oil meets the standards.

Cold-pressed oils also called virgin oils, are purer and has a better flavour than oil expressed with the application of heat. After cold pressing, the remaining meals or cakes may still contain approximately 5–15% of oil which can be further extracted using organic solvents. The solvents could penetrate through the meals and dissolve the oil retained inside. The solvents will then be recovered via evaporation and reused.

Moreover, cold-pressed oils show minimal adverse effects caused by high temperature as compared to hot-pressed oils. The cold-pressing system prevents the degradation of valuable oil components (phytosterols and tocopherols) and produces oils with good oxidative and storage stability, thereby preserving the purity and natural properties of seed oils. Owing to these attractive qualities, there is a growing global demand for cold-pressed oil. Despite the benefits aforementioned, low oil extraction yield remains to be major hindrance for cold-pressing extraction method which inhibits the development of this technology to become commercially feasible.

3.6.2.2 Hot-Pressed Extraction

Hot-pressed extraction is the extraction of oil with the implementation of heat to the process. The hot-pressed process gives higher oil yield compared to cold-pressed due to the decreasing of oil viscosity at high temperature. This enhances oil flow during extraction. Thus, high temperature increases the efficiency of the extraction process and yields of up to 80% of available oil in seeds are possible, but they may also cause oil degradation, with attendant deterioration of oil quality. The first step of the hot-pressed extraction is similar to cold-pressing or expeller methods. However, in the heated extraction process, the cakes or pastes are usually heated using warm water or direct heating elements powered by electricity. In larger operations, heating elements may be driven by natural gas. Heating up the pastes was proven to be able to increase the yield of oil extracted but the heating process degrades the quality. Oil created through a heating process sometimes loses its colour as well.

3.6.2.3 Solvent Extraction

Solvent extraction method is an efficient approach with relatively high yield and consistent performance. The method is capable of extracting most of the oils from the cakes or meals, leaving with minimal residual oil left behind after the extraction. Therefore, the solvent extraction technique is suitable for those oilcrops with low oil content (<20%) such as soybean (Chew 2019). Sometimes, the method can be used

after mechanical expression to extract and recover the remaining residual oil trapped within the pressed cake as mentioned earlier (Gaber et al. 2018). The common solvents used are hexane, diethyl ether, petroleum ether and ethanol. Besides, the oil can be extracted with solvent combination of hexane, 2-methyl pentane and 3-methylpentane. The use of solvent is a cost-effective extraction method with minute amount of oil left (~0.5%) in the solvent after extraction which can be easily redistilled and recycled due to its low boiling point (63–67 °C) (Daun et al. 2011). While after oil recovery by desolventization, the content of solvent remaining in the oil is less than 100 ppm (Gaber et al. 2018). For laboratory purposes, solvent extraction of oil by Soxhlet process is using widely used. The major advantage of the Soxhlet process is solvent recycling during extraction. Solvent extraction is more advantageous than mechanical expression owing to its high extraction yield, resulting in lesser residual oil in cakes or meals besides providing a high accuracy of the oil content of the seed samples.

Unfortunately, this approach has some industrial disadvantages such as long extraction times, relatively high solvent consumption, high capital investment, high energy requirement, possible emission of volatile compounds into the atmosphere, high operating costs, poor product quality caused by high processing temperatures and a great number of processing steps (Gaber et al. 2018; Azadmard-Damirchi et al. 2010). Additionally, the process of desolventization brings additional cost and labour. Also, it may cause plant security problems (Azadmard-Damirchi et al. 2010).

3.6.3 Innovative Extraction Methods

Nowadays, Soxhlet extraction is the most widely used method for seed oil technique in research and industries. Unfortunately, conventional oil extraction method, such as hot-pressing is thermally unsafe and Soxhlet extraction is temperature limited, depending on the boiling point of the organic solvent chosen in addition to long extraction times and large solvent consumption (Hibbert et al. 2019). Tackling the shortcomings with the utilisation of conventional methods have propelled the development of alternative innovative methods, such as supercritical fluid extraction (SFE), microwave-assisted extraction (MAE) and ultrasonic-assisted extraction (UAE).

3.6.3.1 Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction (SFE) is an advanced technology applied in many food processing industries (Akanda et al. 2012). SFE is recognised as a clean and environmentally friendly processing technology and acted as an alternative to solvent extraction (Akanda et al. 2012; Prabhakaran Nair 2013). It separates oil from the cake or meal of seeds by using gases in supercritical form at temperatures

and pressures above critical values. Supercritical fluids exhibit both the solvating properties of a liquid as well as the high permeability and low density of a gas. It is very important in the separation processes based on the physicochemical characteristics including density, viscosity, diffusivity and dielectric constant which are easily manipulated by pressure and temperature (Akanda et al. 2012).

During SFE, the substances that dissolved in the fluid are able to modify their dissolving power under specific conditions, above or near their critical temperature and pressure to recover extracts from solid matrices (Prabhakaran Nair 2013; Prado et al. 2015). The uniqueness of a supercritical fluid solvent is that its density is pressure dependent. The density can be attuned from liquid to vapour condition with continuity. Supercritical fluid has intermediary properties between gases and liquids, making it an effective extraction solvent for several compounds. Previous literatures show that carbon dioxide in the supercritical state is the most popular solvent used for extraction purpose. It shows relatively low critical temperature and pressure around 304.2 K and 7.38 MPa, respectively (Prado et al. 2015). It is a renewable solvent, non-toxic, non-corrosive, non-flammable, inert and cheap.

During the extraction process, the most important regions in the pressure-temperature-composition space are liquid-vapour, solid-vapour or liquid-liquid equilibria in two-phase process; liquid-liquid-vapour, solid-liquid-vapour, solid-solid-vapour equilibria in three-phase process and solid-supercritical fluid mixtures in four-phase process. The solvent can break up a multi-component mixture based on the different volatile capacities of each component. SFE could facilitate the detachment of the extract from the supercritical fluid solvent by simple expansion. Additionally, supercritical fluids exhibit relatively high diffusivity and very low surface tension, allowing them to demonstrate a superior mass transfer rate as compared to other liquid organic solvents. These characteristics enable easy infiltration into the permeable make-up of the solid matrix to reach the solute.

3.6.3.2 Microwave-Assisted Extraction (MAE)

Microwave-assisted extraction (MAE) is one of the innovative techniques that can be employed for extracting high quality oil from oilseeds. MAE method is simple and superior as compared to other heat induced extraction methods. MAE offers rapid heating of the extraction mixture via microwave irradiation. Pre-treatment of oilseed is done in the microwave oven, which uses radio wave to convey energy and convert it to heat at a frequency range of about 300 MHz to 300 GHz (Hibbert et al. 2019). Microwave pre-treatment for the oilseed is receiving interest to increase the oil yield during cold pressing. The use of microwave radiation in oilseeds results in the rupture of cell membranes for higher extraction yield and an increase in mass transfer coefficients. This treatment decreases the moisture content of the seed to crack the cell membrane as the oil migrates through the permeable cell wall during the pressing process.

MAE provides better extraction rates and saves energy because the microwave irradiation can be transmitted directly throughout the seeds, thereby providing a

rapid and more uniform heat treatment onto the seeds that ensures higher oil yield and better oil quality. MAE promotes the recovery of oil yield with an increased shelf life stability and nutritional value from high amounts of bioactive compounds (Chew 2019). The development of MAE method proved to be more efficient, in yielding more oil whilst requiring a fraction of the time and losing less solvent than the conventional solvent extraction method. In terms of quality of oil from MAE, the technique could retain most of desirable nutraceuticals such as phytosterols and tocopherols, canolol and phenolic compounds in the extracted oil. Therefore, MAE represents a new step forward for the production of nutritional vegetable oils with enhanced shelf life and higher antioxidant concentration.

However, MAE may not suitable for all types of plant, since high microwave energy disrupts plant structure. Moreover, it would degrade the PUFA in vegetable oil, resulting in unrepresentative FA profile. MAE may increase the FFAs and phospholipids contents of the oil. This is because of the hydrolysation in TAG that increases the amount of FFAs under the catalysis of lipase by microwave heating. Besides, the increase of phospholipids content is due to the rupture of the cell membrane as phospholipids are found abundantly in cell membrane. Phospholipids are usually extracted together with oil when the cell membranes are disrupted by the microwave pre-treatment process. The method has been applied to extract oils from a wide variety of seeds including soybean, castor, peanuts, olive, sunflower seeds, hazelnuts, rapeseeds and so forth.

3.6.3.3 Ultrasonic-Assisted Extraction (UAE)

Ultrasonic-assisted extraction (UAE) is a new innovative technology which makes use of high-intensity, high-frequency ultrasonic sound waves to increase vibration and heat transfer rate resulting in the destruction of rigid plant cell walls, thereby enhancing contact between solvent and the plant material (Teng et al. 2016; Mello et al. 2015). Although relatively easy to achieve on a lab scale, but scale-up for the industry is challenging (Chew 2019). The extraction efficiency of UAE method can be improved further improved when coupled with solvent extraction. A combination of the two methods could reduce the thickness of the cell walls and enhance the capability of the solvent to dissolve oil and extract them from the meals. The dual approach could be an innovative way to increase the oil extraction yield. According to the finding from Mello et al. (2015), UAE provides a higher content of beneficial unsaturated fatty acids compared to Soxhlet extraction. The outstanding performance of these innovative technologies has recently encouraged researchers to explore the prospects of combining some of these methods, with the aim of synergising the oil extraction.

3.7 Refining of Seed Oil

Refining of crude oil is designed to remove unwanted minor components that make oils unappealing to consumers. It is important to reduce the impurities present in the oil to a level where their deleterious effects on oil stability are minimum and made suitable for human consumption (Pal et al. 2015). Whilst there is oil exemption from the refining process, particularly virgin oils, where the extracted oil receives minimum or no processing in order to maintain their distinctive odour, colour and taste. The refining process could eliminate the undesired compounds present in the crude oil, producing a clear, bright, pale coloured oil with no off flavours or odours and enhanced storage stability. The refining process should be kept from damage to the neutral oil as well as minimum refining loss.

During the refining process, the compounds, such as glyceridic and non-glyceridic compounds which can affect the flavour, colour, storage stability of the oil will be removed (Pal et al. 2015). They are primarily phosphoacylglycerols, free fatty acids, colourants, waxes, volatiles and contaminants (Aluyor et al. 2009; Pal et al. 2015). Some of these contaminants are naturally present in the seeds, formed during harvesting and storage of seeds or oil extraction process. The major steps and main compounds removed during oil refining are shown in Table 3.4.

The processes used can vary depending on the type and nature of the particular oil, most oils are processed in three stages, neutralisation, bleaching and finally deodorization (NEODA n.d.). Oil refining included chemical and physical refining. The main difference between the process is that chemical refining procedure includes neutralization to remove the free fatty acids whereas physical refining, free fatty acids are eliminated by distillation during deodorization. Chemical refining includes degumming, neutralization, bleaching, dewaxing and deodorization stages. Although chemical refining decreased oil yield, higher investment cost, high chemicals used and higher waste, but has less effect on oil desirable components and oil stability (Suliman et al. 2013). However, physical refining reduces the loss of neutral oil, minimises pollution and enables recovery of high quality free fatty acids. Nevertheless, not all oils can be physically refined.

Table 3.4 The major steps and main compounds removed during oil refining

Main compounds removed	Physical refining	Chemical refining
Phospholipids	Degumming	Degumming
Free fatty acids	–	Neutralization
Pigments, metals, soaps	Bleaching	Bleaching
Saturated triacylglycerols, waxes	Dewaxing	Dewaxing
Volatiles compounds, free fatty acids	Deodorization or deacidification	Deodorization

3.7.1 Degumming

Under certain circumstances, the oil may develop a darker colour with an unpleasant flavour. Gums, phosphatides and mucilaginous substances act as emulsifier increasing loss of oil and decompose at high temperature, increasing colour of the refined oil (Pal et al. 2015). The purpose of degumming is to remove phospholipids or gums from the crude oil. During the degumming process, most of the phospholipids are hydrated and are insoluble in the oil after adding water. The hydrated compounds can be separated easily from the crude oil *via* centrifugation or filtration. For the nonhydratable fraction, the crude oil is treated with phosphoric acid, which chelates the calcium and magnesium converting the phosphatides into the hydratable forms. An analysis of the phosphorus content in the pre-treated crude oil is essential to determine the proper acid dosage, especially when the content of calcium and magnesium salts is high. Depending on the oil composition, the degumming step can be omitted as the phosphatides are also removed along with the soaps during neutralisation in chemical refining process. However, degumming is mandatory for physical refining to remove the phosphorus content

3.7.2 Neutralisation

Free fatty acids increase foaming, reduce smoke point and diminish keeping properties of oil (Pal et al. 2015). The crude oil is neutralised using a mild alkaline solution, such as sodium hydroxide during chemical refining in order to remove elements which may have broken away from the triglyceride, also known as free fatty acids (NEODA n.d.). The free fatty acids will be converted into insoluble soaps, which can be easily separated by centrifugation. The free fatty acid content of the oil is the main factor that determines the amount and concentration of sodium hydroxide. Alkali neutralization can improve the oil colour by reacting with polar compounds and solubilization. Alkali neutralization of oil is compulsory in crude oils of high acidity and pigment contents.

3.7.3 Bleaching

Bleaching also called decolouration, it is common in both physical and chemical refining process. It is designed to remove not only pigments but also a wide range of other impurities in the crude oil. Colour and impurities are removed by mixing the oil with a naturally occurring bleaching clay (bleaching earth) which is subsequently filtered out to leave a clear and clean oil. During bleaching, the oil will be heated and mixed with acid-activated bleaching earth and naturally occurring blend of attapulgite and montmorillonite. Bleaching clays are normally comprised of one or

more of three types of clay minerals, namely calcium montmorillonite, attapulgite, and sepiolite (Brooks et al. 2011). Bleaching requires the use of adsorbents to remove the coloured pigments and impurities or contaminants from oil to give it a desirable quality (Chetima et al. 2018). The colour pigments, trace metals, oxidation products, residual soaps and phospholipids will be absorbed.

The removal of chlorophyll is very important since they are not eliminated in any other stage of refining, as carotenoid compounds will be eliminated during deodorization. Thus, the common practice for bleaching process optimization is by monitoring the chlorophyll content after bleaching (Brooks et al. 2011). Besides that, the activated earth must be removed completely after the final filtration. Acid-activated based bleaching clays are commonly used industrially while other types of adsorbents such as active carbons and synthetic silicas are also applied sometimes with more specific goals (Chetima et al. 2018). For instance, the active carbons are effective in eliminating polycyclic aromatic hydrocarbons from some oils such as fish oils and pomace oils (Chetima et al. 2018) whereas synthetic silicas can be utilised for the removal of secondary oxidation products like phospholipids and soaps.

3.7.4 Dewaxing

Dewaxing also knew as winterization, is only applied when the oil is not clear at room temperature because of the presence of waxes or saturated TAGs. The oil will be cooled down to allow crystallization. The waxes and saturated TAGs will be removed by centrifugation at low temperature. This treatment ensures excellent clarity of oils when stored at either room or refrigeration temperatures.

3.7.5 Deodorization

Deodorization also called steam distillation consists of vacuum steam distillation process by heating to high temperatures, typically using high pressure steam, under a tight vacuum and blowing steam through the heated oil. The purpose of this step is to remove volatile compounds, mainly ketones and aldehydes contributing to oil taste and odour, total free fatty acids in physical refining and the residual free fatty acids from neutralized bleached oils. It is to improve the flavour quality, shelf life and stability of the final oil products (Ma et al. 2017). The deodorization conditions also contribute to the removal of contaminants, like polycyclic aromatic hydrocarbons and pesticides and to the reduction of colour of the oil due to the breakdown of the remaining carotenes at high temperature. After the deodorization, the oil will be cooled and the addition of citric acid is recommended to chelate metal traces and increase its stability during storage. Besides, it is crucial to consider the time of

deodorization and too high temperatures may degrade the valued compounds like tocopherols, sterols and hydrocarbons.

3.8 Conclusion

Vegetable oils have been predominantly used for food-based applications. Seed oils provide a wide diversity in fatty acids composition with diverse applications. Besides being edible, they are now increasingly being used in industrial applications such as, cosmetics, paints, lubricants, soaps and biofuels. Moreover, the seed oils which are nutritionally beneficial to human health have been incorporated in dietary intake. Besides that, these oils have potential to substitute ever increasing demand of non-renewable petroleum sources for industrial applications. Thus, various extraction methods were incorporated to maximize the oil yield in order to fulfil the market demand. Also, refining processes are included to produce edible oils with specific characteristic and at the same time maintain their nutritional quality.

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Chapter 4
Vegetable Oil Refining

Nur Azwani Ab Karim, Noor Hidayu Othman, and Masni Mat Yusoff

Abstract Edible oils which include vegetable oils, are the major sources of dietary lipids. Crude vegetable oils and fats are mainly triacylglycerols (around 95%) along with impurities and variable amounts of other minor constituents. To ensure the oils are safe for human consumption, the crude oils are subjected to several purification steps, known as refining process. The aim of this process is to eliminate the unwanted constituents from the oil with the least possible damage to the triglycerides and minimal loss of the desirable constituents. Recently, extensive improvement and innovation have been made on refining technology, using either conventional physical/chemical processes or several unconventional process including membrane and biological process. This paper includes a brief description of general composition of some commonly used vegetable oils, followed by the review of various refining methods and recent development in refining process.

Keywords Refining · Degumming · Bleaching · Deodorization · Esterification · Biorefining · Membrane technology

4.1 Introduction

The oils and fats production and usage can be traced back since ancient time. They served as food, oil lamps, candles and lubricant for Greek wrestlers. The first fats obtained was probably being extracted from the fatty tissue of animal using the a simple rendering process (Dijkstra and Segers 2007).

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In Egypt around 259 BCE, edible oils were obtained through the pressing of from oilseed such as sesame seeds and linseed. After 75 years, the ancient Rome developed a more sophisticated instrument like screw and wedge presses for the extraction of nut and seed oils, in particular, olive oil (Blank 1942). Mechanical screw pressed was introduced by Anderson in the 1900 to crush and press the nuts and oilseeds for the production of edible oil (Hastert 1998).

During the ancient Greece, it is not a common practice to refine the edible fats and oils. Not until in the 1842 that a patent was file for the use of caustic soda for refining of fats and oils by Schmersal. However, the implementation of the causatic soda refining process was only being practiced after 16 years since the first patent was filed, in which, Bareswil used 30% caustic solution to deacidify the cottonseed oil. In the 1891, using steam deodorisation process operating at atmospheric pressure was introduced by Eckstein. Further, an improved version of the vacuum deodorisation processes was developed by Bataille in France and Wesson in the U.S (Blank 1942). Despite for only being used for the past decades, refining of edible fats and oils (neutralisation, bleaching, and deodorisation) remain to have a play a significant role to ensure that quality and palatibility of edible fats and oils.

4.2 Components of Vegetable Oil

Approximately 95% of crude vegetable oils and fats are mainly composed of triacylglycerols whilst the remaining is attributed by free fatty acids (FFA) and partial acylglycerol. Small amounts of minor components such as sterols, triterpene alcohols, tocopherols and tocotrienols, carotenes, chlorophylls and other pigments, hydrocarbons as well as traces of metals, oxidation products and etc. are found in crude vegetable oils and fats. Some of the constituents are desirable due to their beneficiary effects and should be retained in the refined oil; for instance; tocopherols and tocotrienols protect the oil against oxidation, whereas carotenoids are known for their pro-vitamin A activity. Phosphatides and metal traces, on the other hand, will interfere within further processing. To ensure the oils are safe for human consumption, the crude oils are subjected to several purification steps, known as refining process. The aim of this process is to eliminate the unwanted constituents from the oil with the least possible damage to the triglycerides and minimal loss of the desirable constituents.

Majority of the impurities presence in crude palm oil are contributed by the FFA and phospholipids. In addition, the crude oil also contains some suspended materials, such as meal, fiber, dirt, etc. that need to be eliminated through filtration the refining process. Otherwise, it will lead to clogging of the centrifuge bowl that often required frequent shut down to remove theses impurities.

FFA is generated right after harvesting. It is derived from the hydrolysis of the ester bonds in lipid. The hydrolysis reaction is mainly contributed by the action of enzyme lipase or heat treatment or moisture exposure (Akoh and Min 2002). FFA is

Table 4.1 Typical phospholipids and phosphorus contents in most common vegetable oils

Crude oil	Phospholipids content (%)	Phosphorus content (ppm)
Soybean oil	1–3	400–1200
Corn oil	0.7–2.0	250–800
Cottonseed oil	1.0–2.5	400–1000
Peanut (groundnut) oil	0.3–0.7	100–300
Canola oil	0.5–3.5	200–1400
Sunflower oil	0.5–1.3	200–500
Safflower oil	0.4–0.6	160–240
Palm oil	0.03–0.1	12–40
Coconut oil	–0.05	8–20

one of the major constituent that influences the quality of the oil due to is pro-oxidative property.

Phospholipid or also known as phosphatides, can be classified into phosphatidylcholines, phosphatidylethanolamines, and phosphatidylinositols as well as minute amount of phospholipids. Table 4.1 shows the phospholipids and phosphorus contents in common crude vegetable oils (Gupta 2017). The content of phospholipid particularly lecithin is relatively high in soybean oil followed by rapeseed oil and sunflower oil. On the other hand, the lecithin presence in palm oil is insignificant (Schneider 1997; Goh et al. 1985). Therefore, majority of the commercially available lecithin is mainly derived from soybean lecithin. Lecithin has found a wide application to be used in food, animal feeds, and industrial applications. Despite being a valuable components to the phospholipid industry, the phospholipids need to be eliminated during refining process in the degumming stage. This is because existance of the phospholipid in crude oil will not only lead to darkening of oil but also promote oxidative rancidity when exposed to air or light. Henceforth, degradation of the quality of crude oil will further hinder the distillation and deodorisation process during refining (Dijkstra 2009).

Apart from these major contaminants, metal ions and volatile matter should also be removed effectively in order to ensure that the refined oil is able to meet the standard quality set for edible vegetable oil**.**

Carotenoids are another minor constituents presence in many vegetable oils. As compared to other vegetable oils, carotenoids content are relatively higher in crude palm oil. Crude palm oil normally contains 500–2000 ppm of carotenes, about 90% of it consist of α- and β-carotene. Carotenoids especially β-carotene are known for their pro-vitamin A activity which shows purported health benefits in preventing certain types of cancer (Goh et al. 1985). Today, many studies have been made to concentrate the carotenoids in refined palm oil to be used as food-dyes, vitamin additive, and pharmaceutical and cosmeceutical products (Ooi et al. 1994).

Tocopherols and tocotrienols are the fat-soluble vitamin E that exist in α, β, γ and δ isomers. Vegetable oils, especially the seed oils, are rich sources of tocopherols. Soybean oil contains the four isomers of tocopherol and is particularly rich in gamma-tocopherols that constitutes of around 60%. On the other hand, tocotrienols

Table 4.2 Unsaponifiable composition (percent) of some common vegetable oils

Oil	Hydrocarbons	Squalene	Aliphatic alcohols	Terpene alcohols	Sterols
Olive	2.8–3.5	32–50	0.5	20–26	20–30
Rapeseed	8.7	4.2	7.2	9.2	63.6
Corn	1.4	2.2	5.0	5.7	81.3
Soybean	3.8	2.5	4.9	23.2	58.4
Lineed	3.7–14	1.0–3.9	2.5–5.9	29–30	31.5–52.0
Tea seed	3.4	2.6	–	–	22.7
Rice bran	7.0	–	–	24.0	42.0

only exist in few of the vegetable oils like palm oil or cereal oils (barley or rice bran). Out of the 600–1000 ppm of tocopherols and tocotrienols found in palm oil, tocotrienols contributed to around 72–82% while the remaining 18–22% is attributed to tocopherols. Specifically, major tocotrienols include: 46% of gamma-tocotrienol, 22% of alpha-tocotrienol and 12% of delta-tocotrienol (Ravigadevi et al. 2000). Nevertheless, the vitamin E is partially lost during the refining process of palm oil especially during the deodorization stage (Liu 1997).

Sterols are presence in most of the vegetable oils at approximately1000–5000-ppm (1–5 g/kg) either in free form as sterols or esterified form as esterified sterols. Rapeseed oil (5–11 g/kg, mean 7.5) and corn oil (8–22 g/kg, mean 14) have a higher level of sterols as compared to other vegetable oils. Examples of sterols include: sitosterol, campesterol, stigmasterol, and 5-avenasterol. Main sterols constitutes of sitosterol that contributes to around 50–80% of the total sterols. On the other hand, a less frequently seen brassicasterol can only be found in rapeseed oil. Therefore, it is often used as a marker to detect adulteration of rapeseed/canola oils (Przybylski and Mag 2020). Cholesterol is virtually non existance or presence in plant species except in animals species such as animal fats (up to 1000 ppm), fish oils (up to 7000 ppm), dairy fats (2000–3000 ppm), and egg yolks (12,500 ppm). Similar as tocopherols and tocotrienols, the level of sterols are affected by processing. Around 40% of these components were removed from the oil during deodorization (Clark 1996).

Hydrocarbon found in vegetable oil includes paraffins (normal and branched-chain), terpenoids (squalene and its homologues), and polycyclic aromatic compounds. Some other hydrocarbons have also been reported but their presence need further confirmation. There are around 1–14% of hydrocarbon and 2.2–50% of squalene found in the unsaponifiable components in vegetable oil (Table 4.2). Approximately 30–50% of the unsaponifiable matter of olive oil is contributed by the squalene in which the actual amount ranges from 400 to 700 mg/L. The biological action of the hydrocarbon is still unknown except for squalene where the isoprenoid-related terpenoids may potential to reduce the cholesterol synthesis in the body (Stermer et al. 1994).

Table 4.3 Cyclic terpenes in different edible oils (mg/100 g)

Oil	Cyclo-artarnol	Cyclo-arterenol	24-Methylene cyclo-arterenol
Rice bran	106	482	494
Safflower	1	34	7
Corn	4	8	11
Sunflower	–	29	16
Cottonseed	–	10	17
Sesame	4	62	107
Soybean	–	168	8
Groundnut	1	11	16
Olive (crude)	1	18	31
Palm	2	60	34
Coconut	2	55	22
Rapeseed/mustard	1	54	14

Vegetable oils also contain terpene alcohols either in free form or esterified with fatty acids where the fatty acids are presumed to be similar to the fatty acids or triglycerides except for ferulic acid (Jacini and Fedeli 1967). The terpene alcohols identified so far are acyclic terpenes and cyclic di- and triterpenes (Itoh et al. 1973). Cyclic terpenes being the predominant terpene alcohols are found in rice bran oil, wheat germ oil, soybean oil, linseed oil, and olive oil (Table 4.3). Cycloarterenols from rice bran oil demonstrated to have be an effective hypocholesterolemic agents. Two acyclic terpene alcohols phytol and geranylgeraniol which is the precursor of di- and tricyclic terpenes and some forms of carotenes (including lycopenes) have also being identified in vegetable oils.

4.3 Edible Oil Refining

One of the challenges face by the edible oil industry today is to produce an oil with stable and neutral taste during storage while retaining their nutritional components. As such, a well designed refining process is necessary not only to remove the undesirable compounds but also to minimise the loss of the nutritional components. A schematic representation of the various available refining strategies is provided in Fig. 4.1.

4.3.1 Degumming

Crude oil obtained by screw pressing and solvent extraction of oilseeds will throw a deposit of so-called gums on storage. These gums consist mainly of phosphatides with entrained oil and mill particles. They are formed when the oil absorbs water that

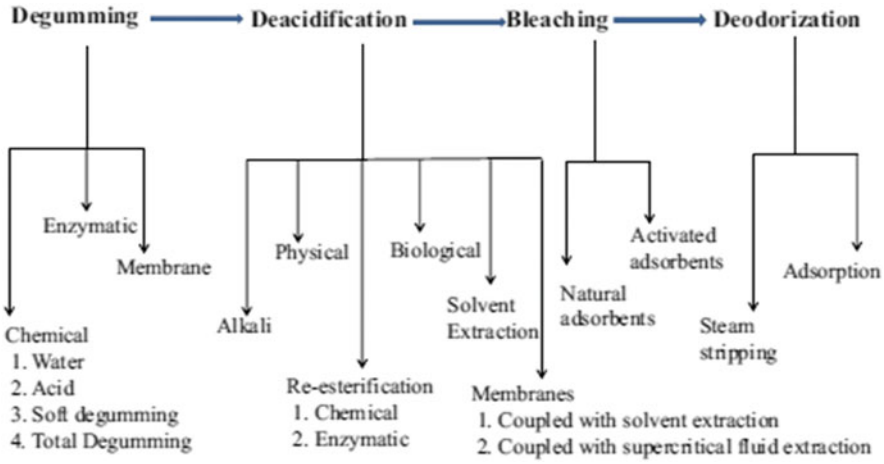

Fig. 4.1 Overview of existing and potential technologies for edible oil refining

causes some of the phosphatides to become hydrated and thereby oil-insoluble. In oil processing industry, the phospholipids (also referred to as phosphatides) content in the oil is expressed in terms of parts per million (ppm) of phosphorus. There is a relationship between the phospholipids content and the corresponding phosphorus level in the oil (Gupta 2017).

Phospholipids are similar to triacylglycerols (TAGs) molecules but differ in that one carboxylic acid or fatty acid chain is replaced by a phosphate group that linked to different substituents. The PLs are diacyl surfactants and are amphiphilic in nature because they have hydrophobic fatty acid groups and hydrophilic glycerol phosphate moieties (Subramanian et al. 2001). Among main PLs, Phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA) and phosphatidylethanolamine (PE) are the major components of PLs. Due to the presence of two long lipophilic fatty acid chains and a hydrophilic phosphate group on the same molecule, the PLs behave like emulsifiers and responsible for oil loss in refining process. PLs have to be eliminated in the early refining stage because they decompose under heating and caused oil darkening at high deodorisation temperature. Among them, PC, PI and PS being more hydrophilic are considered as hydratable phospholipids (HPs) compared to PE, PA which are non-hydratable phospholipids (NHPs).

4.3.1.1 Methods of Degumming Process

The choice to select for a suitable degumming process relies on the nature of the PLs, of which, can be divided into two categories:

1. Hydratable gum removals process
2. Non-hydratable gum removal process

Five basic principles and conventional degumming processes are described as water degumming, dry degumming, acid degumming, TOP degumming and soft degumming. Besides, the gums also can be removed by physical degumming method using membrane separation and biological method by enzymatic degumming. Both methods are considered as an eco-friendly and green technology but very costly at an industrial scale, which need more intensive research for commercial implementation (Sharma et al. 2018).

Hydratable phospholipids can be removed by a simple water degumming technique. The addition of water to crude oil results in the formation of a ternary phase system of the water-phospholipid-oil molecules and hydration of HPs occurs as a result of the weak dipole-dipole interaction (hydrophilic) between the polar head group of PLs and the water molecules. This interaction results in phase transitions, i.e. oil-soluble PL aggregates transform into oil-insoluble lamellar liquid crystals (gum) (Sharma et al. 2018).

Water degumming technique can be used to remove hydratable phospholipids. Addition of water during degumming process to the crude oil leads to the formation of ternary phase system consisting of water-phospholipid-oil molecules. The polar head group of PLs and the water molecules are bonded via weak dipole-dipole interaction resulting in the formation of hydrated phospholipids. Henceforth, the initial PLs that is soluble in oil has transformed into oil-insoluble lamellar liquid crystals (gum) (Sharma et al. 2018).

Regarded as the oldest degumming approach, water degumming is used for the production of commercial lecithin. It is the simplest method for phosphatide reduction (Gibon et al. 2007). During water degumming process, hot water is mixed into heated crude oil (70–90 °C) where HPLs in oil interact with water at an increased temperature. Oil-insoluble gums are separated by settling, filtering or centrifugation and dried by vacuum drying. The classic water degumming process leads to high loss of neutral oil, large amount of wastewater and energy consumption (Vinayak et al. 2014).

Non-hydratable PLs are the salts of Ca, Mg and Fe of PA and PE. They are not able to be hydrated by water nor be separated out from the oil phase. As such, the NHPs usually required the use of chemical agents like acids, bases and complexing agents as well as biocatalyst enzymes to be eliminated (Gunstone et al. 2007). Four conventional chemical degumming methods are dry degumming, acid degumming, TOP degumming and soft degumming (Sharma et al. 2018).

Dry degumming process is mainly for oils and fats where the phosphatides content is relatively low (around 20 ppm of phosphorus). Vegetable oils that met the specification include palm oil, palm kernel oil and olive oil. In physical refining of palm oil, the CPO is degummed by mixing with 0.04–0.1% phosphoric acid (conc. 85%) for about 5–20 min. Subsequently, the degummed oils were bleached using around 1–2% of bleaching earth under vacuum at a temperature of 95–120 °C. The spent bleaching earth is then separated via filtration. The magnesium and calcium complexes of the NHPs were broken down with the incorporation of the phosphoric acid during dry degumming. Then, it coagulates the phosphatides, sequestrates iron and copper, before being removed by adsorption on bleaching

earth (Gibon et al. 2007). Low dosage of phosphoric acid may lead to oil darkening during deodorization and off-flavor problems due to phosphatides breakdown caused by thermal instability (Patterson 1992). An excess of phosphoric acid may promote oil darkening due to the reverse reaction of phosphatidic acid (PA) into phosphatides (Thiagarajan and Tang 1991).

Acid degumming is preferred for processing of rapeseed and sunflower oils (Molik and Pokorny 2000). The hydra-ability of phospholipids is formed with the addition of acid such as acetic acid, citric acid or tartaric acid, whilst the toxic or corrosive acids are usually avoided (Deffense 2011). Dosage of phosphoric acid in the range of around 0.05–0.20% of was found to satisfactory to degum palm oil However, the conditions may not be suitable for majority other vegetable oils (Faessler 1988). For instance, soybean oil was degummed in stepwise manner using concentrated acid such as phosphoric acid and water prior to subsequent degumming with a diluted acid (Ally 1992). As an alternative to the expensive phosphoric acid a more economical At times, citric acid acid can be a better alternative to phosphoric acid in removing the NHPs. Nevertheless, citric acid is relatively more expensive. Crude rapeseed oil that was subject to degumming using 0.1% of citric acid at 70 °C managed to produce a degummed oil containing 2 ppm phosphorus and 0.3 ppm iron (Segers 1994).

TOP or commonly known as the 'total degumming process' employed two steps for degumming. At the initial step, water-degummed oil or crude oil is mixed with 14% of phosphoric acid at 0.1 wt% ratio under elevated temperature. Phosphatide metal complexes are removed during the stage. Subsequently, the degummed oil is neutralised with diluted base, e.g. NaOH, Na_2CO_3 or Na_2SiO_4 to remove the decomposed phosphatic acid. Neutralisation was not until the extend that leads to the formation of soap. A centrifugation process is used to separate the neutralised acid and the metal complex to form a low PL oil (Ohlson 1992; Zufarov et al. 2008; Dijkstra 2013a, b).

Crude oil with a high phosphatide and iron contents can be treated with soft degumming approach (Gibon and Tirtiaux 1998). A 2% solution containing a complexing molecule [ethylene diamine tetraacetic acid (EDTA)] and a wetting compound [sodium dodecyl sulfate (SDS)] is mixed with heated oil (75–85 °C) and treated from 20 min. As chelating agent, EDTA served to chelate metals such as magnesium, calcium and iron. Whilst, SDS acts to attract the iron Soft degumming managed to produced less than 1 ppm of phosphorus and 0.1 ppm of iron (Gibon et al. 2007). The degree of contact between chelating agent (EDTA) and the NHP is important to determine the efficiency of the degumming process. In order to facilitate the mixing and contact of the NHP in the oil phase and the water phase solution, detergent such as sodium lauryl sulfate (SLS) can be added. However, this approach has its setbacks as a highly stable emulsion is usually developed and this hinder the separation of the oil and water phases (Molik and Pokorny 2000).

4.3.2 Deacidification

After oilseed is harvested, the naturally-present lipase enzyme in the crude oil hydrolyses ester bonds and produces free fatty acids (FFA). This lipolysis reaction can be initiated and enhanced in the presence of moisture and heat (Nawar 1996). As compared to glycerol esters of the fatty acids, the FFA is more susceptible to oxidation which negatively affects the oxidative stability of an oil and causes it to turn rancid. Therefore, the FFA needs to be removed along with other impurities, and the oil acidity should be minimized. In a refining cycle, removal of the FFA is termed as deacidification process which determines the efficiency of the subsequent process operations and the quality of the final product (Bhosle and Subramaniam 2005). A number of deacidification methods are available conventionally (Table 4.4), besides other special methods which are gaining interest nowadays and are still under research.

4.3.2.1 Alkaline Refining Methods

Alkaline refining, or commonly termed as caustic soda process, is a conventional way of removing mainly FFA from oil in a refining process (O'Brien 2009). Since only caustic soda is mainly used in the fats and oils industry it gives the name of deacidification. In some industries, potassium hydroxide is used to replace the caustic soda.

An FFA is acid in nature due to presence of H+ of the carbonyl group. Upon addition of caustic soda (NaOH), its OH- group reacts with the H+ producing soap and water. This saponification reaction is visualized in Fig. 4.2. Soap formed is washed up using water and further separated by either static separators for small-scale batch processes or centrifugal separation for continuous and large-scale

Table 4.4 Principles of oil deacidification methods

Deacidification methods	Principles
Alkaline refining	Addition of caustic soda to neutralize and saponify the FFA
Physical Refining	Steam-stripping of FFA from the oil into fatty acid distillates
Re-esterification	
Chemical re-esterification	The use of free hydroxyl groups in the oil or added hydroxyl groups from glycerol to re-esterify FFA into neutral glycerides.
Enzymatic re-esterification and Amidation	The use of microbial lipases to synthesize a triglyceride from FFA and acyl acceptor
Biological refining	The use of a whole-cell microorganism system which selectively removes and/or assimilates FFA for its own growth
Solvent	Separation of FFA based on the different solubility of fatty acids and neutral triglycerides in various organic solvents
Membrane	The use of membrane or semipermeable barrier to separate the FFA from triglycerides

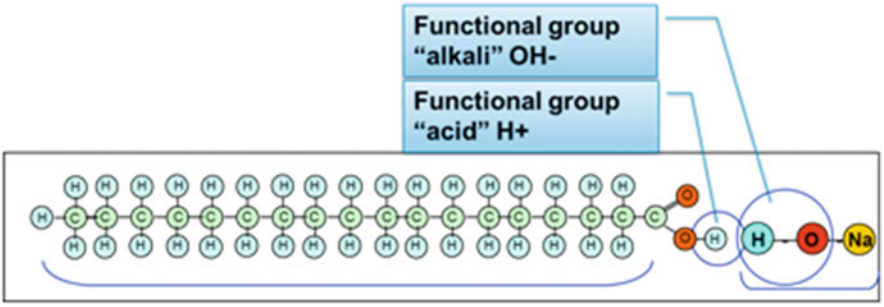

Fig. 4.2 Saponification reaction. (Adapted from Zeldenrust 2020)

processes (Zeldenrust 2020). Besides saponifying the FFA, the alkali is absorbed by phosphatides and gums present in the oil and are coagulated through hydration or degradation. Moreover, the coloring pigments are degraded and converted into water-soluble compounds by the alkali, or absorbed by the gums, while insoluble matters are entrained with the other coagulable materials. Therefore, in overall, other undesirable non-glyceride materials such as phospholipids (gums), oxidized products, metal ions (e.g. iron, copper), colour pigments (e.g. gossypol), insoluble impurities (e.g. meal fines) are also removed in this process besides FFA (O'Brien 2009; Zeldenrust 2020).

This alkaline refining process is applicable for majority of the crude oils, including oils with low quality, except for castor oil (Zeldenrust 2020). Most industries use this process for oil deacidification due to its ability in reducing the FFA to the desired level, regardless of the quality of the crude oil and its initial FFA content (Bhosle and Subramaniam 2005). Table 4.5 shows significant findings from a number of studies which used NaOH solution for deacidification of different type of vegetable oil samples.

The main drawback of alkaline refining is the loss of neutral oil—either been hydrolyzed by the caustic, or loss in the form of occlusion in soapstock. The soapstock can hold up to 50% of its weight of neutral oil (Bhosle and Subramaniam 2005). These undesirable situations take place especially upon extended processing time of high temperature. Therefore, selection of NaOH and the processing condition are critical to minimize the loss of neutral oil (O'Brien 2009). Factors affecting the effectiveness of the NaOH used and the process efficiency include the strength of the NaOH, the mixing time, energy, and temperature, and the amount of excess caustic. In order to produce a good quality oil with acceptable amount of oil losses, the processing steps are normally determined and adopted based on the crude oil quality (Zeldenrust 2020).

Selection of refining process varies with the type and quality of oils processed, the plants, and the countries where the oils are processed, among others. Figure 4.3 summarizes different alkaline refining methods conventionally carried out in the industry, and a number of special processes which also utilize alkaline for the deacidification purpose.

Table 4.5 Alkaline refining of different type of vegetable oils

Vegetable oil	Solvent	Findings	References
Degummed palm oil	NaOH solution	89% decreased in acid value	Franke et al. (2009)
Degummed rape-seed oil	NaOH solution	87% decreased in acid value	
Degummed kenaf seed oil	NaOH solution (12 °Bé)	40% decreased in FFA 35% increased in total carotenoid content 105% increased in DPPH radical scavenging activity 37% increased in ABTS radical scavenging activity	Chew et al. (2016)
Crude kenaf seed oil	NaOH solution (16 °Bé)	94% decreased in FFA 46% decreased in PV	Chew et al. (2017)
Crude soybean oil	NaOH solution (20 °Bé)	99% decreased in acid value	Shi et al. (2018)
Crude palm-pressed fibre oil	NaOH solution	97% decreased in FFA	Nur Sulihatimarsyila et al. (2019)

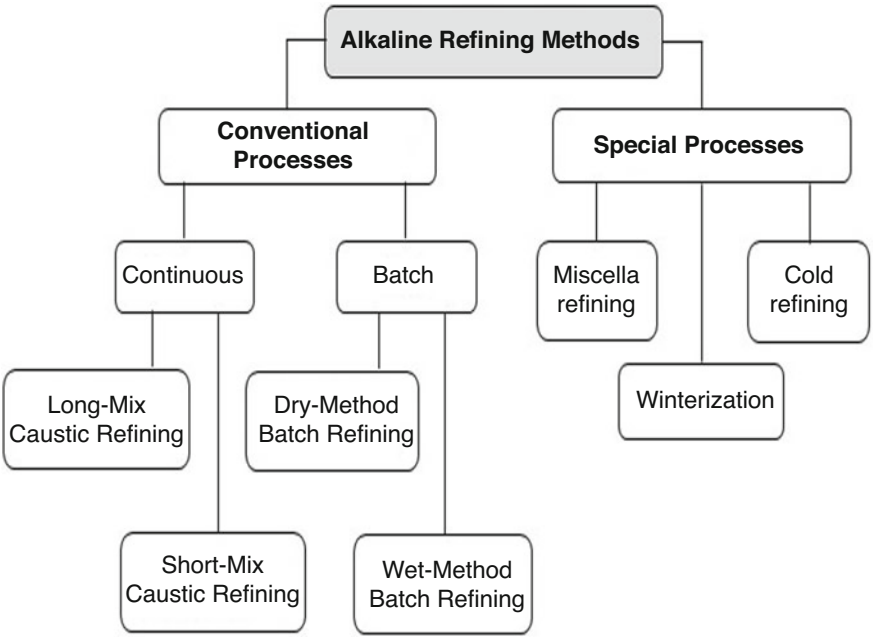

Fig. 4.3 Alkaline refining methods. (Adapted from O'Brien 2009, Zeldenrust 2020)

Prior to the deacidification process, seed oils are normally degummed for removal of phosphatide content which can negatively affect the oil yield. Moreover, the use of clean and dry crude oil can ensure a trouble-free continuous refining operation. The crude oil should be free from air—along the refining process, the soapstock should settle at the bottom of the refining kettle. However, if air is present in the oil, the soapstock can entrain the air and float on top of the oil which is undesirable. Therefore, if the oil contains occluded air after been pumped into the refining kettle, the oil must be settled within adequate time to allow the air to escape (O'Brien 2009). Damages to the pumps and both the recording and metering instruments can be prevented upon incorporation of additional filters in the process line which function in retaining impurities (Zeldenrust 2020).

The crude oil is normally conditioned before being treated with the caustic soda. For this purpose, food-grade phosphoric acid is commonly added at different concentrations based on the type of oil to be refined (300–1000 ppm for soybean oil; 1000–3000 ppm for canola oil). The conditioning process can last for 4 h minimum, up to preferably 8 h in an appropriate pre-treatment or supply tank. The main objectives of this crude oil conditioning are to precipitate phosphatidic materials, precipitate natural calcium and magnesium as insoluble phosphate salts, inactivate trace metals that may be present in the oil such as iron and copper, reduce the loss of neutral oil, destabilize and enhance the removal of pigment chlorophyll in bleaching, and to enhance the color and flavor stability of the finished deodorized oil (O'Brien 2009).

The concentration or the strength of the caustic soda (°Bé) to be used depends on a number of factors, including the type of crude oil, its FFA content, the refining equipment available, the refining 'history' of the oil, and the amount of acid added during the conditioning step. At time, excessive caustic soda is required depending on the type of oil and its quality. In most cases, the caustic soda is prepared at 32 or 50% concentration, kept in a caustic tank, and is likewise circulated. Upon addition of the caustic soda, the FFA and the added phosphoric acid in the oil should completely be neutralized (O'Brien 2009; Zeldenrust 2020).

In the case of cottonseed oil, upon heat treatment which leads to oxidation, the sensitive gossypol pigment forms colored compound which is difficult to be removed except by using caustic. Therefore, for removal of gossypol compound, excess of a more concentrated of NaOH is used, or the caustic refining process is normally repeated. Despite repeating the caustic treatment, the loss of neutral oil is relatively low due the fact that the oil has already undergone degumming and the first caustic treatment (O'Brien 2009). This re-refining process utilizes the caustic soda from the first refining stage. The process control system is used once again to calculate the amount of water needed for the caustic dilution (Zeldenrust 2020).

High-speed centrifuges are commonly used for the soap-oil separation following the caustic treatment. Two different phases are formed, i.e. the neutral oil containing traces of moisture and soap (light phase), and the soapstock which is a mixture of primarily insoluble soap, meal, free caustic, phosphatides, and small quantities of neutral oil (heavy phase). Complete separation however is not possible even under the most optimum conditions; thus, the first separation step is always followed by a

number of water-wash steps. At the end of the refining process, the refined oil is treated with vacuum drying to ensure maximum removal of moisture content. A multi-stage steam jet vacuum pump with mixing or surface condensers equipped with vacuum meter is normally used to produce the vacuum (O'Brien 2009; Zeldenrust 2020).

4.3.2.1.1 Traditional Alkaline Refining

Most conventional alkaline refining methods are conducted continuously, either using long-mix or short-mix process. The choice of method specially depends on the countries where the oil is refined, the available equipment, and the crude oil quality, among others. These continuous refining processes are also adopted in batch systems termed as dry-method which resembles that of long-mix process, and wet-method which resembles that of short-mix process. Batch systems are suitable to be installed and conducted when the amount of refined oil required is low, and in production of specialty oils of good or premium quality. It is still a practice in some developing countries for small- and pilot-scale production lots. Low investment cost is needed. However, the process is time-consuming, generates high refining losses, charges high load into wastewater plants, and is high in operational costs. Therefore, it is normally installed for special conditions only (O'Brien 2009).

Long-Mix Caustic Refining

This process (Fig. 4.4) is normally adopted in the United States and has been established for refining of soybean oil, involving combination of degumming and neutralization. It is commonly installed with two separation stages nowadays, yet single-stage and three-stage are also common. The choice depends on the oil that needs to be treated. In the case where single-stage process is installed, the residual soaps are removed by filtration using silica, silicate, cellulose, or bleaching earth as filter aids (Zeldenrust 2020).

Short-Mix Caustic Refining

In Europe, the oilseeds or crude oils must be imported which vary in quality. Most of the time, the long journey resulted in oils with high FFA. If long-mix process is carried out for these imported oil samples, high amount of caustic and long caustic-oil contact time is needed to decrease the high FFA content. Therefore, to avoid these undesirable situations, the short-mix caustic refining process was adopted in Europe after World War II, and the processing steps are shown in Fig. 4.5. As compared to the 15–30 min caustic-oil mixing time at 30–35 °C in the long-mix process, shorter mixing time of 30 s (maximum) at 85–90 °C is utilized in the short-mix process (O'Brien 2009).

Losses of natural oil can be further reduced by using ultrashort-mix caustic refining as compared to the short-mix process. The caustic soda is introduced

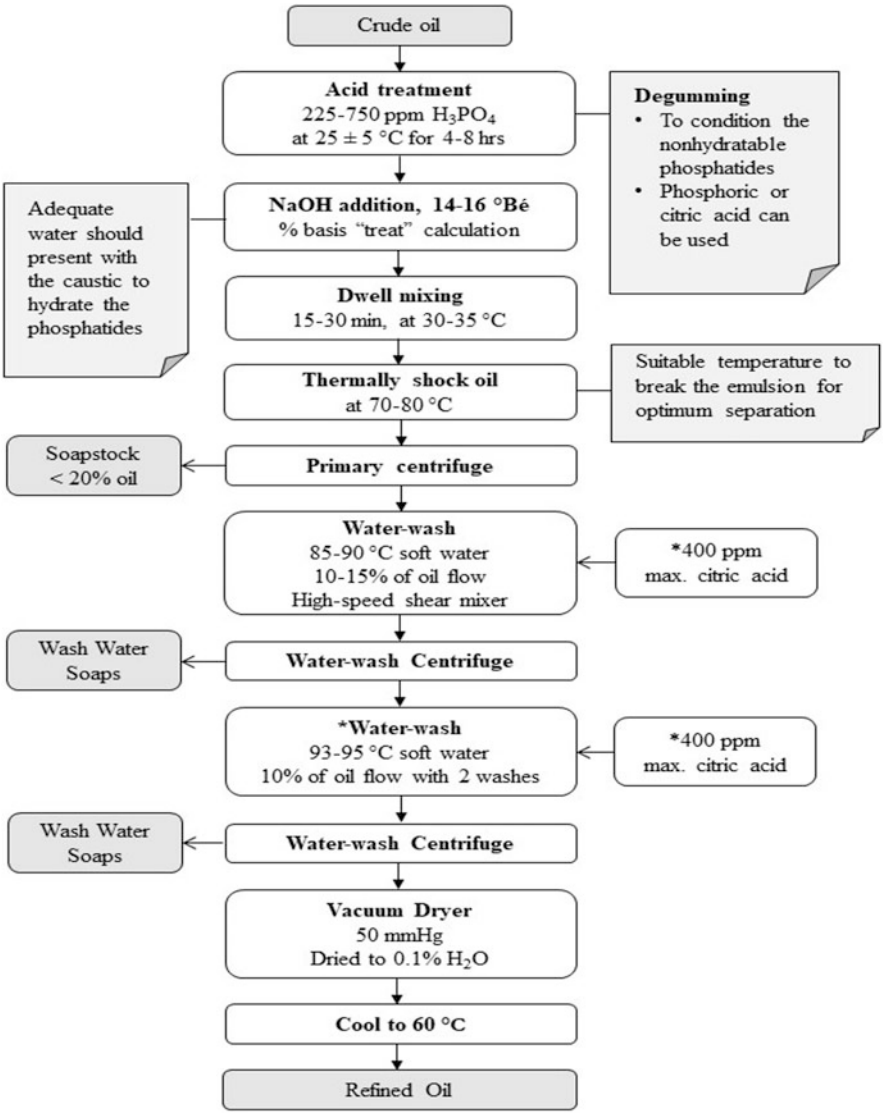

Fig. 4.4 Long-mix caustic refining—a three stage separation process. *Options; addition of citric acid or phosphoric acid can be done in the case where extremely low residual contents is required in the neutral oil. (Adapted from O'Brien 2009, Zeldenrust 2020).

directly in a hollow centrifuge spindle consisting of a special mixing device. The caustic-oil mixing takes place at a very short time in the centrifuge, thus allowing the use of caustic soda with greater strength whilst minimizing excess saponification of

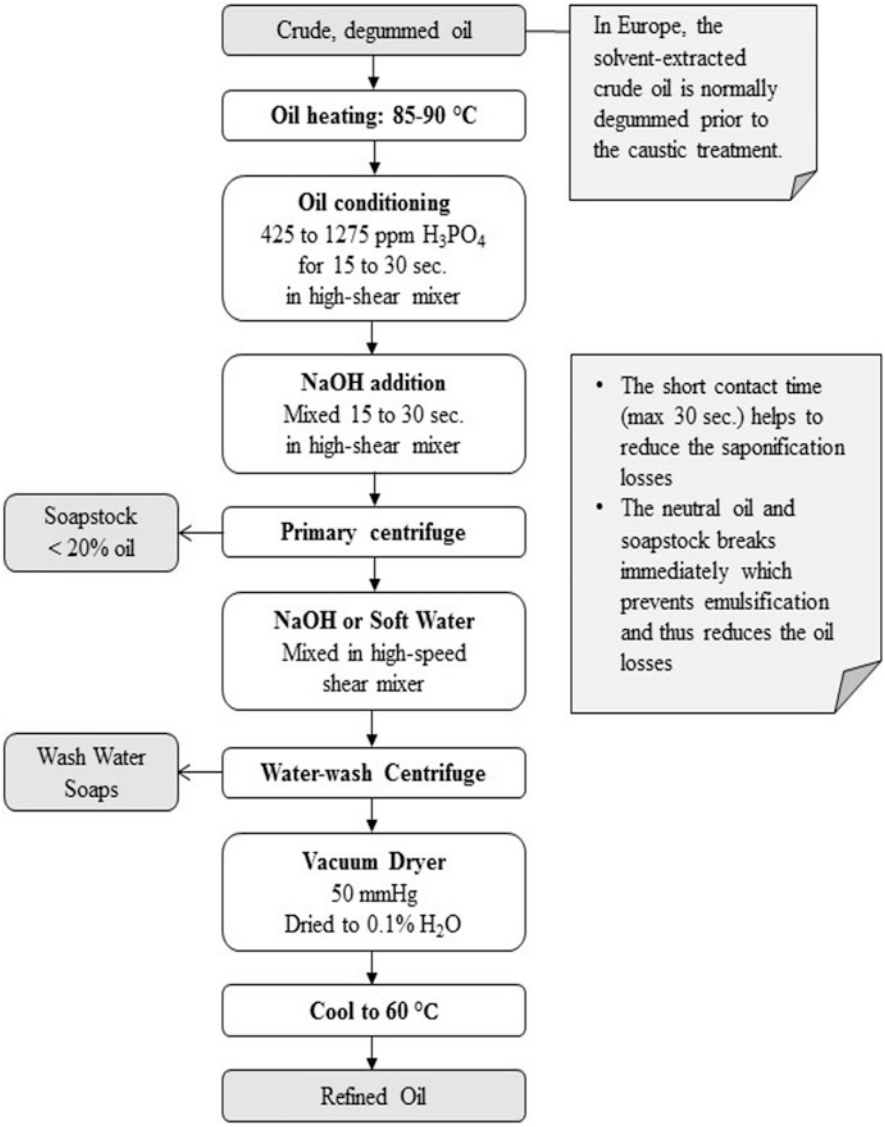

Fig. 4.5 Short-mix caustic refining process. (Adapted from O'Brien 2009)

the neutral oil. This approach is suitable for crude oil with high level of acidity such as palm. As compared to the short-mix process, this process resulted in 7.0–16.5% less loss of neutral palm oil (Braae 1976; O'Brien 2009).

Dry-Method Batch Refining

Figure 4.6 shows the steps involved in the dry-method batch refining process. The process is normally carried out in a conical-bottom tank or kettle with an open top, equipped with a two-speed agitator and steam coils for heating. The agitator's shaft is centered in the vessel and equipped with sweep arms. Each arm is equipped with paddles which push the liquid oil upward during agitation. The agitator is either extended from the bottom or suspended from the top with rates of 8–10 rpm minimum and 30–35 rpm maximum. Before the process is carried out, the crude oil should be in molten or liquid state—the oil can be kept at ambient temperature or at temperature just high enough for this purpose (O'Brien 2009).

Wet-Method Batch Refining

This refining process is applicable for oil samples with high FFA content such as palm and olive oils, besides other lauric oils including coconut oil. The oil is first heated up to 65 °C, and high caustic concentration of 20 °Bé is used with about 0.10% excess treatment. In order to break the soapstock and the oil emulsion, 0.10% NaCl is added per 1.0% FFA in most cases. As indicated by the method's name, a spray of hot water is introduced onto the oil's surface to wash down the precipitated soapstock, followed by several water washes for complete soapstock removal from the oil. Adequate settling time is required between each water-wash step. The equipment used is nearly similar with those of dry-method batch refining, yet for the purpose of vacuum bleaching and water-wash steps, closed tanks are normally used in this process (O'Brien 2009).

4.3.2.1.2 Special Alkaline Refining Methods

Miscella Refining

The use of solvent for oil extraction from oil-bearing materials is commonly followed by stripping off the solvent from the extracted oil. In the case of miscella refining, the process takes place as soon as the oil is extracted, preferably within 6 h, prior to the solvent-stripping step (Bhosle and Subramaniam 2005). Miscella is a mixture or solution containing the solvent-extracted oil. The process can be conducted either continuously or in batch in facilities with an existing oilseed solvent extraction system. The same solvent recovery unit can be used for both oil extraction and oil deacidification from most fats and oils of seeds and animal origin. The crude miscella can be obtained from O'Brien (2009) and Zeldenrust (2020).

- Pre-evaporator of a direct-solvent extraction plant
- A blend of pre-pressed crude oil and solvent-extracted miscella from the press cake
- A reconstituted blend of crude oil with solvent

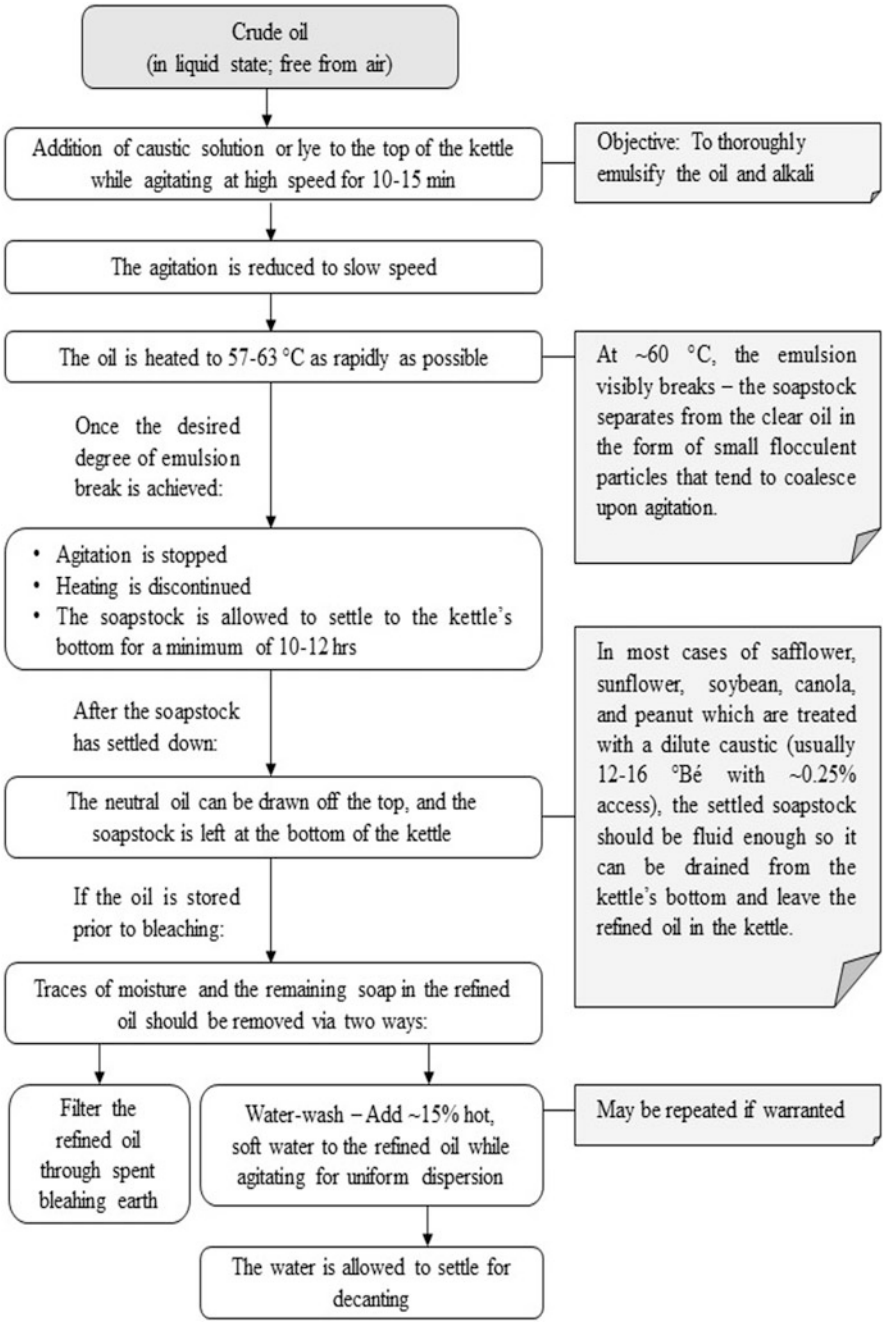

Fig. 4.6 Dry-method batch refining process. (Adapted from O'Brien 2009)

Table 4.6 Advantages and disadvantages of miscella refining method

Advantage	Disadvantage
1. Dilute caustic soda solution of 10–14 °Bé can be used satisfactorily 2. The oil-hexane solution and the caustic added are greatly different in specific gravity which allows efficient separation upon centrifugation 3. Extraction of the color pigments which leads to lighter oil color without bleaching as compared to non-miscella refining 4. The oil's color can be varied by increasing the amount of caustic added without critical loss of neutral oil 5. Higher oil yield 6. Elimination of water-wash step	1. All equipment and facilities must be totally enclosed and explosion-proof for solvent handling, and well-maintained to avoid excessive solvent losses and accidents 2. More elaborate laboratory facilities and staffing are necessary to control this process 3. Higher investment cost due to the requirements on the equipment, facilities, and staffing. The process needs to be carried out at the solvent mills to be economical and effective

Adapted from Bhosle and Subramaniam (2005), O'Brien (2009), Zeldenrust (2020)

This process was originally established and commercialized for neutralization of cottonseed oil since it allows removal of most gossypol, thus producing oil with lighter color with high neutral oil yield. Most of the cottonseed oil mills in the United States have expanded their solvent extraction unit to include the miscella deacidification, thus producing cottonseed oil of lighter color at lower cost and lower refining loss (Wan et al. 1996). The advantages and disadvantages of this process as compared to conventional continuous caustic soda refining process are summarized in Table 4.6, while the processes involved are shown in Fig. 4.7.

4.3.2.2 Winterization

This refining process is applicable in the case of some vegetable oils such as sunflower or corn oil which contain waxes (long chain fatty alcohols and fatty acid esters). The waxes are undesirable, since they crystallize at low temperatures and further cause turbidity in the oil. A neutralization process followed by wet winterization is suitable to remove the waxes as shown in Fig. 4.8. At the end of the process, the residual soap content is washed out using a separator, and the refined oil is further treated with vacuum drying.

4.3.2.3 Cold Refining

This process as shown in Fig. 4.9 is another option for the winterization process. It is more suitable for deacidification of oils with relatively low FFA as the oil losses are otherwise too high.

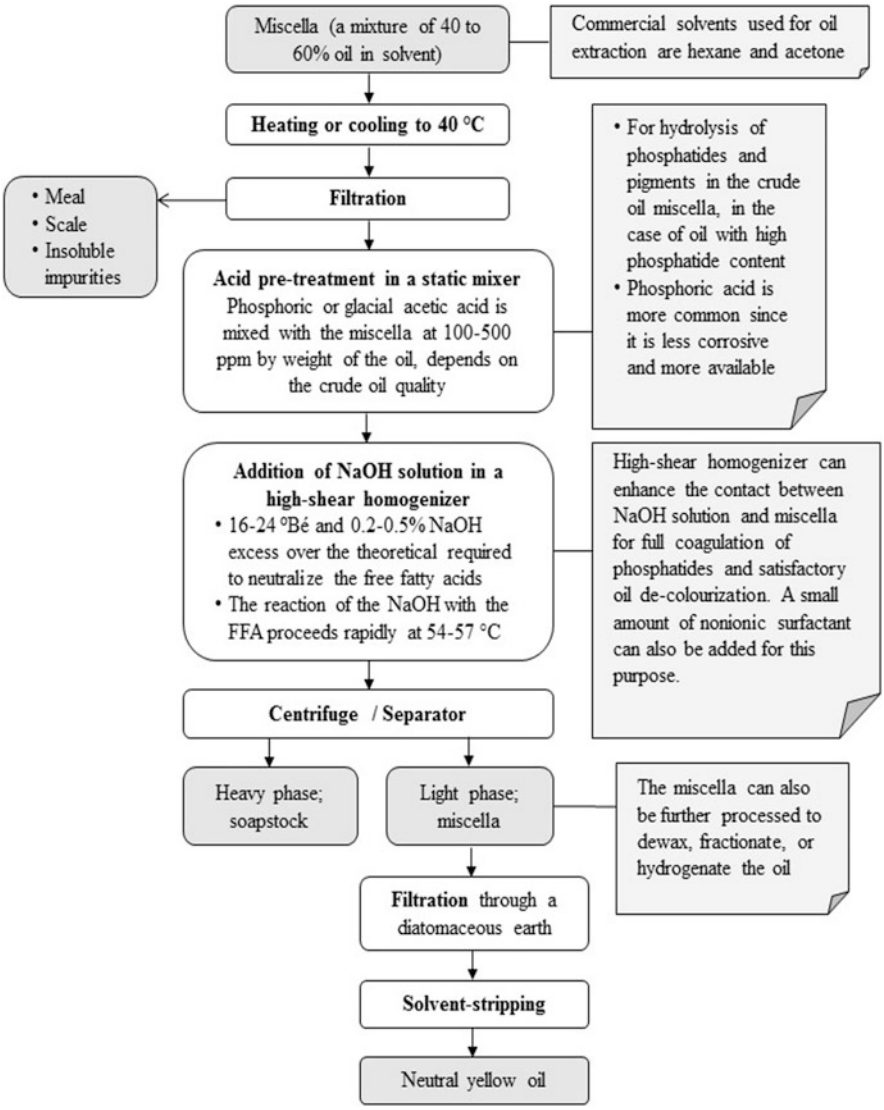

Fig. 4.7 Miscella refining process. (Adapted from Bhosle and Subramaniam 2005, O'Brien 2009, Zeldenrust 2020)

4.4 Bleaching

In edible oil refining, the bleaching treatment is a critical step in order to eliminate pigments and other impurities, such as soaps, trace metals, phospholipids, oxidation products, and polyaromatics in producing a clear edible oil (Mag 1990). Simultaneously the phosphorus and iron are removed during bleaching. By minimizing the

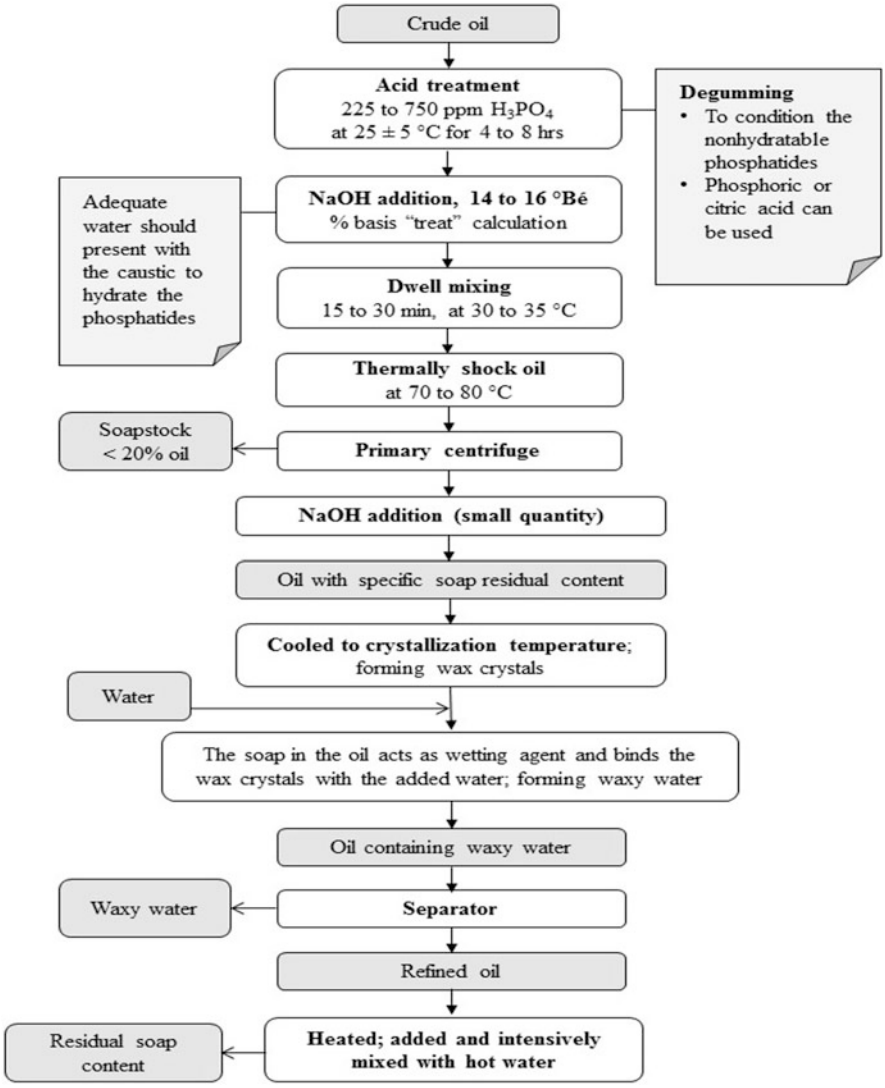

Fig. 4.8 Winterization process. (Adapted from Zeldenrust 2020)

level of phosphorus and iron as well as oxidative products, it will prevent colour fixation that might take place during deodorization process. Removal of these impurities help to improve the sensory attributes and enhance the oxidative stability of the deodorised oil (De Greyt and Kellens 2000; Gibon et al. 2007). Adsorbents is usually used for bleaching. Typically, these impurities are attracted *via* Van der Waal's force of attraction with the active sites of the adsorbent. The strength of attraction relies on a few factors as follows (Gupta 2017):

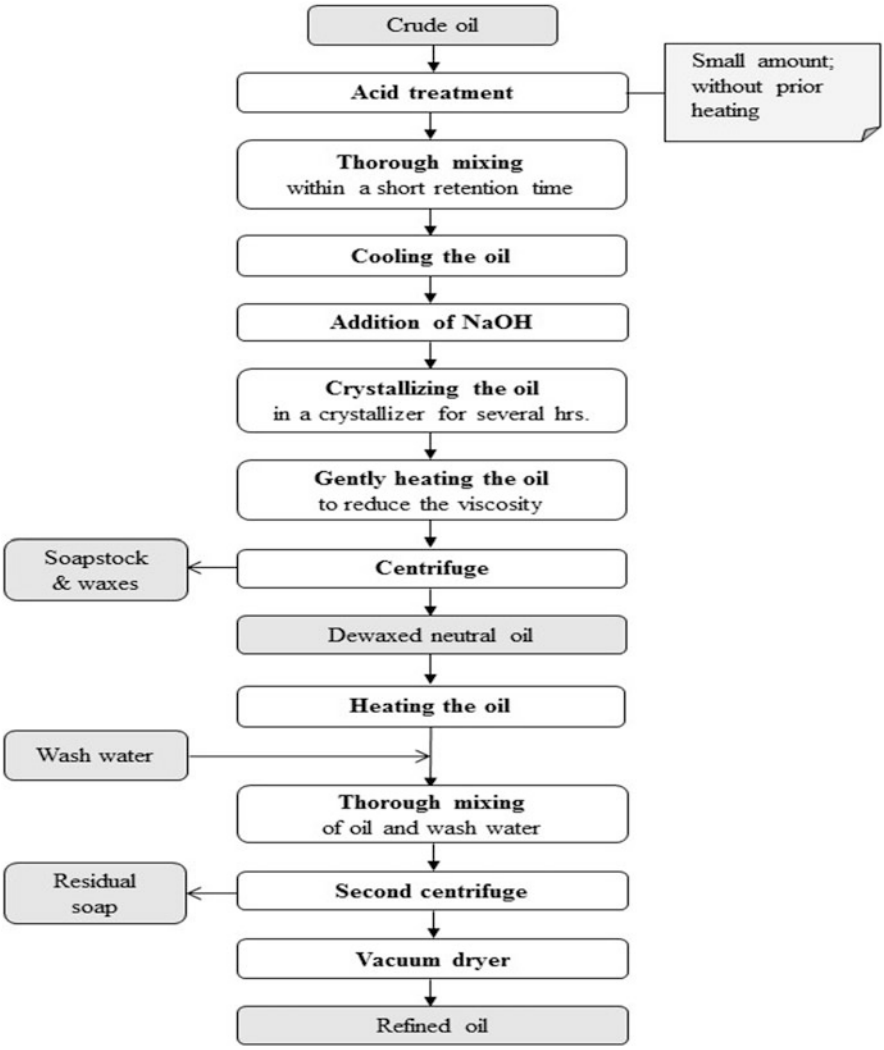

Fig. 4.9 Cold refining process. (Adapted from Zeldenrust 2020)

1. The strength of electrostatic force between the impurities and the adsorbent.
2. The particle size of the absorbent and impurities or pigment.
3. The distance or the contact of mixing between the oil and the adsorbent.
4. Porosity of the adsorbent particles.
5. Surface area of the adsorbent.

4.4.1 Types of Adsorbents for Bleaching

Generally, bleaching earths used derived from three types of clay minerals such as bentonite, attapulgite and sepiolite.

4.4.1.1 Natural Clays

Natural clays/bleaching earth is commonly known as "fuller's earth" since 1880. Usage of natural clays for bleaching was discovered from its usage to bleach olive oil. Natural active clays comprised a number of silicates such as bentonite, palygorskite, hectorite or sepiolite. It was processed in physical rather than chemical way and possess some bleaching activity. Oil refiners used natural clay mainly for color removal. However, this type of clay is suitable for crude oil with having lighter color instead of darker colour oils containing chlorophyll or poor quality crude oil.

4.4.1.2 Activated Clays

Activated bleaching earth is the most common adsorbent used in edible oil bleaching to absorb cationic or polar components. It is usually made from mineral bentonite in which majority is composed of montmorillonite. Bleaching earth is usually treated with mineral acid (sulphuric or hydrochloric acid) at high temperature for a few hours in order to activate it before used. Nature of the raw clay are important to determine the optimum amount of acid and the conditions for activation. Whilst, the bleaching capacity depends on type of oil (Fahn 1973).

4.4.1.3 Activated Carbon

A variety of carbon-containing substances such as coal, coke, saw dust, peat, wood char, coke from coconut shells, etc. can be used for the production of activated carbon to remove particularly polyaromatic hydrocarbons and ensure removal of a wide range of specific pollutants (Zschau 2001). The activation of carbon is performed out by chemical or gas activation under elevated temperature of around 400–800 °C.

4.4.1.4 Mechanism of Bleaching

Bleaching process involves several mechanisms from physical to chemical interactions to improve the quality of oils which includes (David et al. 2020):

(a) Adsorption—the absorbent attracted to the contaminant via three different mechanism: (1) Physically: involving van der waals forces (2) Chemically: by

electromechanical bonding (3) Molecular sieves: entrapment under pressure inside the pores of the clay during filtration

(b) Absorption—mechanism where the intra-granular pores are filled with some fluids mainly oil and it turned the contaminants came along with it. Oil retention depends on a number of variables including clay dosage, clay characteristic (e.g.: particle size distribution and type of minerals), permeability of the filter bed, feed oil quality, cleanliness of the filter screen and the filter conditions before filter cake disposal.

(c) Filtration—Mechanism of trapping and physical act of filtering out the suspended clay had simultaneously removes the minor contaminants adsorbed to the clay particles. Filters used in the bleaching system include (1) processing filters (vertical leaf filter), and (2) polishing filters (pocket and cartridge filters).

(d) Catalyst—Mechanism by which contaminants are degraded by the interaction with the surface of the clay. The oil/clay interaction caused a peroxides decomposed into a volatile oxidation by-products and pigments formed a colour compounds with excessive heat and oxidation. Once the colour fixation occurred, red colour became more difficult to be removed by bleaching clay alone and lead to higher red colour after deodorisation.

4.4.2 Effect of Processing Parameters on Bleaching Process

4.4.2.1 Effect of Temperature

According to van't Hoff rule, the reaction speed doubles with every 10 °C increased in temperature. Bleaching conducted at high temperature is desirable to reduce the viscosity of oils in order to increase the contact between the absorbents and oils and hence reducing the bleaching time. Most of the oils are bleached in the temperature range of 90–100 °C or up to 120 °C for oils that is difficult-to-bleached (Fahn 1973; Eicke 1984).

However, high temperature will also shift the adsorption equilibrium toward desorption. Hence, it is imperative to chose an ideal temperature for bleaching. The optimum temperature depends on type of oil, by-products contaminants in the oils.

4.4.2.2 Effect of Time

Bleaching duration often depends on the bleaching temperature. Stout et al. (1949) reported that a colour improvement could be observed when bleaching was conducted at 40 min at temperature of 70 °C. However, bleaching of 20 min is sufficient to notice the full colour improvement. Similarly, Rich (1964) showed that soybean and cottonseed oil require to be bleached for 35 min at 105 °C and 5 min at 120 °C, respectively to attain the best. However, the colour turns dark with a

prolonged bleaching duration of around 55 min. This effect was more pronounce at 120 °C than at 105 °C (Zschau 2001). Practically, palm oil is usually bleached at temperature range of 95–110 °C the for 30–45 min.

4.4.2.3 Effect of Pressure

Eicke (1984) reported the difference effect of pressure on bleaching where the beef tallow was bleached under atmospheric and vacuum conditions. Under vacuum conditions, the pressure works along with temperature to draw the moisture out from the oil. A vacuum that is too strong can detrimentally affect the bleaching efficacy as it pulled-out the moisture from the system too rapidly thereby driving the moisture below the optimal range of 0.06–0.1% for bleaching (David et al. 2013).

4.4.3 Bleaching Processes

4.4.3.1 Dry-Bleaching Process

Dry bleaching is a conventional method used in fats and oils industry. As dry bleaching is conducted under vacuum condition of about 70 torr that greatly reduces the moisture content of the oil, it is as such called as dry bleaching. Dry bleaching has several distinct advantages which include:

1. Economical compared with other types of bleaching (used of plate heat exchangers for heating and cooling lower energy consumption as compared with shell-and-tube solutions)
2. Easy and simple operation
3. A minimal installation space

4.4.3.2 Wet Bleaching

Wet bleaching is performed by contacting the bleaching agent with the oil that is added with water. Incorporation of water enhance the efficiency of bleaching thereby indirectly reduces the usage and cost of the bleaching earth and lower the oil losses. Water is added either from citric acid solution or using wetted oil obtained from separation line. Adjustment is carried out by careful control of the operating vacuum in the bleaching reactor to control the evaporation of the water.

The advantages of wet bleaching include:

1. More efficient use of the bleaching earth
2. Easy to combine with a silica treatment process
3. Highly flexible

4. Low usage of bleaching earth
5. Reduced oil loss during filtration

4.4.3.3 Multi-step Bleaching

Multi-step bleaching involves two-stage of bleaching process. It is performed with a combination of wet and dry bleaching followed by with a highly efficient two-step filtration. Two stage bleaching is regarded to be more efficient than conventional bleaching process. Adsorption of impurities take place in a continuous manner until an equilibrium reached where the concentration of the pigments and impurities in the absorbent is equivalent to their concentration in the oil. Thereafter, filtration is performed to separate the adsorbent from the oil. At the second stage, the treated oil is treated with a fresh batch of the adsorbent. The absorption process is conducted until a new state of equilibrium is reached. This oil then passes through a second filtration stage. The advantages of two-stage bleaching include:

1. Best bleaching results for oils otherwise often considered difficult
2. Possibilities for using a cheaper bleaching agents at first-stage thus reducing operating costs
3. Reduced bleaching agent dosage resulting in lower costs
4. High degree of flexibility

4.5 Deodorisation

Deodorization is basically a vacuum stripping process in which neutral oil is treated to remove FFA and volatile odiferous compounds in order to obtain a bland and odourless refined oil with a good shelf life. Batch-type deodorizers—'Bataille' and 'Lurgi' deodorizers are most widely used in Europe. The deodorization was conducted under vacuum (protect the oil against oxidation) and superheated steam as a stripping agent (to facilitate stripping and to avoid hydrolysis) (Lee and King 1937).

Over the years, deodorization gradually transformed from a 'simple' process to a crucial unit operations of which free fatty acid (referred to physical refining) and volatile contaminants are stripped-out and unwanted colour pigments been degraded (heat bleaching). Recently, increasing attention on the safety and nutritional quality of oils and fats had urged the industrial to continuously improve deodorisation technology to meet the required organoleptic and nutritional quality standard for safe human consumption (Lee and King 1937).

4.5.1 Theory and Principal of Deodorisation

Basically, deodorisation process is a combination of three different process operations: (1) distillation by vacuum stripping of volatile components mainly free fatty acids, tocopherols and tocotrienols, sterols, and contaminants like pesticides or light aromatic hydrocarbons (2) deodorization by removal of odoriferous compounds to make a bland-taste oil (3) heating effect by thermal destruction of pigments (carotenoids) while maintaining low side reaction of polymerization and cis-trans isomerisation (Gibon et al. 2007).

The intrinsic volatility (vapor pressure curve) and the deodorizing conditions (temperature, pressure and amount of sparge steam) often influence the stripping of volatile components in vacuum stripping. The stripping medium are described by the following mathematical equation derived from "Dalton's" and "Raoult's" laws.

$$S = \frac{P_t}{\mathrm{E}.P_i^0}.\ln\frac{V_a}{V_0} + \left(\frac{P_t}{\mathrm{E}.P_i^0} - 1\right).(V_a - V_o) \tag{4.1}$$

Where; S = total moles of steam or other stripping agent per mole of oil (the factor S has to be multiplied by a factor of 2); Pt = total pressure of the gas phase = system pressure; Pi0 = Vapor pressure of a given fatty acid I; E = vaporization efficiency; Va = initial amount of the volatile component in the oil (moles), V0 = final amount of the volatile component in the oil (moles).

As shown from the Eq. (4.1), it can be deduced that the amount of sparge steam required to strip of a given volatile component (e.g. free fatty acids) is positively correlated to the absolute pressure in the deodorizer and the vapour pressure of the volatile component but inversely related to the overall vaporization efficiency.

From the factor (ln Va/V0), it is impossible to totally removed all volatile components via deodorization. Similar amount of stripping steam is required to reduce the concentration of a given volatile component by half, irrespective of its absolute concentration.

The vaporization efficiency E in Eq. (4.1) measure the degree of saturation of the stripping agent (steam) with volatile components when in contact with the oil. It is usually considered as a deodorizer design-specific factor. E = 1 is assumed to be the ideal case theoretically. However, in most of the cases the industrial deodorizers only managed to have the value of 0.3–0.7, depending on their design (Gibon et al. 2007; De Greyt 2020).

Actual deodorization is mainly focusing on removal of odoriferous compounds that already present in crude oil as well as that formed by thermal degradation of flavour precursors. It is more complicated and longer to conduct a perfect due to the presence of a variety of non-volatile flavour precursors. A certain amount of time needed for deodorization to transform these compound into more volatile off-flavors to be stripped-out from the oil. Insufficient deodorization time can cause some flavour precursors remains in the deodorised oil (De Greyt and Kellens 2005).

Thermal destruction of pigments (carotenoids) also known as "heat bleaching" is purely time-temperature-dependent reaction, not affected by the deodorization pressure. Carotene is degraded during the deodorization/steam refining of palm oil mainly due to heat treatment. Deterioration of carotene is relatively very slow at 210 °C as compared to T > 260 °C which may happened within a few minutes. On the other hand, deodorization has the potential to cause darkening of oil. This phenomenon is also known as "color fixation'. It takes place mainly attributed to the when oils are poorly treated and contains high residual phosphatides, iron, other impurities or traces amount of bleaching earth (De Greyt and Kellens 2005; De Greyt 2020).

Recently, there is a general trend to conduct deodorization at lower temperature in concern to the current awareness on heat treatment on the formation of thermal degradation products such as trans fatty acids, polymeric triglycerides, 3-mcpd esters and glycidyl esters.

4.5.2 Deodorization Process Condition

The deodorization process relies on four processing parameters which are (1) temperature, (2) time, (3) pressure and (4) amount of stripping steam. The optimum process parameters depend on the type of oil (bleached and refined oil specifications) and the refining process applied (chemical or physical). Table 4.7 showed the typical range of the different deodorizing process parameters for general guideline (De Greyt 2020).

Table 4.7 Typical process for edible oil deodorization

Parameter	Remarks
Temperature (160–260 °C)	Low (<200 °C): Thermaliable oils (e.g. fish oil) to avoid deterioration of heat sensitive omega-3 fatty acids (fish oil)
	High (260 °C): Stripping of FFA/heat bleaching (e.g. physical refining of palm oil)
	Latest trend: a mild temperature (≤240 °C) to reduce the formation of 3-MCPD esters and GE formation
Time (5 min–4 h)	Short (5 min): FFA stripping (with packed column) without deodorization
	Short (20–90 min): Deodorization of soybean/canola oil
	Long/full deodorization (2–4 h): Fish oil
Pressure (1.5–5 mbar)	Common range: 2–4 mbar
	Low pressure: Stripping of FFA and volatile contaminants (pesticides, light PAH, etc.)
	Latest trend: a mild deodorizing pressure to allow stripping efficiency at lower temperature
Stripping steam (0.5–3%)	Depend on type of oil and refining mode
	Steam is the most commonly used stripping agent (efficient—Lowest cost)

Table 4.8 Effect of process variables on quality of deodorized oil

Quality parameter	Temperature	Time	Pressure	Steam
Taste	+	++	+	++
Color (heat bleach)	++	+	–	–
FFA removal	++	–	++	+
Trans fatty acid formation	++	++	–	–
Tocopherol/sterol stripping	++	–	++	+
Pesticides, PAH, dioxin	++	–	++	+
Glycidyl ester formation	++	++	–	–

Table 4.8 summarized the effects of deodorization process conditions on the standard quality of deodorized/refined oil as described in the literature (Dijkstra 2007; De Greyt 2020).

Deodorization can be performed in different ways (continuous, semi-continuous, or batch). The selection of most appropriate deodorizer technology depends on many factors, such as the number of feedstock changes, heat recovery, investment, and operating costs.

4.5.2.1 Batch Deodorisation

Batch deodorization is suitable for small capacities (<50 ton/day) production, irregular production or small batches of different oil. Main advantages of a batch deodorizer are more flexible (process parameters can easily be adjusted according to the incoming oil quality) and the minimal intermixing between two consecutive batches. However, batch deodorisation less attractive in current oil refining industry due to high operating costs (high steam consumption and very low heat recovery) and relatively long processing time (can up to 8 h) (De Greyt and Kellens 2005; Gibon et al. 2007).

4.5.2.2 Semi-continuous Deodorisation

Semi-continuous deodorizers are basically batch systems designed for larger capacities. Their main application is in plants with frequent feedstock changes of oils sensitive to cross contamination. The semi-continuous design allows more efficient heat recovery than a batch system by means of indirect economizers. Lower intermixing and shorter time for feedstock changes are the main advantages of semi-continuous deodorizers over continuous deodorizers (De Greyt and Kellens 2005; De Greyt 2020).

4.5.2.3 Continuous Deodorisation

Continuous deodorizers are most preferred for high-capacity plants operation running on single feedstock. The main advantages are moderate investment costs, low utilities cost (high heat recovery), excellent process control and easy maintenance. Continuous deodorizer is performed via vertical tray-type deodorizers and is considered as the most commonly used deodorizers. A series of trays or compartments are stacked on top of one another in a cylindrical shell. Each tray is designed is meant for a specific task from heating, deodorization and heat recovery are combined in a single vessel (Dijkstra 2007; De Greyt 2020).

Dual-temperature deodoriser successfully been adopted on industrial-scale application with the growing demand to perform deodorization at a lower temperature to reduce the thermal degradation of oils that may lead to the formation of undesirable compounds such as the formation of trans fatty acids in soybean and canola oil and processing contaminants glycidyl esters in palm oil. The deodorizer is operated under two different temperatures to achieve the best compromise for stripping volatile components (shorter time at higher temperature) and actual deodorization (longer time at lower temperature) whilst minimising the thermal effect of the deodorizer (De Greyt 2020).

4.6 Recent Development in Vegetable Oil Processing

4.6.1 Esterification

4.6.1.1 Chemical Re-esterification

Chemical re-esterification involves the use of free hydroxyl groups in the oil or added hydroxyl groups from glycerol to re-esterify FFA into neutral glycerides. This method is normally carried out in an inert atmosphere and at a high temperature of 180–200 °C, with or without catalyst (Anderson 1962; Bhosle and Subramaniam 2005). Some FFA remain in the re-esterified oil which will be further removed by chemical deacidification. Studies on this method evolved starting from 1853, and many studies ever since reported its suitability for deacidification of high-FFA oils and rice bran oil. De and Bhattacharyya (1999) managed to decrease the FFA in rice bran oil to an acceptable level of 0.5–3.5% by conducting an autocatalytic chemical re-esterification using monoglycerides. However, the approach was reported to be costly and is not competitive with physical refining processes. Few more studies also reported the technical feasibility and suitability of this method for deacidification of rice bran oil, yet it is still not commercially accepted (Gingras 2000).

4.6.1.2 Enzymatic Re-esterification and Amidation

Enzymatic re-esterification has gained much interest nowadays as a green and sustainable deacidification method. Microbial lipases are used to synthesize a triglyceride from a fatty acid and glycerol. Therefore, glycerides and other lipids are produced and further increases the oil yield which is greatly advantageous. Moreover, it is a low-energy process which does not need to be conducted at high temperature such as in the chemical esterification. This condition leads to refined oil with better quality. On top of that, this process is applicable for de-acidifying oils with high FFA content. Despite these advantages, only a few studies used this method due to the high cost of enzyme (Bhosle and Subramaniam 2005; Sengupta and Bhattacharyya 1992). Factors affecting the efficiency of an esterification process are as follow, among others (Bhattacharyya and Bhattacharyya 1989).

- Enzyme concentration
- Reaction temperature
- Reaction time
- Glycerol concentration
- Amount of moisture in the reaction mixture
- Pressure employed

As compared to enzymatic esterification, the use of enzyme had shown higher efficiency in FFA removal from degummed rice bran oil through an enzymatic amidation reaction (Table 4.9). Enzymatic amidation converts FFA to a bioactive

Table 4.9 Differences between enzymatic re-esterification and enzymatic amidation systems in the case of high-acid rice bran oil

Enzymatic re-esterification (acyl acceptors: MAG and glycerol)	Enzymatic amidation (acyl acceptor: ethanolamine)
Esterification reaction between –OH and –COOH	Amidation reaction between $–NH_2$ and –COOH
Higher molar ratio of acyl acceptor (MAG and glycerol) to FFA (4:1).	Lower molar ratio of acyl acceptor to (ethanolamine) to FFA (1:1) which can possibly enhance the reactants interaction.
A solvent-free method. The process can be improved by adding appropriate solvent (i.e. hexane) to shorten the reaction time, yet the FFA removal is still not improved significantly.	Involves addition of solvent which decreases the viscosity of the reaction system and therefore enhances substrate interaction.
Less FFA-removal within longer reaction time.	Ethanolamine is more effective as an acyl acceptor which allows removal of greater FFA within shorter reaction time.
MAG and glycerol react with TAG for transesterification, and can also forms partial acylglycerols. Presence of partial glycerides can potentially form glycidyl esters which are undesirable harmful factors in edible fats and oils.	Ethanolamine does not react with TAG.

FFA free fatty acid, *MAG* monoacylglycerol, *TAG* triacylglycerol. Adapted from Wang et al. (2017)

Table 4.10 Studies on the use of enzymes for deacidification of vegetable oils

Vegetable oil	Type of enzyme	Type of acyl acceptor	Findings	References
High-acid rice bran oil	sn-1,3-specific lipase from Rhizomucor miehei, immobilized on an anionic exchange resin	Phytosterol	92% decreased in FFA 29.3% increased in phytosterol ester which further enhances the oxidative stability of the oil during storage Most vitamin E content is retained	Wang et al. (2016)
Degummed high-acid rice bran oil	A recombinant lipase from C. antarctica, expressed in Aspergillus niger, and immobilized on Lewatit VP OC 1600	MAG	68.4% decreased in FFA	Wang et al. (2017)
		Glycerol	76.3% decreased in FFA	
		Ethanolamine[a]	92% decreased in FFA	
	sn-1,3-specific lipase from Rhizomucor miehei, immobilized on an anionic exchange resin	MAG	73% decreased in FFA	
		Glycerol	82% decreased in FFA	
Soybean oil	sn-1,3-specific lipase, immobilized on a macroporous ion-exchange resin	Glycerol	79% decreased in acid value	Shi et al. (2018)
		Ethanolamine[a]	83% decreased in acid value	

[a]Amidation reaction. *FFA* free fatty acid, *MAG* monoacylglycerol. Adapted from Wang et al. (2017)

lipid and maintains the oil yield. This advantage contributes to its higher preference as an FFA-removal method as compared to conventional chemical and physical deacidification methods which always resulted in significant refining losses particularly for high-acid rice bran oil. In spite of that, at the end of the process, residual ethanolamine is present in the edible oil which exhibits unfavorable sensory properties. Ethanolamine is high in polarity and water-solubility, with boiling point of 170 °C. Therefore, it can be removed through a water-washing step, or deodorization at 240–260 °C under reduced pressure (Wang et al. 2017) (Table 4.10).

4.6.2 Biological Deacidification/Biorefining

This process involves the use of a whole-cell microorganism system which selectively removes and/or assimilates FFA for its own growth (Bhosle and Subramaniam 2005; Cho et al. 1990) initiated it through identification of Pseudomonas strain (BG1), a microorganism from soil which is able to assimilate and utilize medium- and long-chain fatty acids including lauric, myristic, palmitic, stearic, and oleic acids as carbon sources without secreting extracellular lipases. Fermentation of oleic acid

resulted in highest biomass as compared to other type of fatty acids, due to its higher water-solubility property which contributed to its greater rate of removal. However, the linoleic acid and short-chain fatty acids of less than 12 carbons which were also high in water solubility were not utilized; most likely due to their inhibition effect towards the growth of BG1. Due to these findings, the biological deacidification process is not of common interest to be applied for oil refining purpose.

4.6.3 Solvent Extraction

Solvent extraction, also termed as liquid-liquid extraction, is mainly based on the different solubility of fatty acids and neutral triacylglycerols in various organic solvents (Bhosle and Subramaniam 2005; Rodrigues et al. 2007). Following this treatment is solvent stripping from the deacidified oil and solvent recovery from the extract stream. These processes can easily be carried out at temperatures lower than 80 °C, down to 55 °C in moderate vacuum condition, by using evaporation or distillation (Pina and Meirelles 2000; Rodrigues et al. 2007). Factors affecting the efficiency of a solvent extraction process include (Rodrigues et al. 2007; Gonçalves et al. 2016).

- Type of solvent
- Solvent/oil ratio
- Water content in the solvent
- Phase separation time
- Process efficiency of solvent stripping

This method was discovered decades ago, and a number of studies had been reported ever since in the case of olive oil (Thomopoulos 1971; Turkay and Civelekoglu 1991) cottonseed oil (Sreenivasan and Viswanath 1973), degummed and dewaxed high-FFA RBO (Bhattacharyya and Bhattacharyya 1989), a model mixture of groundnut oil containing 0–50% fatty acids (Shah and Venkatesan 1989), corn oil (Pina and Meirelles 2000) and rice bran oil (Rodrigues et al. 2006) among others. Rodrigues et al. (2007) had thoroughly reviewed the different studies carried out on the use of solvent extraction as a deacidification technique for various type of vegetable oils. Despite these studies, solvent extraction is still considered as a new approach in deacidification of vegetable oils. Following are the advantages of this method (Bhosle and Subramaniam 2005; Rodrigues et al. 2007; Gonçalves et al. 2016).

- Reducing the loss of neutral oil
- Minimizing the loss of natural and nutraceutical compounds in the deacidified oil
- Can be carried out at room temperature and atmospheric pressure
- Less energy consumption
- Minimizing or avoiding the formation of waste products such as wastewater and soapstock.

Table 4.11 Studies on the use of solvents for deacidification of vegetable oils

Vegetable oil	Type of solvent	Findings	References
Palm oil	Aqueous ethanol	99% carotenoids and 80% tocopherols were retained	Gonçalves et al. (2007)
	Aqueous ethanol	96% decreased in FFA	Gonçalves et al. (2016)
	Betaine monohydrate-glycerol natural deep eutectic solvent	99% antioxidants in the palm oil was retained	Zahrina et al. (2018)
Rice bran oil	Aqueous ethanol	98% decreased in FFA	Rodrigues et al. (2014)
Soybean oil	Aqueous ethanol	89% decreased in acid value	Shi et al. (2018)
Soybean oil	Tetrabutylphosphonium phosphate ionic liquid	80% decreased in FFA	Adhami et al. (2019)
Sunflower oil		67% decreased in FFA	
Canola oil		85% decreased in FFA	

Ethanol, particularly aqueous ethanol, has been the solvent of interest in many studies due to its ability in minimizing loss of neutral oil and nutraceutical compounds in the deacidified oil without compromising the solvent capacity in extracting FFA (Rodrigues et al. 2007) (Table 4.11).

Besides aqueous ethanol, the solvents listed in Table 4.6, and the solvents discussed in a review by Rodrigues et al. (2007) ionic liquids are solvents of interest recently with increasing utilization in liquid-liquid extractions of metal and organic compounds. Benefits and applications of ionic liquids are described in details by Adhami et al. (2019) based on a number of related studies. Ionic liquids are known to be selective, have low melting points, low vapor pressure, and are not volatile which make them categorized as green solvents. Ionic liquids can also be separated from crude oil quickly due to their large differences in polarity. Moreover, addition of water is not required in these types of solvents, thus the problems related to emulsification of the oil are avoidable. Ionic liquids have been utilized in a number of studies and applications in industry for removal of naphthenic acids from crude oil, removal of FFA from soybean oil, extraction of phospholipids from extra virgin essential olive oil and hazelnut oil, and simultaneous degumming and deacidification of soybean oil, canola oil, and sunflower oil.

The advantages and potential of using solvent for deacidification purpose are clearly stated. However, there are still important issues that need to be tackled upon adopting this process in an industrial scale. There is an additional use of other type of solvents such as ethanol, methanol, acetone, and/or ionic liquids for the deacidification purpose, besides the hexane commonly used for the oil extraction from oil-bearing materials. These solvents add on to the total capital, energy, and processing costs of the refinery. In the case of vegetable oils having high FFA, Bhosle and Subramaniam (2005) stated that solvent extraction is promising for

partial deacidification yet Adhami et al. (2019) stated that the use of solvent results in incomplete deacidification. Therefore, combining this deacidification process with other refining steps of degumming, bleaching, dewaxing, and deodorization still needs to be considered and further explored. The quality of the deacidified oil is also a critical matter to investigate, in addition to the efficiency of solvent recovery from both the raffinate and the extract streams (Rodrigues et al. 2007).

4.6.4 Membrane Technology

Membrane technology is an emergent technology (Firman et al. 2013) used for deacidification of crude or degummed oil. Membrane is defined as a semipermeable barrier which allows certain components to pass (i.e. the permeate) whilst preventing others from transporting (i.e. the retentate) (Cheryan 1998; Strathmann 1990). Most researches and manufacturers refer the pore size of a membrane based on the molar mass of the smallest component retained with at least 95% efficiency. This is also termed as the molecular weight cut-off (MWCO). Generally, the membranes are classified according to Table 4.12**.**

The selectivity of the membrane depends on (Cheryan 1998; Bhosle and Subramaniam 2005);

- The membrane's pore size
- The nature and dimensions of the particle or molecule of interest to be separated
- The diffusivity of the solute in the matrix
- The associated electric charges

Generally, the molecular weight of fatty acids are less than 300 Da, while the molecular weight of triglycerides are larger than 800 Da. In an ideal process, the membrane is hydrophobic and exhibits pores so precise that it is able to separate the FFA from the triglycerides effectively (Raman et al. 1994). Figure 4.10 shows the pressure-driven system of laboratory scale membrane in crude palm oil refining system (Azmi et al. 2015). The hydraulic pressure acts as the driving force for the mass transport, while the nature of the membrane selectively controls and separates the components to permeate and to retain according to their molar masses or particle size (Cheryan 1998). Therefore, the overall efficiency of the pressure-driven system

Table 4.12 The pressure level and size of separated molecules based on different type of membrane

Type of membrane	Pressure level (MPa)	Size of separated molecules
Microfiltration	<0.2	0.025–10 μm
Ultrafiltration	>1	1–300 kDa
Nanofiltration	1–4	350–1000 Da
Reverse osmosis	4–10	<350 Da

Adapted from Nakao (1994)

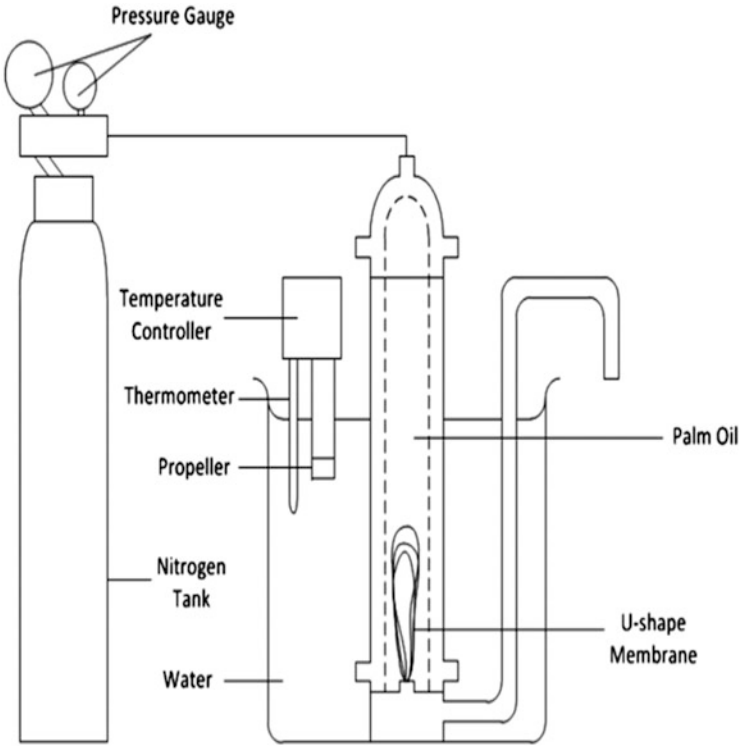

Fig. 4.10 Pressure-driven system of laboratory scale membrane crude palm oil refining system. (Adapted from Azmi et al. 2015)

is mainly affected by the chemical composition of the membrane, besides the processing temperature and pressure, the feed flow, and the interactions between components in the feed flow and the membrane surface (Lin et al. 1997).

The advantages of the membrane technology over the conventional deacidification process are listed as follow (Coutinho et al. 2009):

- Simple to operate
- Applicable in almost all oil processing steps including degumming, dewaxing, solvent recovery, pigment removal, concentration of minor components, and separation of emulsions
- Can be carried out in a continuous or discontinuous way
- Can be combined with other processes
- Can be operated at ambient temperature
- Less number of processing steps required
- Lower energy consumption
- Lower operating, maintenance, and manufacturing costs
- No addition of chemicals

- The oil produced is of better quality with greater retention of nutrients, heat-sensitive and desirable compounds.
- Wastewater treatment is no longer required

These advantages have attracted many researchers and industries to explore on the use of membrane technology in replacement of conventional deacidification methods for different type of oils. Evaluation of such studies had begun since the last few decades. Raman et al. (1994) listed some of the potential applications of membrane technology in vegetable oil processing, which were mainly evaluated at the laboratory or pilot plant scale. Bhosle and Subramaniam (2005) summarized the various approaches made with membrane technology, with and without solvents, by using porous and nonporous membranes, and their limitations. Furthermore, Coutinho et al. (2009) presented the applications and the recent development of membrane technology in the vegetable oil processing.

Despite the advantages and extensive researches carried out, the membrane technology is still inadequate and inefficient to separate the FFAs from the triglycerides. The main limitation is due to the nature of an oil itself—the molecular weight difference between triglycerides (>800 Da) and FFAs (<300 Da) are too small which makes them difficult to be separated by using a membrane (Raman et al. 1994; Firman et al. 2013). This drawback has limited further advancement of membrane technology as a deacidification method in edible oil refining process (Firman et al. 2013). Moreover, there are only few commercial membrane installations in the industries related to edible oil (Bhosle and Subramaniam 2005).

With regard to these issues, the importance of developing highly selective membranes is clearly highlighted (Azmi et al. 2015). Membranes and membrane materials with specific selectivity and good solvent stability can potentially render strong interactions between the membrane surface and the FFA molecules (Azmi et al. 2015). Bhosle and Subramaniam (2005) had presumed the need for alkali treatment to enhance the effectiveness the membrane technology. Without solvent, the permeate flux will be very small (Ladhe and Kumar 2010). Researches on improvement of the membrane technology are still ongoing, and few of them are summarized in Table 4.13.

Table 4.13 Studies on the use of membrane technology for deacidification of vegetable oils

Oil samples	Membrane-based process	Findings	References
Canola oil	Pre-treatment: Addition of chemical agents ($CaCl_2$, EDTA and SDS aqueous solutions) to canola oil miscella Ultrafiltration through PVDF membrane with MWCOs 100 and 50 kDa in a magnetically stirred flat membrane cell at pressure 2 bar and temperature 25 °C.	Reduction of FFA was more in: The miscella filtered without any chemical agents The 50 kDa than that of the 100 kDa The membrane exhibited no appreciable affinity towards tocopherols and carbonyl compounds in the oil samples. The pre-treatment followed by membrane filtration enhanced the quality and stability of canola oil, yet was not as good as conventional refining.	Niazmand et al. (2019)
Soybean oil/hexane miscella	Flat composite nanofiltration membranes of PVDF as a support, and PDMS (10, 12, 15 wt% of Siloc paste in hexane) tor cellulose acetate as coating layer.	The PVDF-12% siloxane composite nanofiltration membrane gives the best result with 80% oil retention and 58% FFA removal.	Firman et al. (2013)
Crude palm oil (CPO)	PVDF crosslinked with PVA solutions of 100–5000 ppm.	Neat PVDF membrane and cross-linked membrane with PVA of >1000 ppm were not capable of removing FFA from CPO feed. PVDF cross-linked with 100 ppm PVA showed highest FFA removal of 5.93% within 3 h operation time.	Azmi et al. (2015)

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Chapter 5
Minor Components in Edible Oil

Wai-Ting Chong, Yee-Ying Lee, Teck-Kim Tang, and Eng-Tong Phuah

Abstract The major component in edible oils is triacylglycerol which consists of 95–98% while the remaining is made up of minor components such as sterols, tocopherols and tocotrienols, carotenoids, squalene, phenolic compounds, chlorophylls, phospholipids and trace metals. Although minor components possess a limited proportion in edible oils, their presence is significant due to the purported health benefits. These minor components are found in abundantly in crude oil but eventually removed during the oil refining process. As some of the minor components such as carotenoids, tocopherols and tocotrienols are beneficial to health, it is important not only to maximize their retention but also to optimize the recovery of these minor components. Other than health benefits, minor components in edible oils also provide a valuable markers for oils and fats authentication. This chapter highlights the properties of various minor components found in fats and oils as well as the recent techniques used for its recovery.

Keywords Tocopherol · Tocotrienol · Sterol · Squalene · Carotenoid · Phenolic compounds · Recovery · Authentication

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5.1 Introduction

The majority of edible oils are sourced from plants viz oil palm, soybean, canola and etc. while some are derived from animals such as cow (butter), swine (lard), and sheep/goat (tallow). The main component in edible oils is triacylglycerol (95–98%). Other than triacylglycerol, edible oils also contain various minor compounds (2–5%) such as sterols, tocopherols and tocotrienols, squalene, carotenoids, phenolic compounds, chlorophylls and traces metals. Composition of minor components in edible oils may vary depending on the species of plants. Moreover, the minor components could be different from the same species of plants due to variation of the growing conditions, geographical locations, milling and refining process.

The presence of minor components influences the physiochemical properties (taste, appearance and nutritional values) of the edible oils. For example, refined red palm oil that does not go through conventional physical refining process is red in colour as a result of the retention of phytonutrients such as carotenoids, vitamin E, and phytosterols (Bonnie and Choo 2000; Nagendran et al. 2000). Minor components are well acknowledged for their health promoting benefits. For example: carotenoids in red palm oil are beneficial in alleviating Vitamin A deficiency (Rao 2000; Zeba et al. 2006). Besides, minor components in edible oils can also act as important and valuable markers to determine the quality, purity and authenticity of edible oils (Tena et al. 2015; Olmo-García et al. 2018). For instance, olive oil can be divided into four different categories which are extra virgin olive oil, virgin olive oil, refined olive oil and commercial olive oil depending on the amount of phenolic components despite sharing similar fatty acid composition (Ramirez-Tortosa et al. 1999; Dais and Hatzakis 2013).

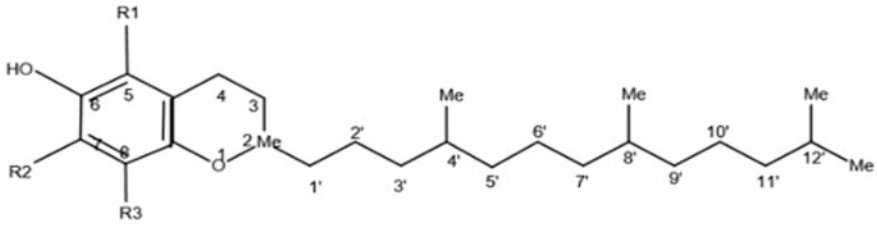
Typically, oil extraction or refining process that is meant to remove objectionable co-constituents of crude oil in order to make them more appealing to consumers in terms of their tastes, appearance and aroma had often resulted in the loss and degradation of minor components. It is therefore important not only to minimize the loss but also to maximize the retention of minor components in the edible oils during these processes as well as to concentrate, recover or isolate them from the various feedstock of oils. This chapter introduces the properties of various important minor components that contribute to the chemical makeup of the edible oils and the current processes or techniques that can be employed to recover the valuable components from the edible oil.

5.2 The Minor Components in Edible Oils

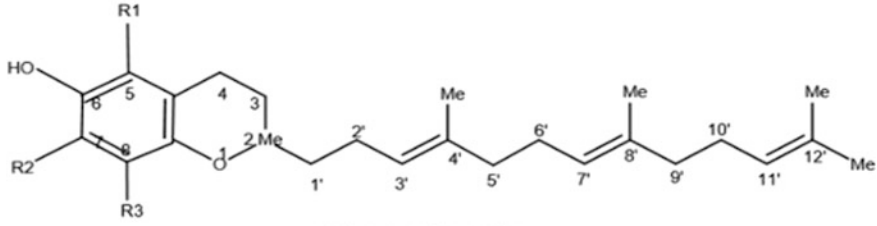
5.2.1 Tocochromanols

Tocopherols and tocotrienols belong to the subfamilies of the Vitamin E. They are different in structure whereby tocopherol have a phytyl side chain without double

bond while tocotrienol possesses double bond at C-3′, C-7′, and C-11′ on the phytyl side chain (Peh et al. 2016). Therefore, tocopherols allow for three stereocenters at C-2, C-4′ and C-8′. Meanwhile, tocotrienols only have one stereocenter at C-2 due to the double-bond configuration (Colombo 2010). Tocochromanols exist in the forms of α-, β-, γ-, and δ-depending on the number and position of the methyl groups in the chromanol ring. Figure 5.1 shows the structure of tocopherols and tocotrienols with the Greek letter prefix in the form of α-, β-, γ-, and δ-. The α -tocopherol can be obtained naturally or chemically synthesised. Synthesized α-tocopherol exist in eight



Tocopherols



Tocotrienols

Isomers	Greek letter prefix	R1	R2	R3
α	2,5,7,8-tetramethyl	CH_3	CH_3	CH_3
β	2,5,8-trimethyl	CH_3	H	CH_3
γ	2,7,8-trimethyl	H	CH_3	CH_3
δ	2,8-dimethyl	H	H	CH_3

Fig. 5.1 The structure of tocopherols and tocotrienols (Sen et al. 2006)

Table 5.1 The tocopherols and tocotrienols content in selected edible oils

Edible oils (ppm)	α-T	β-T	γ-T	δ-T	α-T3	β-T3	γ-T3	δ-T3	Total
Corn oil	260	10	750	30	10	–	20	–	1080
Olive oil	120	–	10	–	–	–	–	–	130
Rapeseed oil	190	–	490	10	–	–	–	–	690
Red palm oil	166	–	–	–	202	–	275	64	707
RBD palm oil	139	–	–	–	163	–	205	54	561
Rice bran oil	324	18	53	–	116	–	–	349	860
Soy bean	101	–	593	264	–	–	–	–	958
Sunflower oil	620	20	30	–	–	–	–	–	670

RBD-refined, bleached, deodorized; T-tocopherol; T3-tocotrienol (Adapted from Nagendran et al. 2000; Ko et al. 2003; Peh et al. 2016)

stereoisomers which are the all-rac-α-tocopherol (RRR-, RRS-, RSS-, RSR-, SRS-, SSR-, SRR- and SSS-α-tocopherol)] (Kiyose et al. 1997). Similar to the synthesized α-tocopherol, α-tocotrienol can exist in the form of cis or trans isomers, which are [2D,3′cis,7′cis (R,cis-cis); 2D,3′cis,7′trans (R,cis-trans); 2D,3′trans,7′cis (R,trans-cis); 2D,3′trans,7′trans (R,trans-trans); 2L,3′cis,7′cis (S,cis-cis); 2L,3′cis,7′trans (S, cis-trans); 2L,3′trans,7′cis (S,trans-cis); 2L,3′trans, 7′trans (S,trans-trans)] (Diplock 1985). The isomers of synthetic tocochromanols are different as compared to the natural tocochromanols. It is convenient to find the existence of synthetic tocopherols. However, synthetic tocotrienols are uncommon as the yields and purity are relatively lower when compared to the synthetic tocopherols (Netscher 2007).

Tocopherols and tocotrienols are available in oils derived from vegetables, fruits and plants. The α -tocopherols are usually found in the green part of the plants such as the leaves while γ-tocopherols are commonly found in the non-green part of the plants such as the flesh and seeds (Zielinski 2008). Common edibles oils including red palm oil, corn oil, rapeseed oil, sunflower oil and soybean oil contain large amount of tocopherols and tocotrienols. Among the selected edible oils, sunflower oil has the richest source of α-tocopherol while soybean oil is rich in γ- tocopherol. Vitamin E in edible oils mainly exist in the form of tocopherol and it is rare to find tocotrienols in the edible oils with the exception of rice bran oil, red palm oil and refined, bleached and deodorised palm oil. The tocopherols and tocotrienols contents of selected edible oils are shown in Table 5.1.

Interestingly, studies showed that tocotrienols have the most excellent antioxidative properties as compared to tocopherols. α-tocopherol was reported to be less effective than tocotrienols in protecting the glutamate-induced neuronal cell from damage (Sen et al. 2000). A research conducted by Serbinova et al. (1991) demonstrated that the antioxidant efficacy of α-tocotrienol was 1.5-fold higher compared to α-tocopherol in liposomal. Another study reported that α-tocotrienol was 40 times more effective in preventing lipid peroxidation induced by iron (II) and nicotinamide adenine dinucleotide phosphate than α-tocopherol in rat liver

Table 5.2 Health benefits of tocopherols and tocotrienols

Health benefits	Type of vitamin E	Bioactivities	References
Heart disease	Tocopherols, tocotrienols	– Decrease lipid peroxidation, monocyte proatherogenicity and platelet aggregation – Reduce LDL level – Delay intra-arterial thrombus formation	Tom et al. (1999), Kaul et al. (2001), Hodis et al. (2002), Mathur et al. (2015)
Cancer	Tocopherols, tocotrienols	– Facilitating apoptosis and impeding angiogenesis of tumor cells – Antiproliferation activity of cancer cells	Shin-Kang et al. (2011), Shibata et al. (2015)
Cognitive function	Tocopherols, tocotrienols	– Strong antioxidant – Free radicals scavenging – Reduce oxidative stress	Masaki et al. (2000), Shichiri et al. (2011), Mangialasche et al. (2012)
Photoprotection	Tocopherols, tocotrienols	– Strong antioxidant – Skin against oxidative stress from UV	Lopez-Torres et al. (1998), Pedrelli et al. (2012)
Wound healing	α-tocopherol	– Free radicals scavenging – Anti-inflammatory effect – Stimulates production of cyclic adenosine monophosphate – Reduce plasma malondialdehyde level – Increase glutathione peroxidase activity	Lin et al. (2012), Musalmah et al. (2002)
Scar reduction	Tocopherols, tocotrienols	– Protects against oxidation – Prevents bacterial proliferation – Reduces inflammation	Gold et al. (2001), Zampieri et al. (2010)
Bone	α-Tocopherol and palm Vitamin E	– Strong antioxidant – Reduced oxidative stress – Regulate DNA expression of bone	Zakaria et al. (2017)

microsomes. Tocotrienols were also found to be seven-fold more potent than tocopherols in the protection against proliferation of mammary tumor cell (Mcintyre et al. 2000). The effectiveness of antioxidant depends on the structure and properties of the chromanol nucleus and hydrophobic side chain. Tocopherol molecules have long tails with no double bond, whereas tocotrienol molecules have shorter tails and possess stronger disordering effect of membrane lipids (Peh et al. 2016). Accordingly, the chromanoxyl radical of α-tocotrienol can act faster and is more efficient to move around cells and penetrate into membranes and lipoproteins than α-tocopheroxyl radical (Serbinova and Packer 1994). Table 5.2 shows the health benefits of tocopherols and tocotrienols.

Notably, tocopherols and tocotrienols have significant antioxidant properties which play a vital role in protecting cells from damage caused by free radicals. In particular, tocopherols are the common ingredient in most skincare products. Studies have shown that the antioxidant from tocopherols can safeguard the skin against oxidative stress from UV or ozone (Lopez-Torres et al. 1998; Podda et al. 1996). The content of tocopherols in the skin was found to decrease rapidly after being exposed to sunlight (Shindo et al. 1994). Meanwhile, tocotrienol possesses powerful neuroprotective, cholesterol lowering and anti-cancer properties which is not commonly exerted by tocopherols.

5.2.2 Sterols

Sterols can exist in more than 250 types. It is made from the hydroxylated polycyclic isopentenoids possessing a 1,2-cyclopentanophenthrene carbon skeleton with a total 27–30 carbon atoms attached to more than six carbon atoms at the side chains. The basic structural make up of sterols consists of a four-fused ring, hydroxyl group at C-3, side chain at C-17 and two methyl groups at C-18 and C-19. Moreover, the position of the C=C in the ring (mostly at C-5) and the length of the side chain beyond C-22 could be different. The structure of selected sterols is shown in Fig. 5.2.

Plants are good natural sources of phytosterols. The major components of phytosterols found in plants are β-sitosterol, campesterol, stigmasterol, brassicasterol, and Δ5-avenasterol (all is 4,4-desmethylsterols) (Verleyen et al. 2002a). This was agreed by a study conducted by Yang and his co-colleagues. According to his study, rice bran oil has the richest source of sterols (Yang et al. 2019). It has the highest content of stigmasterol, β-sitosterol, Δ^5-avenasterol, cycloartanol, cycloartenol and 24-methylene-cycloartanol. Besides, corn oil, rapeseed oil and flaxseed oil are also found to have high sterol content. From Table 5.3, it can be seen that the sterol content for different types of edible oils varies greatly between one another.

Sterols can be present in vegetable oils as free or esterified forms. This is due to the fatty acids or phenolic acid which can form steryl esters, steryl glycosides, and acylated glycosides (Piironen et al. 2000). Table 5.4 shows the free and esterified sterols contents in a few crude and refined edible oils. The difference in the individual sterols proportion between the crude and refined edible oils was negligible. According to the data shown in the Table 5.4, sitosterol was the major component of the free and esterified sterols in corn oil in both crude and refined forms. Similar observation was found in the other edible oils. However, refining process reduces the proportion of the free sterols and increases the proportion of the esterified sterols (Verleyen et al. 2002b). A reduction of free sterol during oil refining increased the esterified sterol fraction significantly.

In recent years, phytosterols have been found to be effective in reducing blood cholesterol which relate to the prevention of heart diseases (Kamal-Eldin and Moazzami 2009; García-Llatas and Rodríguez-Estrada 2011). Many studies had shown that phytosterols could minimize heart disease by reducing the absorption

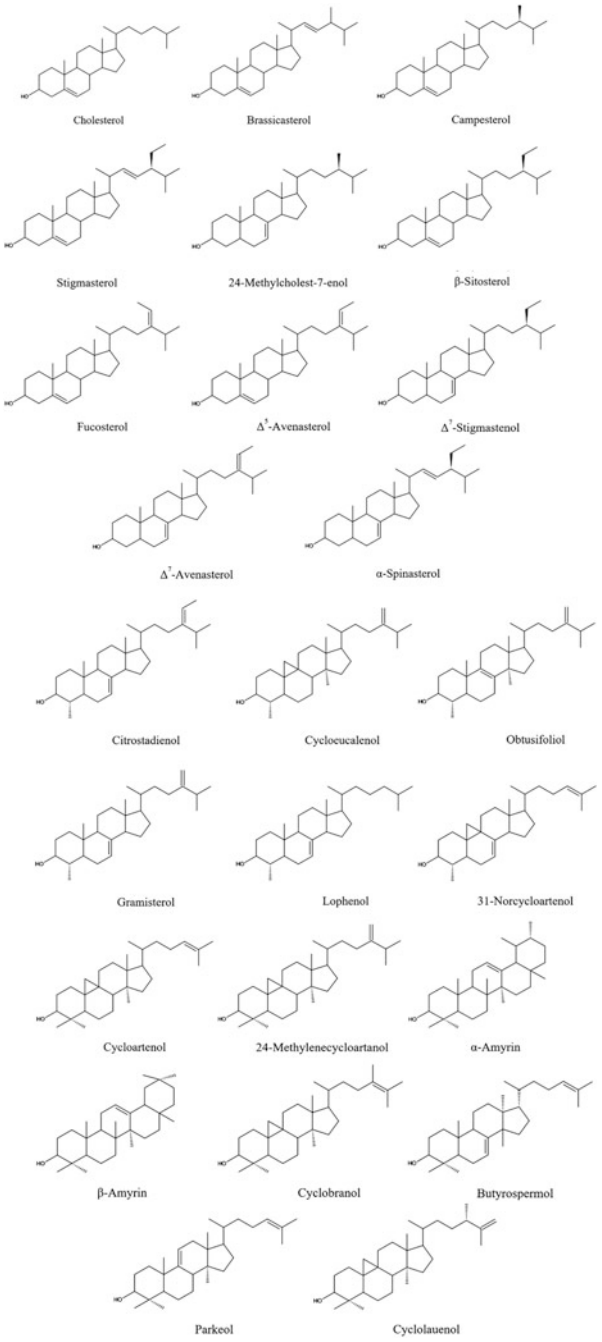


Fig. 5.2 The structure of selected sterol (Kochhar 1983)

Table 5.3 Phytosterol composition in selected edible oils

	Phytosterol content (ppm)								
	4,4-Desmethylsterols				4,4-Dimethylsterols				
	I	II	III	IV	V	VI	VII	VIII	Total
Camellia oil	–	165.2	221.1	500.9	18.1	291.1	178.4	23.3	1398.1
Corn oil	41.3	1973.2	455.3	5399.3	979.2	18.9	158.5	129.7	9155.4
Flaxseed oil	16.6	1155.2	126.2	1577.9	560.1	9.9	786.7	392.9	4625.5
Grapeseed oil	21.5	292.5	357.7	1466.3	161.8	27.1	125.1	233.6	2685.6
Olive oil	–	258.5	211.3	1520.5	297.3	27.9	194.4	345.8	2855.7
Peanut oil	–	411.9	481.6	1891.2	197	7.3	94.2	49.2	3132.4
Rapeseed oil	1366.4	2675	256.7	3941.1	409.2	11	172.6	52.8	8884.8
Rice Bran oil	63.3	2264.3	1329	7351.7	1574.1	310.8	1562.5	2228.8	16684.5
Sesame oil	–	903	868.9	3227.3	987.9	8.3	237.9	47.5	6280.8
Soybean oil	128	626.8	872.8	1660.3	72.1	27.2	47.1	43.4	3477.7
Sunflower oil	5.8	283.6	186.9	1709.1	124.5	1.8	88.5	112.7	2512.9

I = Brassicasterol; II = Campesterol; III = Stigmasterol; IV = β-Sitosterol; V = Δ^5-Avenasterol; VI = Cycloartanol; VII = Cycloartenol; VIII = 24-Methylene-Cycloartanol. Adapted from Yang et al. (2019)

Table 5.4 Free and esterified sterols content in selected crude and refined edible oils

Edible oils	Free sterols					Esterified sterols				
	I	II	III	IV	Total	I	II	III	IV	Total
Corn (crude)	1126	321	3408	–	4855	764	334	3028	107	4233
Corn (refined)	708	226	2421	–	3355	773	295	3191	91.7	4351
Palm (crude)	104	49	358	–	758	55	23	169	–	247
Palm (refined)	63	41	185	–	289	60	32	171	19	282
Rapeseed (crude)	974	–	1706	682	8116	1930	–	2566	258	4754
Rapeseed (refined)	933	–	1585	265	2783	1911	–	2547	389	4847
Soybean (crude)	492	523	1379	–	2394	89	60	525	113	787
Soybean (refined)	439	392	1086	–	1917	107	32	513	101	753

I = Campesterol; II = Stigmasterol; III = Sitosterol; IV = Δ5-Avenasterol. Adapted from Verleyen et al. (2002a, b)

of cholesterol through various mechanisms (Alemany et al. 2014; Shuang et al. 2016). This can be credited to its similar molecular structures as cholesterol (Plat and Mensink 2001; Choi et al. 2007; Kamal-Eldin and Moazzami 2009). In short, cholesterol competes with the phytosterols to integrate into the mixed micelles in the body resulting in increased faecal excretion of cholesterol (Marangoni and Poli 2010). Aside from heart-related diseases, phytosterols also contribute to anti-inflammatory, immunomodulatory and anti-carcinogenic effects (Calpe-Berdiel et al. 2005; Woyengo et al. 2009; Fraile et al. 2012; Aldini et al. 2014).

5.2.3 Hydrocarbons

Hydrocarbons are one of the components that form from a variety of combinations of carbon and hydrogen atoms. They are the least polar and unsaponifiable components of oils. There are many types of hydrocarbons and the most popularly discussed hydrocarbons in edible oils are *n*-alkane, squalene and carotenoids. Other hydrocarbons such as sesquiterpenic (α-farnesene) and terpenic (kaurene) as well as low-molecular-mass aromatics hydrocarbon ranging from benzene to tetramethylbenzene including styrene, and lower molecular mass of polycyclic aromatic are present in minute amount in edible oil (Moreda et al. 2001).

5.2.3.1 *n*-Alkane

Crude vegetable oils have *n*-alkane hydrocarbons in the range of C_{10}–C_{35} (Moreda et al. 2001). Among the selected edible oils, sunflower oil was found to contain the highest amount of alkanes from C_{15} to C_{33} followed by the olive oil and safflower oil. The *n*-alkane proportion in sunflower oil was mainly composed of C_{29} and C_{31} (Table 5.5). A significant amount of the *n*-alkane was discovered between C_{21} and

Table 5.5 *n*-Alkane hydrocarbons content in the range of C_{15}–C_{33} in selected edible oils

	Edible oils (ppm)				
Carbon no	Sunflower	Virgin olive	Sesame	Peanut	Safflower
nC_{15}	0.17	0.16	0.16	0.38	0.41
nC_{16}	0.13	0.06	0.11	0.16	0.48
nC_{17}	0.16	0.12	0.15	0.15	0.52
nC_{18}	0.9	0.08	0.09	0.18	0.52
nC_{19}	0.12	0.13	0.1	0.17	0.55
nC_{20}	0.02	0.08	0.07	0.17	0.7
nC_{21}	0.04	0.81	0.23	0.18	0.72
nC_{22}	0.04	1.24	0.14	0.19	6.4
nC_{23}	0.15	18.54	0.54	0.2	2.92
nC_{24}	0.17	9.54	0.3	0.35	1.15
nC_{25}	1.52	17.98	1.08	0.77	2.78
nC_{26}	0.41	2.04	0.59	0.37	1.22
nC_{27}	11.19	15.72	6.29	3.35	13.52
nC_{28}	2.38	1.84	1.63	0.77	2.1
nC_{29}	49.63	12.38	18.45	12.67	27.05
nC_{30}	5.52	1.7	–	0.81	1.98
nC_{31}	47.96	9.41	14.19	6.2	15.2
nC_{32}	1.79	1.54	1.44	0.39	0.89
nC_{33}	3.6	5.66	6.6	0.85	1.54
Total	125.9	99.03	52.16	28.31	80.65

Adapted from Moreda et al. (2001)

C_{33} and this result was in line with the other literatures which shows that the *n*-alkane between C_{21} and C_{35} was abundantly found in crude vegetable oils (McGill et al. 1993; Lanzón et al. 1994).

5.2.3.2 Squalene

Squalene is a saturated hydrocarbon with six double bonds. It is an important component for biosynthesis of sterols. The molecular structure of a squalene is shown in Fig. 5.3. It was first discovered in shark liver and was later found in plants (Xiao et al. 2016). Among the edible oils as shown in Table 5.6, crude olive oil contains the highest content of squalene followed by rice bran oil and peanut oil. The squalene content in edible oils could be affected by the oil extraction and refining process. Notably, the squalene content obtained from cold-pressed method was similar to crude oils. A reduction in the squalene content after refining process was observed. Studies showed that squalene has the potential in reducing risk of cancers, decrease cholesterol level, improve immune responses and promotes anti-aging (He et al. 2003; Nergiz and Çelikkale 2011; Xiao et al. 2016).

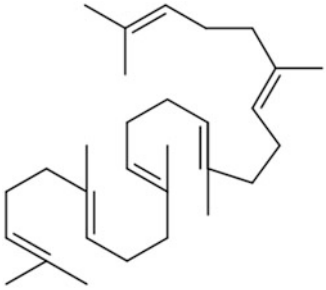


Fig. 5.3 The structure of squalene (Adapted from Reddy and Couvreur 2009)

Table 5.6 Sqaulene contents in selected edible oils

Edible oils	Squalene content (ppm)
Olive (crude)	4910
Olive (refined)	2900
Peanut (cold-pressed)	1329
Rapeseed (crude)	262
Rapeseed (refined)	211
Rice bran (cold-pressed)	3189
Sesame (black, cold-pressed)	572
Sesame (white, cold-pressed)	607
Soybean (crude)	181
Soybean (cold-pressed)	184
Soybean (refined)	125
Sunflower seed (crude)	138
Sunflower seed (cold-pressed)	144
Sunflower seed (refined)	92

Adapted from Podda et al. (1996), Nergiz and Çelikkale (2011)

5.2.3.3 Carotenoids

Carotenoids serve as retinol precursors which will turn into Vitamin A in a human body. Carotenoids are specifically responsible for the absorption and metabolism of Vitamin A. Carotenoids are mainly categorized into two groups, namely carotenes (α,β-carotene, and lycopene) and xanthophylls (astaxanthin, lutein, and zeaxanthin) (Shete and Quadro 2013). Most of the carotenoids are structured with 40 carbons with two rings at the two ends. The chemical structure of carotenoids is as illustrated in Fig. 5.4. Carotenoids can be produced and modified endogenously by a series of biosynthesis process which include hydrogenation, dehydrogenation, cyclization and hydroxylation. The presence of the conjugated double bonds allows the carotenoids to be readily isomerized and oxidized (Oliver and Palou 2000).

Carotenoids cannot be synthesised in a human body, hence it needs to be consumed and obtained from other sources. Carotenoids are primarily found in fruits

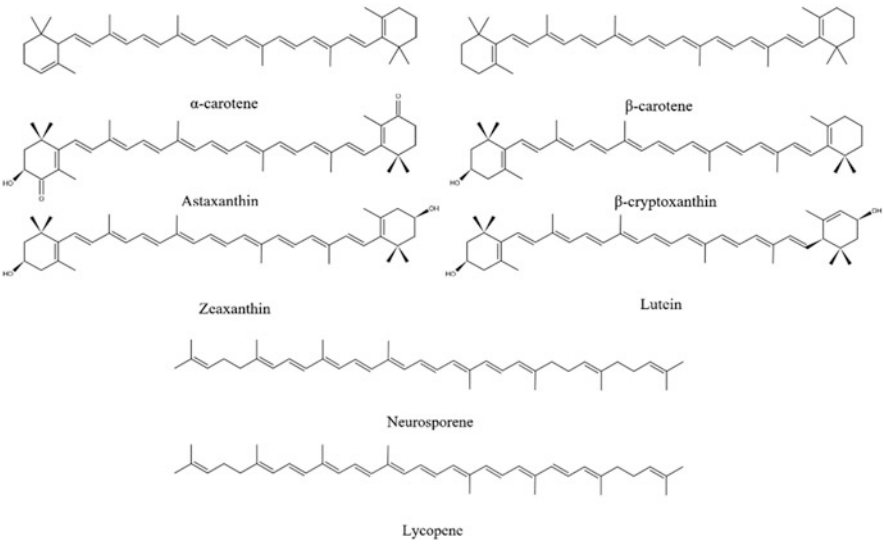


Fig. 5.4 The structure of some of the main carotenoids (Adapted from Britton et al. 2012)

and vegetables. As carotenoids have orange-reddish colour, they are abundantly present in fruits and vegetables which are yellow, orange, and red in colour such as tomatoes, carrot, red pepper, and yellow pepper. They can also be obtained from dark green leafy vegetables which are good sources of lutein and zeaxanthin (Rao and Rao 2007). However, it is rare to find carotenoids in oils or otherwise it would usually exist in a very low amount. The small amount of carotenoids would usually be removed during the oil refining process. Among the vegetable oil, crude palm oil has the richest source of carotenoids. The carotenoids content in the crude palm oil is equivalent to approximately eight times of carotenoids found in carrots (55.04–101.31 ppm α- and β-carotene) and more than 50 times in raw tomatoes (10 ppm β-carotene) (Bozalan and Karadeniz 2010; D'Evoli et al. 2013). Choo and colleagues developed a modified physical refining process which could retain as much as 80% of carotenoids in crude palm oil, which was around 500–700 ppm (37% α-carotene ; 50% β-carotene) (Yap et al. 1991; Choo 1995).

Carotenoids are reported to help in improving Vitamin A status. Zeba et al. (2006) succeeded in demonstrating that Vitamin A deficiency could be enhanced by consuming red palm oil which is rich in α- and β-carotenes. Supplementation with red palm oil effectively increase the serum retinol. An increase in retinol concentration in plasma and breast milk was observed when the diet of lactating women were supplemented with red palm oil (Lietz et al. 2001). Consequently, the consumption of red palm oil did not only improve the mothers' Vitamin A status but also benefited the infants through the breast milk. The consumption of carotenoids has been associated with many other health benefits concerning the eyes, brain, skin, improve

Table 5.7 Health benefits and uses of carotenoids

Health benefit	Type of carotenoid	Bioactivities	References
Vision, ocular disease	Meso-zeaxanthin, lutein, zeaxanthin	– Reduce reactive oxygen species	Johnson (2014), Moeller et al. (2006), Moschos et al. (2017), Nolan et al. (2015)
Cancer	Fucoxanthin, lycopene, neoxanthin	– Able to target cancer cells – Reduce cell viability that can induce the growth of cancer cells	Levy et al. (1995), Kotake-Nara et al. (2001), Kumar et al. (2013)
Cognitive function	Lutein, zeaxanthin, fucoxanthin	– Protect against scopolamine – Induce cognitive impairments – Increase acetylcholinesterase activity – Decrease activities of choline acetyltransferase and brain-derived neurotrophic factor expression	Johnson (2014), Lin et al. (2016)
Nutrition to improve vitamin A status	β-Carotene	– Increase retinol serum and improved vitamin A status of children, lactating women, pregnant women and breast-fed infants	Lietz et al. (2001), Zeba et al. (2006)
Skin	Lycopene, β-carotene	– Strong antioxidant – Protect against skin photodamage from UV-light – Improve skin roughness	Biesalski and Obermueller-Jevic (2001), Darvin et al. (2008), Freitas et al. (2015), Shah and Mahajan (2014)
Cardiovascular disease	Carotene, lutein, zeaxanthin, lycope	– Reduce oxidative stress – Decrease LDL cholesterol – Increase HDL cholesterol	Wang et al. (2014)

fertility and has positive impact on cancer patients. Other health benefits of carotenoids are shown in Table 5.7.

5.2.4 Phenolic Compounds

Phenolic compounds have always been the popular compounds due to their superior antioxidant activity. Phenolic compounds can be categorized into two major groups: phenolic acid and polyphenols (flavonoids, stilbenes, coumarins, lignans and tannins). The basic structure of phenolic compounds consist of one or more six-carbon aromatic rings that are attached to one or more hydroxyl groups (Velderrain-

Rodríguez et al. 2014). The major classes of the phenolic compounds in plants and their respective examples are shown in Table 5.8.

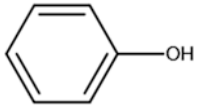
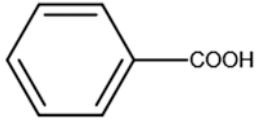
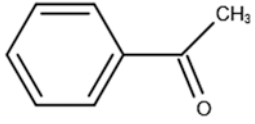
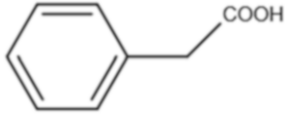
Olive oil is an exceptional source of phenolic compounds. Literature evidence had revealed that at least 30 types of phenolic compounds could be discovered in olive oil, especially in the extra-virgin olive oil (Visioli and Galli 1994; Visioli et al. 1995; Kohyama et al. 1997; Saija et al. 1998; Owen et al. 2000a). The major phenolic compounds in olive oil are oleuropein and its derivatives (Montedoro et al. 1992; Montedoro et al. 1993). Olive oil contains 200–500 ppm of phenolic content. Out of this 60% is contributed by oleuropein and its derivatives (Owen et al. 2000b; Boskou 2015). Oleauropein causes the bitter taste in olives. Oleauropein belongs the major phenolic component found in the olives while hydroxytyrosol which is the derivative of oleuropein found primarily in olive oils (Amiot et al. 1986; Tsimidou et al. 1992). During maturation, it was observed that the oleuropein content was likely to reduce while the content of hydroxytyrosol was found to increase (Climato et al. 1990; Ryan et al. 1999). Table 5.9 shows the phenol content of some of the edible oils.

According to a previous epidemiological study, it was found that there is a profound association between Mediterranean diet and cardiovascular diseases (Hertog et al. 1993). Low incidence of heart diseases has been found among those who adopt the Mediterranean diet and this is strongly related to the regular consumption of the extra-virgin olive oil commonly used in Mediterranean cuisines which is rich in phenolic compounds that could reduce the risk of heart disease (Nocella et al. 2018; Estruch et al. 2018; Pintó et al. 2019). To some extent, the antioxidant properties of the phenolic compounds could lower the risk of heart diseases and other complications caused by atherosclerotic. The phenolic compounds in an olive oil impedes the metabolism of arachidonic acid that leads to platelet aggregation (Nocella et al. 2018). Other than that, phenolic compounds have been shown to have favourable effects such as being anti-allergenic, anti-inflammatory, anti-carcinogenic and antimicrobial (see Table 5.10). The exact mechanism of action as antimicrobial have yet to be fully established. However, Kemperman et al. (2010) have concluded that phenolic compounds inhibit the growth of microbial through interaction of phenolic and membrane further interrupting the DNA of bacteria. Interestingly, not all phenolic compounds are effective against all bacteria. The grape seeds extracts contain high amount of catechin, epicatechin and trans-resveratrol was discovered to succeed in inhibiting all the gram-positive bacteria but not gram-negative bacteria. Conversely, another study conducted by Puupponen-Pimiä et al. (2001) discovered that all gram-negative bacteria showed susceptibility towards berry extracts with a high anthocyanins but not gram-positive bacteria.

5.2.5 Other Minor Components

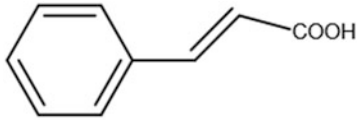
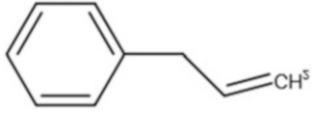
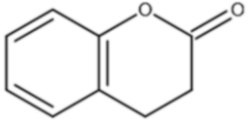
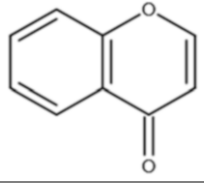
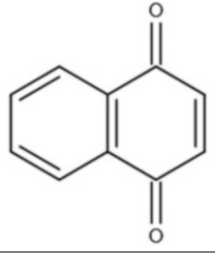
Phospholipid is primarily found in crude oil but was eliminated during oil refining process. Elimination of phospholipid is necessary as it could affect the physical and chemical properties of oil: (1) it darken the colour (darkening) of oil during oil

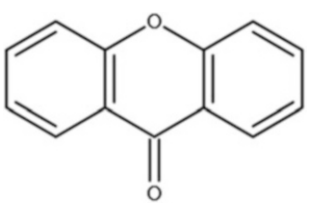
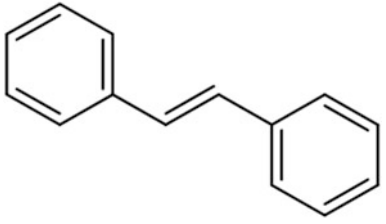
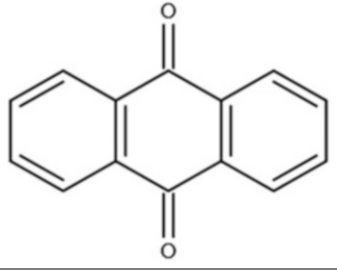
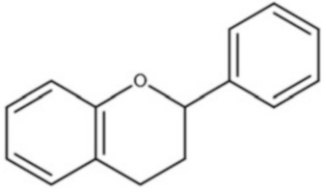
Table 5.8 Major classes of phenolic compounds in plants

No. of carbon	Basic skeleton	Phenolic class	Basic structure	Examples
6	C_6	Simple phenols		Catechol, hydroxyquinone
		Benzoquinones		2,6-Dimethoxybenzoquinone
7	C_6-C_1	Benzoic acid		Gallic, salicylic
8	C_6-C_2	Acetophenones		3-Acetyl-6-methoxybenzaldehyde
		Phenylacetic acid		p-Hydroxyphenylacetic

(continued)

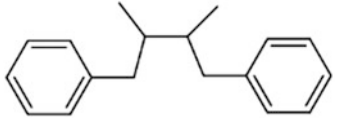
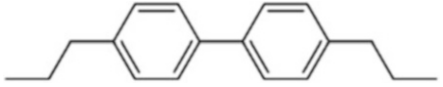
Table 5.8 (continued)

No. of carbon	Basic skeleton	Phenolic class	Basic structure	Examples
9	C_6-C_3	Cinnamic acid		Caffeic, ferulic
		Phenylpropene		Myristicin, eugenol
		Coumarins		Umbelliferone, aesculetin
		Chromones		Euenin
10	C_6-C_4	Naphthoquinones		Juglone, plumbagin

13	C_6-C_1-C_6	Xanthones		Mangiferin
14	C_6-C_2-C_6	Stilbenes		Resveratrol
		Anthraquinones		Emodin
15	C_6-C_3-C_6	Flavonoids		Quercetin, cyanidin

(continued)

Table 5.8 (continued)

No. of carbon	Basic skeleton	Phenolic class	Basic structure	Examples
16	$(C_6\text{-}C_3)_2$	Lignans		Pinoresinol
		Neolignans		Eusiderin
>16	$(C_6\text{-}C_1)_n$	Hydrolysable tannins	Heterogeneous polymer composed of phenolic acids and simple sugars	
	$(C_6\text{-}C_3)_n$	Lignins	Highly cross-linked aromatic polymer	

Adapted from Harborne (1990), Garcia-Salas et al. (2010)

Table 5.9 Total phenolic contents in selected edible oils

Edible oils	Total phenolic (mg CAE/100 g)
Corn	1.26
Grapeseed	0.51
Pumpkin	2.46
Rapeseed	1.31
Rice bran	1.44
Soybean	1.48
Sunflower	1.2

Adapted from Siger et al. (2008)

refining process and (2) it enhances the chelation of metals (Cert et al. 2000). Lecithin is a well-known example of phospholipid. It is a valuable by-product of water degumming which is a part of oil refining processes. Soybean is a main source of lecithin. 90% of the phospholipid in crude soybean oil is eliminated during oil refining and could be used to produce lecithin (Nash et al. 1984). The amphiphilic characteristic of lecithin makes it a great emulsifier to stabilize emulsion.

Studies have evaluated phospholipid for their effectiveness in inhibiting cancer growth (Sakakima et al. 2007; He et al. 2009; Abalsamo et al. 2012; Gándola et al. 2014). Sakakima et al. (2007) have investigated the effect of phosphatidycholine on cancer growth inhibition. In the study, rats were supplemented with phosphatidycholine and the analysis was compared with the control group. The results showed that in the phosphatidylcholine administration group, the number of liver tumour cells significantly reduced. Besides, diets that incorporated with phospholipid could indirectly protect hearts against atherosclerosis. In a study performed by O'Brien and Corrigan (1988), diet-induced hypercholesterolemia guinea pigs were fed with soybean and egg lecithin. The group that was fed with soybean lecithin had a 51 % lower the total plasma cholesterol without reducing HDL cholesterol. Conversely, the group that was fed with egg lecithin showed to have 177% increased in HDL-cholesterol without affecting the total plasma cholesterol. Aortic cholesterol in soybean lecithin-feed group was significantly decreased by 41%.

Chlorophyll (e.g. phophytin) is the colour pigments found in plants and crude oils. It acts as an indicator to evaluate the quality of oils such as rapeseed, soybean and olive oils. Oxidation of chlorophyll could occur in the presence of light (Pokorny et al. 1995). During oil refining process, chlorophyll is removed during bleaching. It is challenging to remove chlorophyll in rapeseed oil as it is naturally exist in higher amount than the other oils (Niewiadomski 1990).

Trace metals (e.g. Cu, Fe, Zn, PB and so on) are also found in edible oils. These metals can enter the edible oils *via* agronomic and environmental conditions (e.g. soil, fertilisers and pesticides), oil refining and manufacturing process (Karadjova et al. 1998; Zeiner et al. 2005). They are indicators to determine the quality of edible oils (Huang and Jiang 2001; Llorent-Martínez et al. 2011). The trace metals in edible oils could promote oxidation process producing peroxides, aldehydes, ketones, acids and epoxides. The oxidation process and their by-products are harmful which could lead to oxidative stress in human body enhancing

Table 5.10 Health benefits and uses of phenolic compounds

Health benefits	Type/Sources	Bioactivities	References
Antimicrobial	Berries, peanut skin and meal from dry-blanched peanuts)	– Inhibit the growth of bacteria	Puupponen-Pimiä et al. (2001), de Camargo et al. (2017)
	Caffeic acid	– Free radical scavenging – Enhance intracellular components release	Božič et al. (2012)
Anti-inflammatory	Honey	– Inhibit albumin denaturation – Protect membrane again lysis –Inhibit enzyme catalytic activity	Ruiz-Ruiz et al. (2017)
Anti-tumor	Gallic acid	– Inhibit proliferation and invasive effect of tumor cells	Hu et al. (2015)
	Extracts from Santlaceae	– Decrease tumoral cellular growth	Melo et al. (2018)
Antioxidant	Caffeic acid	– Free radical activities – Inhibit lipid oxidation	Aytekin et al. (2011)
	Resveratrol from grapes	– As antiarrhythmic agent to protect heart – Scavenger of peroxyl radicals	Ray et al. (1999), Hung et al. (2000)
Anti-allergic	Garlic acid-chitooligosaccharides	– Impede the release of histamine – Inhibit the expression and production of cytokine	Vo et al. (2012)
	Salvianolic acid	– Suppress antigen-induced degranulation – Decrease inflammatory cells – Decrease the expression and secretion of cytokines	Son et al. (2019)

carcinogenic effect (Castillo et al. 1999). Canola oil contain sulphur that could produce unpleasant odour at high temperatures. Therefore, sulphur is removed during refining. The sulphur content is reduced from 15–35 ppm to 2–7 ppm (Mag 1983; Wijesundera et al. 1988). The removal of phospholipid during oil refining leads to the elimination of trace metals (Fe and Cu) which further improve oxidative stability of oils (Cert et al. 2000). The maximum levels for the trace metals in edible oils have been established in legislation to meet the standard and quality of edible oils (Commission 2008).

5.3 Recovery of Minor Components

The sales and price of vegetable oils depend on its quality. Four major processes namely the degumming, neutralization, bleaching and deodorization are involved in oil refining process. Figure 5.5 shows the process of oil refining and the minor components that are removed at every stage. Deodorization process that is conducted under high temperatures (250–270 °C) removes most of the undesirable components (Young 1981). Other than the undesirable components, valuable components such as phytonutrients are lost during the refining process. Previous studies showed that tocopherol loss in edible oils could be as high as 40–50% at deodorization stage. A loss of 73% of β-carotene occur during the bleaching stage (Kreps et al. 2014; Erickson 2015). Over the past decades, researchers have shifted their focus towards the improvement and advancement of oil refining process to minimize the loss of desirable components. In Malaysia, Choo and team had developed a modified method to refine crude palm oil. In the proposed method, crude palm oil was treated at a lower temperature (<170 °C) under a lower pressure which was at below 100 mTorr. This method successfully removed most of the free fatty acid and impurities and at the same time retained approximately 80% of the carotenoids (Choo et al. 1993). Other researcher, such as Wei et al. (2015) evaluated the most suitable temperatures for oil refining to retain essential fatty acids and bioactive compounds in tea seed oil.

Typically, the desirable and undesirable components in the crude vegetable oils are trapped as deodorizer distillates (ODD) during deodorization. ODD are the by-products of the deodorization process which contain free fatty acid (FFA), hydrocarbons, aldehydes, ketones and acyl glycerol (Ramamurthi and McCurdy 1993). The by-products also contain abundance amount of important bioactive components such as tocopherols, carotenoids and sterols (Verleyen et al. 2001; Chu et al. 2003). Therefore, ODD is a common raw material to isolate or concentrate the bioactive components. There are a few numbers of methods to recover these nutritional bioactive components from ODD namely by pretreating the sample to produce ethyl or methyl ester catalysed by chemical or enzyme followed by separation via molecular distillation, crystallization and or supercritical carbon dioxide.

5.3.1 Enzymatic Transesterification as Pre-treatment Process

Enzymatic transesterification method is treated as a pre-treatment step to ease separation of the bioactive components from the oils prior to molecular distillation or solvent extraction. Free fatty acids (FFA) are the major components in ODD. Transforming FFA into FAME by transesterification facilitates the subsequent process of separation via molecular distillation or supercritical carbon dioxide. There are several advantages of using enzymatic pretreatment. The enzymatic process is milder and specific in their reaction (Dumont and Narine 2007). Torres

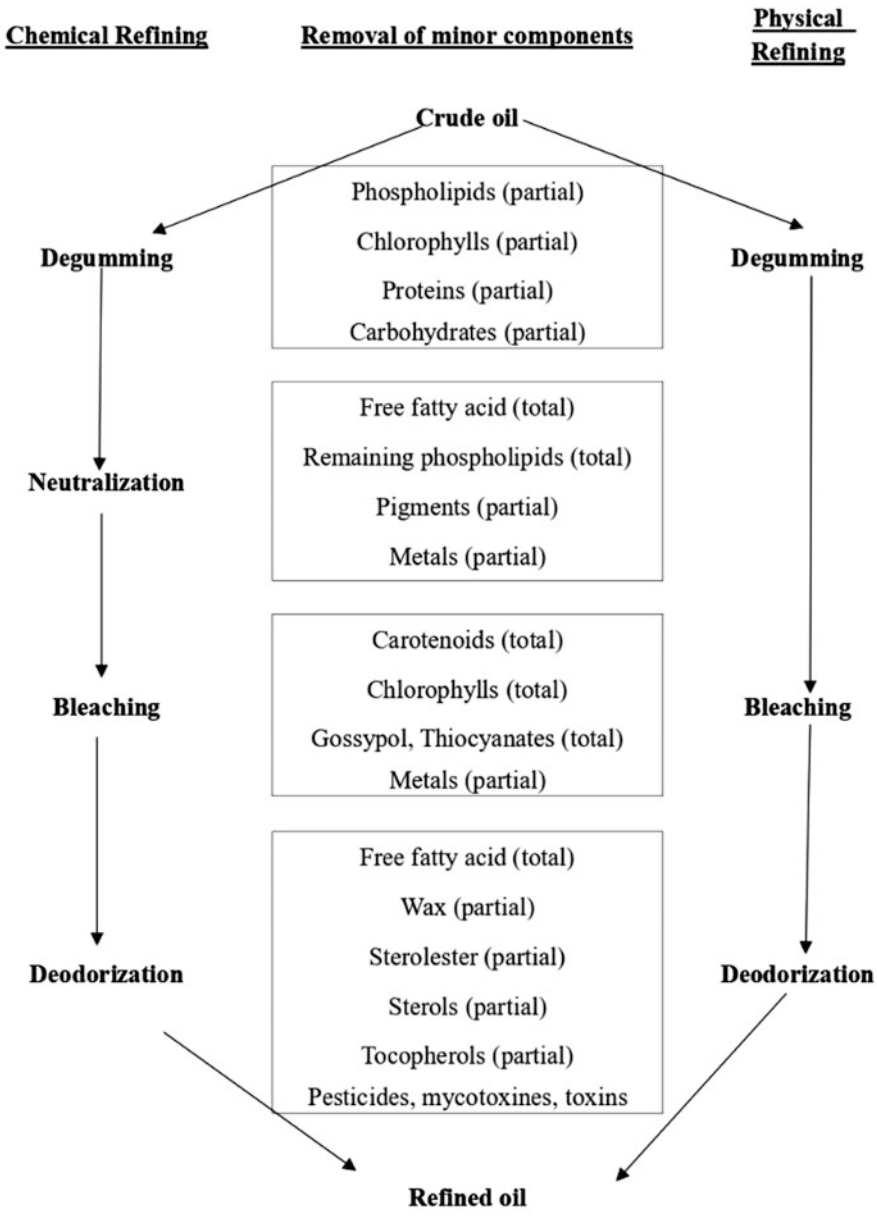


Fig. 5.5 The process of chemical (left) and physical (right) refining of edible oil and the removal of minor components in each stage (Kamm et al. 2001; Maniet et al. 2019)

et al. (2007) applied two steps of enzymatic processes to recover tocopherols and sterol esters from soybean ODD. The enzymatic reaction system involved two kinds of lipase; *Candida rugosa* and *Candida antarctica*. Firstly, sterols with FFA were

esterified followed by a subsequent ethyl esterification to esterify the residual FFA and acylglycerol. These two steps of enzymatic treatments effectively transformed more than 90% of the sterols and FFA within three to five hours. Weber et al. (2002) managed to recover steryl esters from rapeseed oil or ODD (derived from a mixture of soybean and rapeseed). The researchers used *Candida rugose* lipase to transform sterols to steryl esters in vacuum pressure of 20–40 mbar and at the temperature of 40 °C. Steryl esters were recovered with a purity more than 90% and conversion of 87–97%.

5.3.2 Molecular Distillation

Technically, molecular distillation operates through the difference in terms of molecular weights and vapour pressures for the separation of a complex mixture of components and is meant for the purpose of separating, concentrating or purifying valuable components. During the process, the components with low molecular weights at a certain vapour pressure are expected to be removed through the distillate stream while the component with high molecular weights stay in the residue stream. Molecular distillation involves short-path vacuum distillation (20–50 mm distance between the evaporator and the condenser) that is subjected under low temperatures and low vacuum pressure (Lutišan et al. 2002). The distribution of the sample on a uniformly thin film can ensure that the liquid get into contact with the evaporating cylinder for less than one minute. The distillation temperature is controlled by the non-condensable gas in the evaporator and it decreases the temperature by lowering the pressure to less than 0.1 Pa in the evaporator. It has been used for recovery of complex and heat-sensitive components such as tocopherols, squalene and sterols. As compared to other technologies, molecular distillation is superior and it is able to minimize solvent related toxicity issues (Lutišan et al. 2002).

Martins et al. (2006) recovered tocopherol from soybean ODD by removing FFA through molecular distillation. The study evaluated the parameters of the molecular distillation process to maximize the removal of FFA content and minimize the loss of tocopherol content in soybean ODD. The parameters used were: evaporator temperature of 100–180 °C and feed flow rate of 1.5–23.0 g/min. It was observed that higher FFA removal in the distillate stream led to higher tocopherol content in the residue stream. Evaporator temperature of 160 °C and feed flow rate of 10.4 g/min were able to remove 96.16% of FFA and recovered 81.23% of tocopherol in soybean ODD. Jiang et al. (2006) extracted tocopherols from fatty acid methyl esters (FAME) of rapeseed ODD by three stages process. In the first stage, hydrocarbons, ketones and aldehydes were collected at the temperature of 50 °C and pressure of 2.66 Pa. Secondly, the FAME was collected at the temperature range of 100–140 °C and pressure of 5.32 Pa. In the third stage of the distillation process, tocopherol was collected at the temperature range of 170–230 °C and pressure of 2.66 Pa. The tocopherol and FAME recovery following the three stages of distillations were around 50% and 90%, respectively.

5.3.3 Supercritical Carbon Dioxide

Undesirable components such as the FFA and glycerides are converted into FAME prior to its separation at the later stage using supercritical carbon dioxide. Supercritical carbon dioxide method is suitable to extract fat-soluble and thermally sensitive components such as vitamin E. There are many studies that utilized supercritical carbon dioxide to efficiently separate tocopherols from ODD. Fang et al. (2007) and Yun et al. (2010) obtained more than 80% of tocopherols from soybean ODD at the pressure of 16 MPa and 14 MPa, respectively. The pressure used in these studies were relatively low but generates a high yields of Vitamin E. Pressure and temperature are important parameters to control the supercritical carbon dioxide extraction as it can affect the separation factor. The effect of temperature was found to be in the opposite of pressure whereby high temperature resulted in low separation factor. With supercritical carbon dioxide, a low separation factor is desired as it enhances the separation of FAME from other components. A low separation factor can be achieved using low pressure and high temperature (Fang et al. 2007). Supercritical carbon dioxide method offer several advantages. It operates under mild temperature processing conditions and uses less toxic solvent (Temelli 2009; Rocha et al. 2014). Ruivo et al. (2008) have adopted the supercritical carbon dioxide technology with membrane separation to enhance separation efficiency. Around 88% of the squalene was obtained from olive ODD at 18.0 MPa/40 °C. Combination of different methods can improve separation efficiency of supercritical carbon dioxide. Tenllado et al. (2011) succeeded in purifying 70% of alkyglycerols from shark liver oil with a combination of supercritical carbon dioxide and molecular distillation. Rocha et al. (2014) efficiently recovered squalene and eliminated more than 97% of FFA from olive ODD using choline hydrogen carbonate and supercritical carbon dioxide.

5.3.4 Crystallization

Crystallization method usually works in a combination with other separation methods such as supercritical fluid extraction and molecular distillation to yield good separation and recovery. Crystallization is applied on either before or after the separation methods. The studies by Charlemagne et al. (2005) and Hattori et al. (2007) involved saponification as their initial step to remove FFA and acylglycerols, followed by crystallization to purify and extract sterol. The recovered sterols were recorded to be more than 87% and the purity was at 96%. Moreira and Baltanás (2004) evaluated the operational variables namely: solvents and co-solvent, cooling rate, crystallization temperature and ripening time on the extraction of phytosterols from sunflower ODD through crystallization. The research started by converting FFA into ethyl esters through esterification using hydrochloric acid as the catalyst. The optimum conditions of the operational variables were achieved when hexane was used as solvent, water and ethanol as co-solvents, crystallization temperature

was set in a range of –20 to 0 °C and crystallization time was set in a range of 4–96 h. Around 84% of sterols were satisfactorily obtained but with a low purity of 36%. A study by Yan et al. (2011) utilized solvent coupled with crystallization approach to recover sterols. The study showed that increasing the number of crystallization improved the recovery of sterols. Sterols of around 84.7% were managed to recover after first crystallization. Subsequently, the second and third round of crystallization increased the recovery of sterols to 92.73% and 97.17%, respectively. Crystallization is a simple method that requires low temperature and consumes less energy as compared to other approaches. The downside for crystallization is that it sometimes requires a relatively high amount of solvents that could increase the cost of the final product (Johansson et al. 1977; Lin and Koseoglu 2003).

5.4 Techniques for Characterization

It is hard to recommend a suitable method to analyse the minor components in edible oils. In this regard, there is no one size fit all techniques that is regarded as perfect for the identification and characterization of a component in edible oils. One major challenge is the insignificant amount of minor components presence in edible oils as compared to the major constituent which is the triacylglycerol. Taking this challenge into account, methods and techniques used need to be highly specific and sensitive for minor components identification. The standard analytical techniques that are more commonly used are gas chromatography (GC), high-performance liquid chromatography (HPLC), mass spectrometry (MS) and others. HPLC uses liquid as mobile phase while GC uses gases. GC was limited to volatile components, while HPLC has no volatility issue and can perform well for the analysis of both volatile and non-volatile organic components (Feng et al. 2019). However, the accuracy of GC is better than HPLC (Dzah et al. 2020). LC–MS and GC–MS are more advanced techniques of LC and GC that combined with MS detector LC–MS and GC–MS are more accurate, sensitive and specific) in their analysis. Studies used LC–MS to analyze triacylglycerides, phenolic compounds, tocopherols and carotenoids, while GC–MS was utilized to analyze volatile components such as squalene and sterols (Lang et al. 2019; Navarro-Reig et al. 2016; Podda et al. 1996; Sales et al. 2019; Shi et al. 2019; Xie et al. 2013). MS was found to be difficult in identifying phenolic compounds as the incomplete metabolome lead to the limitation of MS data collection speed (Cui et al. 2018). It is recommended to couple LC–MS/MS and GC–MS/MS with another instrument to overcome the limitation of phenolic compound identification, such as nuclear magnetic resonance (NMR) (Forino et al. 2016; Said et al. 2020; Yeo and Shahidi 2020). NMR allows the analysis of structural information from the vibration of the molecule. The molecule can be identified through the NMR libraries; however, the scope available in the libraries is limited (Dzah et al. 2020). Supercritical fluid chromatography (SFC) is the latest separation method that functions similarily to HPLC and GC, but uses supercritical carbon dioxide as mobile phase. Compared with HPLC and LC, SFC is more versatile,

provides better resolution, and requires short analysis time (Ignat et al. 2011). SFC separation and analysis is carried out at or slightly above room temperature; thereby, it is suitable for thermal sensitive compounds (Cert et al. 2000). Table 5.11 shows the advantages and disadvantages of selected analytical techniques used for the characterization of minor components in edible oils.

5.5 Roles of Minor Component

5.5.1 Antioxidant and Pro-oxidant

5.5.1.1 Tocopherols and Tocotrienols

Tocopherols and tocotrienols are well-known antioxidants. Although both the tocopherols and tocotrienols are subfamilies of Vitamin E, the former is more popularly discussed compared to the latter. According to the database of PubMed, out of more than 24,000 research on Vitamin E, only approximately 1% of the research was related to tocotrienols (Sen et al. 2006). Tocopherols are good at scavenging free radicals. It reacts with lipid peroxyl radicals faster than unsaturated lipids, thereby preventing radicals from attacking the lipids and inhibiting oil oxidation. At low peroxide level, one tocopherol molecule is capable to defend around 10^3–10^8 polyunsaturated fatty acid from being attacked by free radicals (Kamal-Eldin and Appelqvist 1996). Tocopherols inhibit oil oxidation by different pathways: reacting with lipid peroxyl radicals and reacting with $^1O_2$ (Liebler et al. 1990).

Apart from this, tocopherols may also act as pro-oxidants. Some research has found that the antioxidant activity of tocopherol was suppressed when the concentration of tocopherol was too high. It was found that when the concentration was above the optimum concentration, tocopherol acted as pro-oxidant and produced peroxyl radical, tocopherol radical, hydroxyl radical and singlet oxygen (Huang et al. 1995; Evans et al. 2002; Rietjens et al. 2002; Kim et al. 2007). Oil oxidation was induced due to the oxidized compounds from tocopherols, as they reduced surface tension of edible oils and accelerate oxygen diffusion from atmosphere (Kim et al. 2007). However, other research discovered that despite having high concentration of tocopherols, the pro-oxidant effect of tocopherol was diminished under high temperature (Saucy et al. 1990). The explanation made by the research was that the solubility of oxygen in edible oils decreased at high temperatures which slowed the autoxidative peroxide formation (Saucy et al. 1990). Other than that, in the presence of trace metals (e.g., Cu^{2+} or Fe^{3+}) and without protections of metal chelators (e.g. EDTA and citric acid), tocopherols act as pro-oxidants by recycling transition of metal ion (Cort et al. 2006). The pro-oxidant effect of tocopherol can be diminished by the presence of ascorbic acid (Yamamoto 2001). Ascorbic acid reacts with tocopheroxyl radical and in turn regenerating the tocopherol and producing dehydroascorbic acid.

Table 5.11 Selected analytical techniques used for characterization of minor components in edible oils

Apparatus	Advantages	Disadvantages	Analysts	Reference
High performance liquid chromatography (HPLC)	– Rapid and precise quantitative analysis – Automated operation – Quantitative sample recovery	– Detection limit – Time consuming – Poor resolution – No universal detector	Tocopherols, sterols, squalene, phenolic compounds, chlorophyll and carotenoids	Zapata et al. (2000), Tasioula-Margari and Okogeri (2001), Sánchez-Machado et al. (2004), Dong (2006), Ignat et al. (2011), Kakade and Magdum (2012), Yuan et al. (2015)
High speed counter current chromatography (HSCCC)	– Dual-mode capability of counter current chromatography	– Time consuming – Risk of losing valuable compounds – Require gram-size sample injection	Carotenoid, phenolic compounds, sterols	Degenhardt et al. (2000), Aman et al. (2005), Sethi et al. (2009), He et al. (2009), Di et al. (2011), Ignat et al. (2011), Schröder and Vetter (2012)
Supercritical fluid chromatography (SFC)	– More versatile – Cost efficient – High output – Good resolution – Short analysis time – Multiple detection	– Limited mobile phase for high polar compounds – Not suitable for high polar solutes analysis – Require CO_2 tank which could occupied space – High power consumption – Complex equipment	Carotenoids, tocopherol, sterols, squalene, phenolic compounds	Choo et al. (2005), Yuan and Olesik (2006), Ignat et al. (2011), Méjean et al. (2015) Pilařová et al. (2016), Liu et al. (2018), Al Khawli et al. (2019)
Thin-layer chromatography	– Low cost – Short analysis time – Multiple detection – Specific derivatisation on the same plate	– Apply to only non-volatile compounds – Limited resolution capability – Cannot fully automated system	Tocopherols, phenolic compounds and sterols	Copius Peereboom and Beekes (1962), Medić-Šarić et al. (2004), Sherma (2006), Rybakova et al. (2008), Ignat et al. (2011)

(continued)

Table 5.11 (continued)

Apparatus	Advantages	Disadvantages	Analysts	Reference
Gas chromatography (GC)	– Great separation capacity – Offer high sensitivity and selectivity – Able to combine with mass spectrometry	– Complicated samples preparation – Limited to volatile compounds	Tocopherols, sterols, squalene and phenolic compounds	Verleyen et al. (2001), Liu et al. (2008), Mercy et al. (2014)
Near infrared spectroscopy	– Minimum sample preparation – Time and cost saving – Non-destructive – Real-time and on-site monitoring – Excellent repeatability – High sensitivity	– Broad peaks – Time-consuming data analysis	Chlorophyll, carotenoids, phenolic compounds	Moh et al. (1999), Lu et al. (2011), Toledo-Martín et al. (2018), Shi et al. (2020)
Mass spectrometry (MS)	– Provide qualitative and quantitative analysis – Able to combined with chromatographic separation, e.g. LC–MS, GC–MS – High specificity – High sensitivity and accuracy	– Time consuming sample extraction – Large consumption of solvent – Sample destruction – Technically trained operators – Complex chemical analysis	Phenolic compounds, tocopherols, carotenoids, volatile components, squalene and sterols	Podda et al. (1996), Ellis et al. (2012), Cao et al. (2017), Sales et al. (2019), Lang et al. (2019), Shi et al. (2019, 2020)
Nuclear magnetic resonance (NMR)	– Simple sample preparation – Simple measurement procedures – Extreme speed – Excellent repeatability – Minor reagents used	– Expensive equipment – Low sensitivity	Squalene, tocopherols, free fatty acid, phenolic compounds	Dayrit et al. (2008), Christophoridou and Dais (2009), Ignat et al. (2011), Nam et al. (2017), Martin-Rubio et al. (2018), Shi et al. (2020)

5.5.1.2 Carotenoids

Carotenoids are antioxidants that inhibit oil oxidation by free radical scavenging, light filtering and $^1O_2$ quenching (Choe and Min 2009). Carotenoids were reported

to filter light with short wavelength (400-500nm) (Fakourelis et al. 1987). In the presence of chlorophylls, carotenoids enhance light stability by providing strong protective effect against light. Warner (1987) found that the addition of β-carotene in soybean could inhibit flavour deterioration initiated by lights. Study explained that this could be due to carotenoid acting as the $^1O_2$ quenchers (Lee and Min 1988). Carotenoids also scavenge free radical by donating hydrogen and electron to radicals. Carotenoids react with lipid peroxyl radicals and produce non-radical and inactive products (Beutner et al. 2001; El-Agamey et al. 2004). In the process of anti-oxidation, the depletion of Vitamin E could occur and carotenoids are suggested to be beneficial in replenishing the depleted Vitamin E (Böhm et al. 1997). Carotenoids particularly transfer electrons to tocopheroxyl radical and regenerate the tocopherols (Böhm et al. 1997). However, certain conditions such as high oxygen pressure, high carotenoid concentration and UV light-induced photo-oxidation can enhance the pro-oxidant effect of carotenoids (Ryu et al. 2005; Choe and Min 2009).

5.5.1.3 Phenolic Compounds

Phenolic compounds are excellent antioxidants that scavenge free radicals to protect edible oils from lipid oxidation. Phenolic compounds impede lipid oxidation by donating hydrogen to lipid radicals like other antioxidants. The phenolic compounds inhibit oxidation at the initial stage by scavenging free radicals and chelating metals. Phenolic compounds inhibit the volatile formations such as aldehydes and ketones in oils that may lead to rancidity of oils (Kiokias et al. 2008; Nanditha and Prabhasankar 2008; Alamed et al. 2009). Sunflower oil has been discovered to be rich in phenolic compounds such as chlorogenic acid (CGA), caffeic acid, cinnamic sinapic and many other compounds. Leonardis et al. (2003) compared the antioxidant effectiveness between phenolic compounds in sunflower oil with synthetic phenol which is butylated hydroxyanisole (BHA). The phenolic compounds in sunflower oil (CGA) was found to be 118% more stable in stabilizing the oil oxidation compared to the control. The researchers concluded that the CGA from the phenolic compounds of sunflower oil stabilized oil oxidation more effectively than BHA at both low and high temperatures. Olive oil is stable as it contains many phenolic compounds which can work against autoxidation (Guillén and Cabo 2002). Glycoside oleuropein, hydroxytyrosol and tyrosol are the three main phenolic compounds in olive oils (Tuck and Hayball 2002). Among the phenolic compounds in an olive oil, hydroxytyrosol provides the most antioxidant effect in oil oxidation (Baldioli et al. 1996; Chimi et al. 1991; Deiana et al. 2002).

5.5.1.4 Phospholipids

The role of phospholipid in edible oils is complex as it can act as a pro-oxidant as well as an antioxidant. Phospholipids impede oil oxidation by chelating metals. However, the antioxidant activity depends on the content of the phospholipid in

edible oils. For example, ferrous ion (positive charge) is attracted to the negative charges on phosphate head group of phospholipid and suppresses oil oxidation by chelating iron (Cui and Decker 2016). A research found that adding more than 10 ppm of phospholipid in soybean oil would facilitate oil oxidation while lesser phospholipids addition would act as antioxidants (Yoon and Min 1987). Conversely, the absence of metals in purified soybean oil facilitates oil oxidation as phospholipid acts as oxidants. Many researches have reported on the synergism effect between phospholipid and tocopherol. Studies also reported that phospholipid alone does not show any antioxidant effect but it tends to act differently when tocopherols are available (Hidalgo et al. 2004; Takenaka et al. 2007; Lee and Choe 2011). Phosphatidylethanolamine which is a type of phospholipid has been proposed to facilitate the antioxidant activity of tocopherols. Phosphatidylethanolamine replenishes the "used-up" tocopherol by regenerating oxidized tocopherol quinone to its original form continuing with the free radical scavenging activity (Dziedzic and Hudson 1984; Judde et al. 2003; Doert et al. 2012; Cui et al. 2015). Inhibition of lipid oxidation was observed only when tocopherol was added with phosphatidylethanolamine in the edible oil, but was not available when the phosphatidylethanolamine existed alone (Cui et al. 2015). Conversely, phosphatidylcholine which is another type of phospholipid creates reverse micelles in edible oils, thereby facilitating oxidation without increasing the antioxidant activity of tocopherol (Cui et al. 2015). Besides, due to the amphiphilic character of phospholipids, it tends to reduce the surface tension of edible oils and enhances oxygen diffusion from the atmosphere into oil, thereby accelerating the oxidation of edible oils (Yoon and Min 1987). Overall, the phospholipid acts as an antioxidant due to its chelating properties, possesses synergism effect with tocopherol and acts as a pro-oxidant.

5.5.2 Authenticity Markers for Edible Oils

Authentication of edible oils is important from perspective of the commercial purposes. Authenticity includes adulteration, mislabelling, and misleading source or origin. Premium edible oils such as extra-virgin olive oil and camellia oil are most susceptible to adulteration with other vegetable oils by unscrupulous merchants. Adulteration of premium edible oils with other oils such peanut oil or other poor quality oil also imposes the risk of several health related issues including allergies that could lead to fatality. Other than fatty acid composition and triacylglycerol profile analysis, authentication of edible oils could be detected based on the minor components such as sterols, alkane and aliphatic alcohol analysis (Arvanitoyannis and Vlachos 2007). Minor components play important markers to determine oil quality, purity and authenticity (Tena et al. 2015; Olmo-García et al. 2018). Edible oils have their own characteristics and features, for example sterols. Rapeseed oil is high in brassicasterol (100–1100 mg/kg), olive oil contains high amount of β-sitosterol and Δ^5-avenasterol (683–2610 mg/kg and 34–266 mg/kg, respectively) and sunflower has high levels of Δ^7-stigmastenol (150–500 mg/kg)

Table 5.12 The sterol content for the authentication of edible oils

Vegetable	Sterol	Detection
2% rapeseed oil	brassicasterol	≤0.1% of olive oil 12–13% rapeseed oil 5–13% canola oil
10% soybean	campesterol	15–24% soybean oil ≤4% for olive oil
	stigmasterol	16–19% for soybean oil Less than campesterol for olive oil
>5% sunflower oil	campesterol	7–13% for sunflower oil 8–10% for high oleic sunflower oil
	stigmasterol	8–11% for sunflower oil
	Δ^7-stigmastenol	7–13% for sunflower oil 14–22% for high oleic sunflower oil ≤0.5% for olive oil
>10% grapeseed oil	campesterol	9–14% for grapeseed oil
	stigmasterol	9–17% for grapeseed oil

Adapted from Grob et al. (1994), Aparicio and Aparicio-Ruíz (2000)

(Aparicio and Aparicio-Ruíz 2000). These features could be used to detect adulteration. Another example, adulteration of sunflower or groundnut oil with rapeseed oil of more than 5%, can be evaluated by detecting the brassicasterol content (Aparicio and Aparicio-Ruíz 2000). Adulteration of safflower and sunflower oil with other edible oils can be detected by determining the Δ^7-stigmastenol as the contents of the Δ^7-stigmastenol are different in safflower and sunflower oils which are 16–23% and 7–13%, respectively (Aparicio and Aparicio-Ruíz 2000). Other examples of authentication of edible oils by detection of sterol content are listed in Table 5.12. Gumus et al. (2017) have succeeded in authenticating the geographical origin of virgin olive oils from six locations of the west part of Turkey. The results showed that there were significant differences in the trace metals such as iron and zinc in olive oils originated from different geographical locations.

Chen et al. (2011) used tocopherols as markers for authentification study of extra virgin olive oil. Reversed-phase high performance liquid chromatograph method was utilized for the detection of tocopherols. According to Chen's et al. (2011) results, extra-virgin oil (EVOO) has higher α/β tocopherol ratio value compared to other edible oils such as the sunflower, hazelnut and peanut oil. Other than tocopherol, study also used chlorophyll and carotenoids to establish the authenticity of a virgin-olive oil (Gandul-Rojas et al. 2000). Around 50 mono-variety virgin olive oils were able to be differentiated using the lutein, violaxanthin and total pigment as markers. However, factors such as species, geographical origin, storage, agricultural practices, agricultural conditions, manufacturing conditions and storage could also affect the physiochemical properties of the edible oils that might indirectly impact the results of authenticity (Bajoub et al. 2018; García-Vico et al. 2018; Conte et al. 2020).

5.6 Conclusion

Vegetable oils are made up of approximately 90% of the triacylglycerol while the remaining constitute of a minute amount of minor components. Minor components may vary depending on the plant species, growth condition, milling and refining process, extraction/or concentration process. The minor components include Vitamin E (tocopherols and tocotrienols), hydrocarbon (sterol and carotenoids) and phenolic compounds (polyphenols and phenolic acid). These are considered as phytonutrients that are beneficial to health attributed to their potent anti-oxidative properties or hypo-cholesterolemic characteristics. These valuable phytonutrients are being isolated, extracted or concentrated to be sold widely around the globe. Apart from that these phytonutrients can also act as authenticity markers for adulteration determination of vegetable oil. Seeing its valuable property of the phytonutrients, many attempts have been developed to recover the phytonutrients. Vegetable oils often being pre-treated to convert the free fatty acid or acylglcyerol into esters prior to separating using molecular distillation, supercritical carbon dioxide or crystallization. However, there some undesirable minor components are also present in vegetable oil and need to be removed during the refining process such as metals viz copper and ferrous ion as their presence will affect cause auto-oxidation and affect the quality of oil.

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Chapter 6
Blending, Hydrogenation, Fractionation and Interesterification Processing

Wan Jun Lee and Yong Wang

Abstract The lipid industry is constantly researching and developing techniques to improve the quality, stability, nutrition, and technological features of edible lipids. This chapter encompasses some of the recent advances reported on the four major lipid modification technologies, i.e., blending, hydrogenation, fractionation, and interesterification. Oil blending is a cost-effective method for modifying the oils' physicochemical and nutritional profiles, leading to the desired modulation of lipids and lipoproteins in human systems, improved thermo-oxidative stability, increased bioactive compounds' concentration, and potentially reduced food safety risks. Oil saturation can be increased *via* hydrogenation, resulting in the conversion of liquid into a semi-solid or solid state that is more stable in use. The formation of the dreaded trans-fatty acids during hydrogenation is an existing major drawback, but their concentration has been lowered with the progressive efforts in search of new catalysts, support materials, and technique innovation. Fractionation, a fully reversible process, has been utilized to separate lipid into fractions with greater application value, enrich oil with targeted triacylglycerol species or fatty acids, remove undesirable minor compounds, and detect foreign fat adulteration. Last but not least, interesterification can alter the positions of fatty acid moieties within or between triacylglycerols, leading to the modification in overall fatty acid composition while the degree of unsaturation or the isomeric state of the lipid system remains unchanged. Much attention has been paid to the enzymatic interesterification compared to the chemical method for the apparent advantages of higher reaction specificity, which allows better control of the reaction and production of structured lipids suitable for use in a wide array of food products.

Keywords Lipid modification · Blending · Hydrogenation · Fractionation · Interesterification · Structured lipid

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6.1 Introduction

Fats and oils are crucial components of our daily diets for they provide energy for the human body, transport fat-soluble vitamins, and perform a variety of other tasks. Extensively, they have been incorporated into a wide array of food products. The quality, stability, nutritional trait, and application features of the fats and oils are the utmost key factors that should be emphasized. Fats and oils can be acquired from diverse sources, e.g., plants, animals, marine life, and microorganisms. Nonetheless, fats and oils in their original forms have restricted technological application attributed to their specific chemical compositions and until now, no single fat or oil has been identified that has excellent properties. Lipid modification technologies, *viz*., blending, hydrogenation, fractionation, and interesterification, are therefore being introduced to modify the physical and chemical profiles of these fats and oils, depending on their specific end-use. The modified lipids have optimized technological application in addition to the improved quality, thermo-oxidative stability, and nutritional values. This chapter discusses the utilization and recent advancements of blending, hydrogenation, fractionation, and interesterification in modifying the physicochemical properties of fats and oils intended for food application.

6.2 Blending

Vegetable oils from different sources are consisted of distinguishing fatty acid compositions, comprising of both saturated fatty acids (SFA) and unsaturated fatty acids (UFA). In UFA, at least one double bond exists within the fatty acid chain in monounsaturated fatty acids (MUFAs) and multiple double bonds in polyunsaturated fatty acids (PUFA). Vegetable oils can be categorized into oils which are high in SFA, MUFA and PUFA, respectively, as shown in Fig. 6.1. This classification is based on the fatty acid compositions issued in the International food standards (Codex Alimentarius 2019).

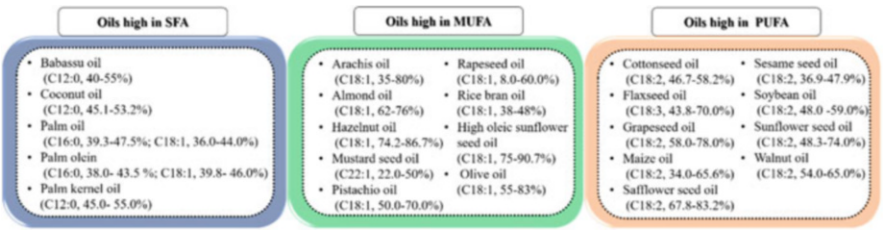

Fig. 6.1 Vegetable oils which are high in saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids. The fatty acid compositions are adapted from Standard for Named Vegteble Oils from International Food Standards (Codex Alimentarius 2019)

Consumption of UFA-rich oils is encouraged over SFA-rich oils since the intake of SFA has been associated with various health issues such as obesity and an increased risk of coronary heart diseases (CHD). However, UFA-rich oil has lower thermo-oxidative stability than its counterpart due to the presence of double bonds in the molecular structures. A correct blend of oils can modify and improve the overall fatty acid composition by taking the advantage of the desirable characteristics from each type of oil, resulting in a new oil blend with favorable characteristics in terms of physicochemical properties, nutritional qualities and improved industrial applicability. Physical oil blending has then emerged as an economical way to modify the physicochemical and nutritional qualities of oils. The blending procedure is straight forward; it entails simple mechanical stirring and, in some cases, nitrogen is bubbled during the blending to prevent oxidative damage caused by the mechanical stirring. Oil blending results in the change of the fatty acid compositions which are favorable for modulating lipids and lipoproteins in human systems, improving thermo-oxidative stability, increasing the concentration of bioactive compounds, and reducing the concentrations of food safety risk components.

6.2.1 Modulation of Lipids and Lipoproteins

Consumption of oils high in SFA has been linked to the incidence of an increase in total blood cholesterol level and the ratio of low-density lipoprotein-cholesterol/high-density lipoprotein-cholesterol (LDL-C/HDL-C), and the development of cardiovascular diseases, obesity, type II diabetes, and cancer (Pedersen and Kirkhus 2011). However, the intake of SFA should not be entirely omitted as SFA has a heterogenous structure and function and are synthesized de novo by the human body. Only certain SFAs in excess have been associated to CHD (Nettleton et al. 2015). Reduction in SFA intake or replacement of SFA with vegetable oils high in UFA did not result in a significant reduction in CHD risk although it did result in a lower risk of stroke and a decrease in LDL-C levels (Duarte et al. 2020). Hence, the effects are largely dependent on the source and chain lengths of the SFA. For instance, medium-chain SFAs are linked with higher HDL-C levels than long-chain SFAs (Panth et al. 2018). A balanced consumption of SFA, MUFA, and PUFA is encouraged. The World Health Organization recommends a total intake of SFA of not exceeding 10%, and the American Heart Association recommends a 1:1:1 ratio of SFA: MUFA: PUFA for a balanced diet. Sufficient intake of both SFA and PUFA is required for the ideal LDL-C/HDL-C ratio in the blood. By blending oil high in SFA with oil rich in UFA, the saturation level can be modified, thereby lowering the atherogenic potentials caused by the consumption of SFA.

Examples of oils which are high in SFA include palm oil (PO), palm olein (POO), and coconut oil (CNO). By virtue of the balanced SFA and UFA compositions, PO exhibits versatility in the food industry whereby it is suitable for cooking, frying, and application as baking fats. The complex interplay of PO fatty acids on blood lipids has been reviewed by Hayes and Khosla (2007), and a systematic review and

meta-analysis of dietary intervention trials have been published by Fattore et al. (2014), concluding that both favorable and unfavorable changes in CHD risk markers occurred when PO was substituted for the primary dietary fats. Blending PO with other vegetable oils is shown to lower its saturation level and its lipid modulating effect. A high-oleic cooking oil blend consisting of POO and canola oil (CO) comprising of 50% MUFA showed no significant difference in terms of the anthropometric indices, waist circumference, waist-to-hip ratio, serum biomarkers of obesity and inflammation, in comparison to that of extra virgin olive oil (EVOO) and CNO (Lee et al. 2018). In a study conducted by Adeyemi et al. (2015), PO (20%) and CO (80%) blend was fed to goats as dietary supplement to increase UFA and minimize SFA in the ruminant meat without causing negative effects on its physicochemical properties or shelf life. CNO contains high SFA and has been incorporated into various oil blends. Antithrombotic effects such as reduction in the rate of adenosine diphosphate-induced platelet aggregation were reported in blends of CNO/rice bran oil (RBO) and CNO/sesame seed oil (SSO) (SFA: MUFA:PUFA of approximately 1:1:1) (Reena et al. 2010). In oil blends comprising of CNO/sunflower oil (SFO)(1:1) and CNO/soybean oil (SBO) (1:1), a reduction in the serum cholesterol levels and significant change in triglyceride (TAG) concentration was reported (Chandrashekar et al. 2010). Significant reduction in the serum total cholesterol, LDL, and TAG, as well as a significant increase in serum HDL was observed in rats fed with oil blends prepared from CNO/mustard seed oil (6:4) (Seneviratne et al. 2011).

Modulatory effect on lipid metabolism was also observed in oil blends comprising of UFA-rich oils. The blend of SBO/SFO/flaxseed oil (FO) was reported to be effective in the treatment of hypercholesterolemia as the blend was able to lower the total cholesterol, TAG, and LDL-C levels, while increasing the HDL-C levels (Ramadan et al. 2009). A similar effect was reported by Umesha and Naidu (2012) whereby the modification of fatty acid composition aimed at improving the n-6/n-3 PUFA ratio (2.3–2.6) in different vegetable oils such as SFO, RBO, and SSO through blending with α-linolenic acid (ALA)-rich Garden cress oil, was able to lower the total cholesterol, TAG, and LDL-C levels. Besides, a significant increase in the ALA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) levels was observed in the test subjects's tissues. Effect of oil blends consisting of 5 different oils, i.e., CO, corn oil (CRO), olive oil (OO), peanut oil (PNO) and SFO, on the cardiovascular health, blood pressure and body weight in rats were determined by Uriho et al. (2019). The prepared blend contained 50.93% of oleic acid and a reduced n-6/n-3 PUFA ratio with 5.41% of ALA. It was found that the growth parameters, oxidative stress, inflammation, lipid metabolism, blood lipids, blood pressure and cardiovascular function were improved with this functional blend (high oleic acid and low n-6/n-3 PUFA ratio) in comparison to that of lard and PNO (high n-6/n-3 PUFA ratio). Therefore, oil blending to reduce the n-6/n-3 PUFA ratio could be a new nutritional strategy for better prevention and management of high blood pressure and cardiovascular risk. Devarajan et al. (2016) found significant anti-hypertensive and lipid-lowering action, as well as a noteworthy additive effect with anti-hypertensive medication with the oil blend of unrefined cold-pressed SSO

with physically refined RBO (20:80). These effects were attributed to the high PUFA (35.6%) and MUFA (42.97%) content. Thus, oil blends that are high in UFA, particularly PUFA, are sought for their valuable health properties.

6.2.2 *Enhanced Oxidative and Frying Stability*

6.2.2.1 Stability as Affected by the Change in Fatty Acid Compositions

Lipid oxidation is a vast challenge in the food industry as not only it limits the shelf life and affects the sensorial quality of the edible oils, it causes the formation of hazardous compounds. One of the most decisive aspects which influence oil oxidative stability is the fatty acid compositions. In general, SFA-rich oils are more stable than UFA-rich oils. It can be anticipated that the higher the unsaturation (MUFA and PUFA), the least stable the oil is. Thus, ΣPUFA/ΣSFA could be a strong indicator to evaluate the oil's stability and deterioration. Kerrihard et al. (2015) quantified the relationship between fatty acids and the stability of 50 types of plant-based oils and fats. It was found that the concentration of MUFA, di-unsaturated fatty acid, and tri-unsaturated fatty acids demonstrated very strong correlation to the magnitude of oxidation, approximately 1:3:12, which was substantially greater than the 1:2:3 ratio of their relative unsaturation. Hence, SFA-rich oils were blended with UFA-rich oils to lower the level of saturation and address the dreaded health issues while still contain sufficient SFA levels to meet our dietary needs and ensure the oil's stability.

Oils used for frying should have a long frying life and impart desirable organoleptic properties on the fried foods. There are some tests or indicators that are commonly used to monitor the deterioration of frying oils, such as free fatty acid (FFA) value, peroxide value (PV), *p*-anisidine value (*p*-AV), conjugate diene and triene, total polar compounds (TPC), and polymeric TAG. Almost all vegetable oils are suitable for frying due to the higher smoke point ($> 200\ ^{\circ}C$) than the frying temperature. Oils such as POO, SBO, CRO, SFO, PNO, and CO which are frequently used in the frying industry or commercially in food outlets or restaurants are available in bulk and at a lower cost. Other oils that can be used for frying but are not readily available in bulk for industrial use (normally for household use) include CNO, grapeseed oil, and RBO. The physiochemical properties of different oil blends have been extensively reviewed by Hashempour-Baltork et al. (2016).

Table 6.1 shows some of the more recent literature reporting on the blending of different oils for frying.

6.2.2.2 Modification of Bioactive Compound Profiles

Vegetable oils contain a high concentration of bioactive compounds, which is beneficial to both human health and the stability of the oils. Through blending, the desirable effects can be attributed to the increment in the concentration of

Table 6.1 Examples of the thermo-oxidative stability of various oil blends

Oil blend	Findings	References
POO/SSO	POO:SSO blend (60:40) with SFA:MUFA:PUFA of 1.00:1.05:1.15 was used for vacuum frying of pear chips at 110 °C, 74–98 mbar, 4.0–4.2 min. Blended oil retained higher PUFA concentration after 20 frying cycles, with FFA≤ 2.84%, PV ≤ 5.28 meq O_2/kg, IA ≤ 0.57, and IT≤1.08.	(Juvvi et al. 2020)
POO/MO	MO contains high MUFA (76%) with MUFA/SFA ratio of 4.73 and bioactive compounds such as β-sitosterol, squalene and tocotrienols. The ratio of MUFA/SFA in the optimal blends of POO:MO of 25:75 (2.81) was significantly higher than that of pure POO (0.97). Oxidative stability tests (65 °C, 15 days) for the optimal blend showed FFA = 0.42%, PV = 17:39 meq O_2/kg, *p*-AV = 1.37, and TOTOX value = 36.96. This blend can be introduced as a stable frying medium for its high smoke point of 221 °C.	(Koohikamali and Alam 2019)
SFO/SSO	SSO is high in ω-fatty acids and contains lignans (sesamolin, sesamol, sesamin). Optimized blend of SFO:SSO of 50.8:49.2 with a balanced ratio of ω-6 and ω-9 (49:34.5) showed improved thermal and oxidative stability, showing PV = 9.65 meq O_2 /kg, AV=0.79 mg KOH/g, *p*-AV =3.33, and TPM =6.19%.	(Ghosh et al. 2019)
SFO/MOO	MOO is rich in MUFA (66.5–81.7% oleic acid), sterols and vitamin E. SFO:MOO (80:20) for deep-frying of fresh potatoes at 180 °C, 90 min for 5 days showed significant reduction in the formation of polymers (<43–85%) and oxidized triglycerides (<20 to 60%), 25–60% reduction in *p*-AV and total volatile aldehydes (alkadienals).	(Boukandoul et al. 2019)
PO/SBO/SFO	Binary and ternary blends were used for deep-fat frying of French fries. Pure PO was a better frying medium than the blended oils but the blended oils have better fatty acid composition for human health and was more stable than pure SBO and SFO.	.(Rudzińska et al. 2018)
POO/CSO	CSO contains approximately 70% UFA (18% MUFA, 52% PUFA). Blend of POO:CSO of 50:50 showed to have the least increment in the TPC value and polymeric compound at the end of 10 h successive frying of frozen French fries at 170 °C; ΣPUFA/ΣSFA= 1.74, TFA = 0.35%, TPC=11.62%, polymeric compounds = 4.34%, PV = 1.46 meq O_2/kg, FFA=1.13%, IV= 80.49. Through blending, the ratio of ΣPUFA/ΣSFA in CSO decreased and the oxidative and frying stability were significantly improved.	(Arslan et al. 2017)
SBO/CMO	CMO has high levels of oleic acid (75–80%), vitamin E, squalene and flavonoid. Blending significantly increased the MUFA content.	(Wang et al. 2016)

(continued)

Table 6.1 (continued)

Oil blend	Findings	References
	SBO/CMO blend (60:40 and 50:50) showed better oxidative stability and radical scavenging activity during deep frying of French fries at 180 °C for 5 consecutive days; FFA = 0.40, 0.32% and *p*-AV = 69.78, 60.55.	
OO/PO/SBO	Frying performance of blends of refined oils (OO:PO and SBO: PO) during a 50 successive deep-frying sessions of potato fries at 180 °C was evaluated. OO:PO blend had the highest chemical stability whereby the TPC increased to> 25% after 40 successive frying whereas SBO: PO blend exceeded the limit after 30 frying sessions.	(Zribi et al. 2016)
Crude PO/refined CO	Blend of crude PO: refined CO of 1:1 was used for 20 h successive deep-fat frying at 170, 180 and 190 °C. FFA and PV accumulation followed the kinetic first-order model, while *p*-AV, TPC, and CI followed the zero-order kinetic model. The Arrhenius model was used to evaluate the temperature effect; PV had the least activation energy whereas the highest activation energy requirement was observed for FFA. The overall activation energy values showed that the stability of the blend was superior and not just intermediate of crude PO and refined CO.	(Mba et al. 2016)

POO palm olein, *SSO* sesame seed oil, *FFA* free fatty acid, *TFA* trans-fatty acid, *PV* peroxide value, *p-AV p*-anisidine value, *AV* acid value, *IA* atherogenicity index, *IT* thrombogenicity index, *MO* macadamia oil, *TOTOX* total oxidation, *SFO* sunflower oil, *TPC/TPM* total polar content/total polar matter, *MOO* Moringa oleifera oil, *PO* palm oil, *SBO* soybean oil, *CSO* cottonseed oil, *CMO* camellia oil, *OO* olive oil, *CO* canola oil, *CI* color index, *SFA* saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid

bioactive compounds and the possible synergistic effect between the different types of bioactives.

Vitamin E (tocopherols, T and tocotrienols, T_3) has been reported to increase oil oxidative stability during frying. CO predominantly contains α-, β-, γ- and δ-T, whereas POO contains α- and γ-T_3. Through blending POO with CO (1:1), the concentrations of T and T_3 were altered, thus increasing the stability of oil (lower TPC after 28 h of frying) whilst minimizing the formation of degradation products (Al-Khusaibi et al. 2012). This is probably due to the greater antioxidant activity of T_3 as reported by Ramadan (2013), whereby the α-T_3 was more effective at reducing lipid peroxidation than α-T. Cold-pressed oil is a good source of bioactive compounds for there is no refining process involved. Oil blends prepared from high linoleic SFO and several other cold-pressed oils (black cumin oil (BCO), cumin oil, coriander oil, and clove oil) showed better oxidative stability due to the high concentration of T and T_3. There is a significant increase in the oxidative stability of oil blends of rapeseed oil (RSO), BCO, and RBO attributed to the increase in the

concentration of bioactive compounds (T and T_3 in blend containing BCO and T_3, β-sitosterol and squalene in blend containing RBO) (Rudzińska et al. 2016). Incorporation of unconventional oil such as bene kernel oil and its unsaponifiable matter fraction, which have been known to be very effective natural sources of antioxidative compounds, were used to improve the frying stability of CO blended with POO and virgin olive oil (VOO) (75:15:10) at 180 °C (Sharayei and Farhoosh 2016). Tavakoli and Sorbi (2018) determined the effect of adding Kolkhoung hull oil, Beneh hull oil, and SSO as natural antioxidants in a blend with refined SBO at various concentrations, on the oil stability during a 32 h frying at 180 °C. The blends showed antioxidative effects that were comparable to that of the synthetic antioxidant tert-Butylhydroquinone attributed to the high concentration of T_3, Δ-7-avenasterol, and Δ-5-avenasterol. Blending oils with commercially trademarked oil that is specially produced for certain applications has also been reported. For instance, Clarinol® G-80 is an oil mixture that contains high concentration of conjugated linoleic acid, has provided stability against oxidation of POO (10% Clarinol® G-80) during 5 days of frying (Alavijeh et al. 2015). However, more studies are needed to validate the hypothesis of the CLA antioxidant effect in the frying oil.

Synergistic effects between the different bioactive compounds acquired through oil blending can be observed. Synergism occurs when there is a more pronounced effect when more than one type of component is present in the same system compared to the sum of their individual effects. Mba et al. (2016) suggested on the synergistic radical scavenging activity between the antioxidants present in the crude PO and refined CO (β-carotene, T, and T_3), which make the oil less prone to oxidation and deterioration. Synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, and dimethyl polysiloxane added into the commercially available refined CO (1:1) during a 20 h of successive deep-fat frying slowed down the deterioration of oil. However, an in-depth study on this synergistic effect is still lacking.

6.2.3 Reduction of Food Safety Risk Factors

Oil blending for the reduction of food safety risk factors is a relatively new research topic introduced in recent years. 3-Chloropropane-1,2-diol esters (3-MCPDE) and glycidyl esters (GE) are the two representatives of food contaminants that have gained attention over the years. These contaminants are found in edible oils in various concentrations (Cheng et al. 2017) and are also formed during the oil refining and heating processes. With oil blending, the levels of these food contaminants can be reduced. A lower concentration of 3-MCPDE and GE in oil blends (mixture of olive oil (OO) with another vegetable oil such as SBO, CO, SFO, refined OO, or olive pomace oil) compared to that of EVOO and OO was reported (Kamikata et al. 2019). In a work by Ben Hammouda et al. (2017), the levels of 3-MCPDE and GE in oil blends consisting of refined olive pomace oil with refined PO during frying of French fries at 175 °C for 16 h has been measured. Fresh PO contains a high

concentration of 3-MCPDE and GE (1.3 and 4.0 mg/kg, respectively), but when blended with refined olive pomace oil (olive pomace oil to PO ratio of 75:25), the concentrations of 3-MCPDE and GE were decreased to 1.2 mg/kg and 2.3 mg/kg, respectively. Post frying, the 3-MCPDE level in the oil blend was slightly higher (0.90) compared to the pure olive pomace oil (0.60) but with lower GE (0.20 vs. 0.50). There was no endogenous formation of 3-MCPDE and GE during deep-frying when refined olive pomace oil /refined PO oil blends were used under typical frying conditions. Another potential food safety risk that is developed under prolonged exposure to heat is the formation of acrolein, which is also carcinogenic. Blending EVOO with salad oil effectively reduced the amount of acrolein formed during repeated frying (Kishimoto and Kashiwagi 2018).

6.2.4 Blended Oil for Food Application

Considering the nutritional qualities and enhanced stability, oil blends have been incorporated into the preparation of various food products. Dollah et al. (2020) produced an oleic acid-rich (67%) table margarine from an oil blend consisting of Moringa oleifera seed oil (MOO) and palm stearin (PS) (70:30). Compared to two commercially available margarine, the oil blend showed to be of superior quality for it is comprised of higher oleic acid and melting temperature with better textural properties even after 8 weeks of storage. Human milk fat substitutes (HMFS) were successfully formulated from oil blends consisting of refined POO, SBO, refined OO, virgin CNO, and fish oil (Mohammadi et al. 2019). The best formula for HMFS was 55% POO, 13.5% SBO, 16% refined OO, 15% virgin CNO, and 0.5% fish oil. Optimal blends of palm mid-fraction, refined bleached deodorized palm kernel oil (PKO), and refined bleached deodorized PS can be used for the preparation of cocoa butter alternative (CBA) for their comparable physicochemical properties (Biswas et al. 2016).

6.3 Hydrogenation

Hydrogenation is the process of converting liquid oil into semi-solid or plastic fats using hydrogen in the presence of a catalyst. The hydrogen atoms bind to the double bond to facilitate the conversion into a single bond, reducing the oil's unsaturation level. The reaction is influenced by the reaction conditions (temperature, time, pressure, and agitation), amount and type of catalyst used. Typically, the reaction is carried out at a temperature of 100–250 °C and a pressure of 1–10 bar, while the catalyst's load varies depending on the type of catalyst used. Tank, bubble column, and fixed bed reactors are the three primary types of reactors that can be used for edible oil hydrogenation, among which autoclaves (tank reactor) operated in batch-wise being the most frequently used. Hydrogenation takes place when oil substrate,

hydrogen gas, and catalyst are mixed under reaction conditions. In general, oil substrate for hydrogenation process should meet the requirements of FFA <0.05%, moisture < 0.05%, PV < 0.5 meq O_2/kg, and *p*-AV < 10. As for the hydrogen gas, the purity should be >99.8%. There are two major categories of catalysts that are suitable for the hydrogenation process, namely, homogeneous and heterogenous catalysts. Homogeneous catalysts exist in the same phase as the reactant and are stereotypically in liquid form (e.g., Wilkinson's catalyst, Crabtree's catalyst, Schrock–Osborn catalyst), whereas heterogenous catalysts are of different phases from the reactant and are commonly in solid form. Most of the edible oil hydrogenation processes employ heterogenous catalysts (e.g., noble metals such as palladium and platinum, and base metals such as nickel, cobalt, and copper) for they can be separated with ease from the reaction mixture via simple procedures and the recovered catalysts can be reused. Depending on the application and aim of the process, a supported or unsupported catalyst is employed. In a supported catalyst, the metal is deposited or supported on an inert material such as silica, carbon, graphite, alumina, or inorganic salts.

6.3.1 Trans-Fatty Acids Formation

Hydrogenation is often associated with the formation of trans-fatty acid (TFA), which is feared among consumers for its detrimental health effects (Levy et al. 2019; Oteng and Kersten 2020). TFA is the UFA that is comprised of at least one non-conjugated double bond in the *trans* configuration. Strict laws and regulations have been imposed worldwide in banning and limiting TFA in food (not more than 2 g per 100 g of fat). Also, the USFDA issued a regulation banning artificial TFA in food applications in 2018. TFA is found naturally in ruminant animals, but the main source of intake is from processed food that utilizes partially hydrogenated oils such as margarine, vanaspati, vegetable shortenings, baked and fried products. During a complete or full hydrogenation process, the liquid oil is entirely converted to a solid form with little or no TFA formed. In the partial hydrogenation process, the short retention time of UFA on the metallic catalyst causes the shifting of the double bond from the *cis* to *trans* configuration through an isomerization reaction, producing semi-solid partially hydrogenated oil. Albeit the detrimental effects, partially hydrogenated oils are useful in many ways in the food industry such as providing improved physical properties (texture, solid fat content, and melting profiles) and oxidative stability. Substituting partially hydrogenated oils with an alternative that can achieve similar desirable properties can be a great challenge.

6.3.2 Reduction and Elimination of Trans-Fatty Acids

Research efforts on eliminating TFA from the partial hydrogenation process while preserving the desired physical properties and stability are still progressing. For instance, the utilization of new catalysts and support materials, innovations in the hydrogenation process, integration of full hydrogenation process with oil blending, fractionation (see Sect. 6.4), and interesterification (see Sect. 6.5).

6.3.3 New Catalysts and Support Materials

A highly selective and active catalyst can be designed to significantly reduce TFA content via the selection of a suitable catalyst, promoter, and carrier. The specific surface area of the catalyst can be improved using a suitable carrier and by adding promoters to improve the selectivity in the preparation of the main catalyst (Yu et al. 2019). Over the years, various supported catalysts have been investigated for their effects in improving the reaction activity and reduce the TFA levels produced during hydrogenation. Table 6.2 shows some of the more recent examples of new catalysts and/or supports used for the hydrogenation process that could decrease the TFA content.

6.3.4 Innovation in the Hydrogenation Process

During the conventional hydrogenation process, the high reaction temperature is one of the reasons in driving the formation of TFA. The high reaction temperature is applied to facilitate better contact between the hydrogen, catalyst and substrate, influencing the activity, selectivity, and yield. Supercritical fluid state hydrogenation, electrochemical hydrogenation, and catalytic transfer hydrogenation can be operated as alternative techniques for the reaction. These processes can be performed at low-temperature conditions, thereby reducing the TFA content in the hydrogenated products.

6.3.4.1 Supercritical Fluid State Hydrogenation

Integration of supercritical fluid (SCF) technology during the catalytic hydrogenation reaction has been introduced due to the properties of SCF as an excellent solvent, i.e., high diffusivity, liquid-like density, solvating properties, and mild reaction conditions. For further reading, readers can refer to the detailed chapters on hydrogenation under supercritical conditions by King and List (2011) which covers the fundamental knowledge, merits, and advances in SCF state hydrogenation

Table 6.2 Catalysts and supports used for the hydrogenation process aiming at reducing the formation of trans-fatty acids

Catalyst/ Metal	Support/Promoter	Oil	Effect on the formation of trans-fatty acid	Reference
Ni-(Ag[a])	PVP-DB-171/SiO_2/ Fe_3O_4	SBO	Ag (molar ratio 0.15) improved the dispersion of Ni on the PVP-DB-171/SiO_2/Fe_3O_4. Compared to Raney Ni and Ag-free magnetic NP catalysts, catalysts with the addition of Ag showed a reduction of TFA content to 10.4%. This magnetic NP catalyst can be separated by applying an external magnetic field effectively and efficiently after reaction from the end product.	(Wang et al. 2018)
Cu, Cu-Ag, Cu-Pd, Ni, Ni-Ag, Ni-Pd	SBA15	SBO	The total TFA content in descending order: Cu/SBA15 (16.01%) > Ni/SBA15 (14.70%) > Cu-Pd/SBA15 (12.18%) > Ni-Pd/SBA15 (11.66%) > Cu-Ag/SBA15 (11.57%) > Ni-Ag/SBA15 (10.55%). The addition of Ag reduced the TFA formation to a higher degree compared to that of Pd. The addition of noble metal promoter enabled the catalyst to be more evenly dispersed on the molecular sieve carrier, increasing the H_2 contact area and the adsorption of H_2 on the catalyst surface, thereby enhancing the hydrogenation and reducing the chance of fatty acid isomerization. Cu catalysts produced higher TFA.	(Zhao et al. 2018)
Pt, Rh, Pd	TPPTS	LSO	Partial hydrogenation of polyunsaturated methyl esters of LSO using water-dispersible metal-nanoparticle-TPPTS-lecithin-stabilized catalytic systems produced a low concentration of $trans_{C18:1}$ (0.1 Mol%). Low $trans_{C18:1}$ (2.4 Mol%) was formed using Pt/TPPTS catalyst compared to the traditional method using Ni-based catalyst ($trans_{C18:1}$ of 30 Mol% in SBO). The $trans_{C18:1}$ formation was the	(Stathis et al. 2017)

(continued)

Table 6.2 (continued)

Catalyst/ Metal	Support/Promoter	Oil	Effect on the formation of trans-fatty acid	Reference
			lowest when Pt/TPPTS was used, followed by Rh/TPPTS and lastly, Pd/TPPTS complexes.	
Pt	TiO_2, ZrO_2, AC, MCNTs	SBO	Pt-supported catalysts showed lower selectivity towards $trans_{C18:1}$ (25.48–27.41%) compared to that of commercial Raney Ni (31.42%). Pt/ZrO_2 catalyst had the lowest selectivity towards the formation of $trans_{C18:1}$ (25.48%) and stearic acid.	(Wang et al. 2017)
Pd, Co, Ni	Al_2O_3, SiO_2	SFO	The reaction catalyzed by Pd/Al_2O_3 had the highest $trans_{C18:1}$ (44.7%), while Pt/SiO_2 resulted in the lowest $trans_{C18:1}$ (6.5%). At 150 °C, 3.5 MPa, the TFA content in descending order, Pd > Ni > Pt (SiO_2 supported), Pd > Pt > Ni (Al_2O_3 supported); At 180 °C, 4.5 MPa, Cu > Co > Ni (SiO_2 supported), Co > Ni = Cu (Al_2O_3 supported). Pd catalyst produced the highest $trans_{C18:1}$ compared to Pt and Ni, attributed to the higher activity and formation of conjugated dienes on the catalyst surface. Pt and Ni had lower trans selectivity due to the subsequent hydrogenation of the intermediate trans-monounsaturated to saturated compounds. Al_2O_3 allows for more active Pd, Pt, and Co catalysts than SiO_2. The influence of the support is much more pronounced for supported Pt catalysts. A binary mixture of Pd/Al_2O_3–Co/SiO_2 had a good balance between activity and selectivity, resulting in a very low production of $trans_{C18:1}$ (11.8%) and a moderate amount of stearic acid (13.5%).	(Cepeda et al. 2016)
Ni-Al-(Ce[a])		CO	Ce impeded the formation of all-trans isomers ($trans_{C18:1}$ and $trans_{C18:2}$).	(Konkol et al. 2016)

(continued)

Table 6.2 (continued)

Catalyst/ Metal	Support/Promoter	Oil	Effect on the formation of trans-fatty acid	Reference
			In combination with hydrogenation pressure, both factors reduced all TFA isomers from 37.7 to 20.9%.	
Pt, Ni	Al_2O_3, ZrO_2, CeO_2, TiO_2, MgO, MoO_{3-x}, SiO_2, CaO, $BaSO_4$, C	SBO	The electronic interactions between the support and Pt significantly affected the TFA levels in partially hydrogenated oils. The TFA levels were the highest for the medium electronegativity metal ion supports. TFA in commercial Ni (23.5%), reference catalysts Pt/C (24.6%), and the highest TFA was produced using Pt/ZrO_2 as the catalyst (35.1%). TFA levels in descending order: $Pt/ZrO_2 > Pt/Al_2O_3 > Pt/TiO_2 > Pt/C > Pt/SiO_2 > Ni > Pt/MgO > Pt/CeO_2 > Pt/CaO > Pt/MoO_{3-x} = Pt/BaSO_4$. $Pt/BaSO_4$ catalyst was the most effective for reducing both TFA (11%) and saturated fatty acids.	(Iida et al. 2015)
Ni-Mg-(Ag[a])	Diatomite	SBO	The addition of Ag (5.88%) to NiMg/diatomite catalyst decreased both the selectivity towards TFA formation and the selectivity towards SFA, forming the least TFA (26.3%) in comparison to the Ag-free catalysts (61.2%). Catalysts with large surface area favor isomerization reaction due to the greater accessibility to the active sites.	(Stanković et al. 2015)

Pd palladium, *Co* cobalt, *Ni* Nickel, *Pt* platinum, *Rh* rhodium, *Al* aluminium, *Ce* cerium, *Ag* silver, Al_2O_3 aluminium oxide, SiO_2 silica, *C* carbon, *TPPTS* trisulfonated triphenylphosphine, TiO_2 titanium dioxide, ZrO_2 zirconium dioxide, *AC* activated carbon, *MCNTs* multi-walled carbon nanotubes, CeO_2 cerium oxide, *Mg* magnesium, *MgO* magnesium oxide, MoO_{3-x} molybdenum oxide, *CaO* calcium oxide, $BaSO_4$ barium sulfate, *SBA15* mesoporous silica, *SFO* sunflower oil, *SBO* soybean oil, *LSO* linseed oil, *CO* canola oil

[a]Metal added as a promoter or additives

for fats and oils and also from Wang et al. (2020a), which includes the principle and fundamentals of SCF hydrogenation, equipment, process developments and challenges, and some insights into future potentials.

In a SCF state hydrogenation, addition of SCF provides a homogenous phase and enhances the transfer of hydrogen to the catalyst surface, thereby increasing the reaction rate and reduces the TFA formation. Hydrogenation of SBO using mixtures of supercritical carbon dioxide ($scCO_2$) and hydrogen was reported by King et al. (2001). The reaction was performed at 2000 psi, 120–140 °C, and using nickel as the catalyst. The addition of carbon dioxide (CO_2) to the fluid phase containing hydrogen slowed the overall reaction rate while lowering the TFA content. For a plant simulation study, Ramírez et al. (2011) hydrogenated SFO in different SCF solvents (supercritical propane, hexane-modified CO_2, pure liquid hexane), catalyzed by two different catalysts (Pd/C, Pd/Al_2O_3). An end product with a selectivity of 400 (oleate/stearate mole ratio) and trans-isomer of $<3\%$ was obtained at optimal conditions (multi-tubular reactor, 170 °C, 3 mol% of hydrogen, and 20 MPa), which was better than the conventional process. However, the author suggested that in order to attain lower TFA content, a significant catalyst adjustment may be necessary to achieve appreciable reaction rates at low temperatures in the presence of co-solvent. Zhao et al. (2018) used six different types of supported catalysts to hydrogenate SBO in a $scCO_2$ system (100 °C, 12 MPa), resulting in an end product containing up to 50.27% oleic acid and 10.43% of TFA.

6.3.4.2 Electrochemical Hydrogenation (ECH)

ECH is another alternative hydrogenation technique that can yield fats with minimal TFA content for the low operating temperature. This process does not require high pressure and can be performed under mild reaction conditions, although at a slower hydrogenation rate. ECH processes are often heterogeneous and the electrochemical reactor contains a cathode for reduction reactions (i.e. electrically conducting catalyst such as Raney Ni or platinum black), an anode for oxidation reactions and with an electrolytic solution (a solvent and supporting electrolyte salt) between the electrodes. This system can yield products with less TFA under controlled conditions, and since hydrogen is generated in situ over the catalytic surface, there is no need to enhance the hydrogen transfer rate, thereby eliminating the requirements to operate at high temperatures and high pressures (Mondal and Lalvani 2003). Hydrogen donors can be transferred through different pathways. It can be accomplished through a proton-exchange membrane with the source of protons derived from the electrolytic water at the anode or by using a mediator-assisted system whereby the electrolytes (formate and formic acid) carry protons to the double bonds of UFA (Fu et al. 2011). These are the earlier mode of ECH. Different types of reactors have been progressively introduced to carry out ECH to increase the efficacy of the hydrogenation process and also to obtain end products with minimal TFA. For instance, the utilization of a diaphragm reactor introduced the regeneration of formate ions as the shuttle occured directly at the cathode area in situ. At the same time, the hydrogen proton donors travel from anode to cathode across the proton exchange membrane, resulting in less formic acid addition and products with lower acid values than that of the mediator-assisted system (Fu et al. 2011). Besides, a

reactor that allows the ECH reaction to be carried out under hydrogenated conditions improved the solubility of hydrogen in oil and enhanced the diffusion onto the catalyst surface, resulting in an end product with a TFA of 4.3% (Wang et al. 2019b). A solid polymer electrolyte reactor fabricated using Pt/graphene oxide nanocomposites as cathode catalysts were used for the hydrogenation of SBO, resulting in hydrogenated products with a TFA content of 1.53% (Ding et al. 2019).

A combination of technologies, in this case, the combination of $scCO_2$ with ECH, can be carried out to produce fats with low TFA. Yu et al. (2017) carried out electrochemical hydrogenation of SBO with $scCO_2$ on electrolytes and under optimal conditions (8 MPa, 48 °C, 125 mA, 300 rpm, 8 h), the process was able to reduce the TFA content by 35.8% in addition to the reduction in reaction time by 4 h compared to the traditional hydrogenation process. Another integrated process of ECH of SBO under mixed gas of CO_2 and hydrogen was reported (Wang et al. 2019a). Fats with a TFA content of 3.32% were produced apart from the reduced reaction time by 5 h, showing great potential for industrialization.

6.3.4.3 Catalytic Transfer Hydrogenation (CTH)

Hydrogen gas is not used in a CTH process; instead hydrogen donors, e.g., ammonium formate, cyclohexadiene, formic acid, hydrazine, phosphinic acid, and sodium formate, are employed in the presence of a catalyst. Compared to the conventional hydrogenation process, the CTH process is safer and more environmentally friendly. Sancheti and Gogate (2017) intensified the CTH reaction by employing ultrasound during the hydrogenation of SBO using Pd/C as the catalyst. This process was found to offer high reaction selectivity and controlled *trans*-isomer formation. A similar approach of intensifying CTH by using ultrasound on CRO was reported (Goyal et al. 2019). Most of the research on CTH of vegetable oil utilized a single metal catalyst, however, in a study by Yu et al. (2019), the effect of a bimetallic (nickel-silver) catalyst immobilized on PVP-DB-171/SiO_2/Fe_3O_4 magnetic particles on the hydrogenation of SBO was evaluated. The addition of silver prevented the aggregation of nickel in the magnetic particles, and the process exhibited a high mass transfer rate and low TFA formation.

6.4 Fractionation

Fractionation is a fully reversible lipid modification technique that involves the physical fractionation of a multi-component mixture into two or more distinctive fractions based on a thermo-mechanical separation process. Fats and oils are not chemically homogenous for they are comprised of a mixture of TAGs with distinctive physical and chemical properties. As a result, fractions can be separated according to their varying solidification, solubility, or volatility, acquiring fats and oils with specific properties. TFA is not formed during this process, which is a huge

advantage. The complexity of the fractionation process is largely dependent on the desired fractions.

6.4.1 What Can Fractionation Do?

Fats and oils can be separated into two or more fractions, each with a different application and value, allowing for greater flexibility and application range. PO, which is the most fractionated oil, can be fractionated into defined fractions of oleins and stearins intended for different applications via multistage fractionation. For instance, stearin (IV 33, vanaspati), mid stearin (IV 45, margarine), super stearin (IV <15, animal feed), olein (IV 56, frying oil), soft PMF (IV 47, margarine), hard PMF (IV <36, confectionary), super olein (IV 65, cooking oil), mid olein and top olein (IV 60, IV 70, salad oil) and, mid olein (IV 54). Fractionation has also been performed on other oils to explore and widen their application values. A new high-stearic-high oleic (HSHO) SFO variety was developed recently from seeds obtained through conventional breeding techniques. This HSHO SFO lacks adequate solid content at high temperatures. Via fractionation, the concentration of di-saturated fatty acid content can be enhanced, obtaining soft and hard stearins (capillary melting points at 30 °C and 35 °C, respectively). Rincón-Cardona et al. (2013) and Herrera et al. (2015) studied the potential application of this HSHO SFO as a trans-fat replacer or as cocoa butter equivalent (CBE) according to the real time crystallization profiles and crystal polymorphisms. The hard and soft stearins were crystallized under different conditions to obtain different crystal polymorphs for various industrial applications. CNO cannot be incorporated directly in margarine, chocolate coatings, or coffee whiteners prior to modification due to its low melting temperature, plasticity and hardness. Sonwai et al. (2017) solvent-fractionated CNO into several fractions with varying crystallization profiles, fatty acid and TAG compositions. Compared to the initial oil, the liquid fractions were softer and crystallized more slowly, whereas the solid fractions were harder and crystallized rapidly. The solid fractions could be used as specialty fats (coating fats, margarine) and the liquid fractions had higher cold stability, which was beneficial for long-term storage.

Fractionation can be used to enrich oil with a targeted TAG species or fatty acids in order to improve its application properties. Solvent fractionation has been employed to produce pinolenic acid concentrates from pine nut oil fatty acids which is an appetite suppressant for use in functional foods and nutraceuticals (Chung et al. 2018). The starting material contains only 18% of pinolenic acid, and post fractionation, fractions with 69% pinolenic acid were obtained. Low-temperature solvent crystallization was carried out to increase the n-6 docosapentaenoic acid (DPA n-6, C22:5 n-6) and docosahexaenoic acid (DHA, C22:6 n-3) from *Schizochytrium* sp. oil (Mu et al. 2016). Bambang kernel fat has a high content of symmetrical monounsaturated TAGs, making it suitable for the preparation of chocolate products. However, because the tri-unsaturated and

di-unsaturated TAGs can influence the end products' physicochemical and thermal properties, fractionation was performed to obtain symmetrical di-saturated TAG (SUS)-rich fractions for application in chocolate formulation (Norazlina et al. 2020). A three-stage fractionation was performed on mango kernel fat to obtain fractions rich in high-melting symmetrical monounsaturated TAG that could be used as a cocoa butter improver while the fractions rich in di-unsaturated and tri-unsaturated TAGs can be used as specialty fat ingredients (Jin et al. 2017a). Selective enrichment of symmetrical monounsaturated TAG such as 1,3-dipalmitoyl-2-oleoyl-glycerol (POP) and 1,3-distearoyl-2-oleoylglycerol (SOS) from palm stearin can be attained using the double solvent fractionation, producing palm stearin fraction suitable for preparing CBE (Kang et al. 2013). 1,3-dioleoyl-2-palmitoylglycerol (OPO)-rich structured lipid for potential use in infant formula was obtained from basa catfish oil via a two-step process (Zou et al. 2015). The fish oil was first fractionated to produce TAG with a high content of sn-2 palmitic acid (from 49% in the initial oil to 60%), followed by subjecting the fractionated products to enzymatic acidolysis to increase the OPO content (79%).

Fractionation can also be used to remove undesirable minor compounds from oils such as waxes, diacylglycerols, and free fatty acids, thereby improving the oils' application properties and stability (Kellens et al. 2007; Maes et al. 2015). Also, fractionation has been performed to detect adulteration of foreign fats in oil samples. For instance, Upadhyay et al. (2017) coupled complete liquification time test with solvent fractionation to detect adulteration of foreign fats in ghee.

6.4.2 Fractionation Techniques

Various fractionation techniques are practiced such as fractional crystallization, fractional distillation, short-path distillation, supercritical extraction, liquid-liquid extraction, adsorption, urea complexation, and membrane separation. Generally, all the fractionation processes include two major steps; selective crystallization and separation. Crystallization occurs in stages, starting with the supercooling of the melt, nucleation, and crystal growth. After achieving the desired crystallization, a separation or filtration process is performed to produce the solid (stearin) and liquid (olein) phases. Different equipment such as vacuum filters (rotary drum or belt filters), membrane or hydraulic press filters, and centrifuges, can be used during the separation step, depending on the required separation efficiency. Advances in the fractionation technology are driven by the massive increase in PO fractionation, but the application of fractionation to other fats has also generated considerable interest and development effort. There are numerous techniques employed for fats and oils fractionation as abovementioned, and some of the techniques are discussed in the following.

6.4.2.1 Fractional Crystallization

Fractional crystallization is widely employed in the oils and fats industry. This process is based on the distinctive solubility of solid fatty molecules, (TAGs, FAs, and fatty acid methyl esters) in the liquid phase, which have different molecular weights and degrees of unsaturation. There are three techniques based on the commonly practiced fractional crystallization, namely, detergent fractionation, solvent fractionation and dry fractionation.

6.4.2.1.1 Dry Fractionation

Dry fractionation is widely used to separate stearin and olein from various vegetable oils and animal fats. The process begins with a complete melt of fat, followed by a controlled cooling process to produce targeted fractions via crystallizing the most saturated TAG in an oil substrate without the use of solvents. This method has the advantages of simple operating procedures, environmentally friendly (no need for chemicals as processing aids), has a lower operational cost, and has no effluent output. Conventionally, dry fractionation is employed in PO refineries to produce various olein and stearin fractions. The application of dry fractionation on new types of oils such as the high-oleic high-stearic SFO (Bootello et al. 2011), refined hoki fish oil (Tengku-Rozaina and Birch 2015), Macauba kernel oil (Magalhães et al. 2020), tea seed oil (Zarringhalami et al. 2012), and basa catfish oil (Zou et al. 2015), has been reported.

Some aspects should be noted while performing dry fractionation as these factors can influence the process efficiency and the properties of the attained fractions. Gupta (2017) has summarized seven critical points which influence the characteristics of end products obtained from dry fractionation, i.e., initial oil temperature, pre-crystallization, cooling rate, crystallization time, agitation, final crystallization temperature, and separation/filtration. The oil feed is first heated to destroy any crystal memory from earlier crystallization in the oil, followed by the pre-crystallization process to initiate nucleation formation, thereby reducing the residence time in the crystallizer. Under certain circumstances, seeding crystals are added to expedite crystallization, facilitate secondary nucleation, and improve crystal growth. In the industrial PO multi-stage fractionation process, seeding practices often involve blending PO into its derived olein fraction, resulting in the facilitation of bulk crystallization attributed to the high-melting tripalmitoyl-glycerol (PPP) in the melt. Calliauw et al. (2010) reported the effect of adding PPP-TAG (0.6%, 0.8% and 1%) on the bulk crystallization properties of POO in an industrial setting. The PPP-TAG served as a template for enhanced crystallization of POP in the form of β′, and this compound hindered the polymorphic transition of POP during the tempering stage, which then influenced the crystal growth and morphology in the subsequent crystal growth stages. Bootello et al. (2011) reported that the addition of seeding crystals (high-melting-point stearin powder, 1.6 mm) with a minimum amount of

0.25% induced changes in the nucleation mechanism. The total crystallization time was decreased, and crystallization selectivity was improved, thereby enhancing the filtration and fractionation efficiency. Also, the addition of seeding crystals increased the amount of di-saturated TAG recovered in the precipitate from 23% to 30%. A controlled cooling rate is needed to produce desirable crystal types and sizes, and to ensure an efficient separation and filtration process. The cooling rate used is dependent on the targeted fractions. A fast cooling rate results in small crystals with a lower amount of incorporated oil but with a relatively large surface where liquid oil can attach; a slower cooling rate leads to the formation of fewer nuclei resulting in larger crystals. There should be sufficient residence time for oil feed to remain in the crystallizer in order to allow crystals to develop into hard crystals of uniform size for optimum filtration efficiency. Often, intense yet non-destructive agitation (5–15 rpm) during the crystallization process is required to prevent settlement of crystals to the bottom of the crystallizer besides of allowing a continuous and uniform crystallization. The final crystallizer temperature is generally based on the targeted olein and stearin fractions. Finally, rapid separation and filtration of the fractions is carried out to prevent re-melting of the crystals.

A multistep process is often required to produce products with desired properties. It has been revealed that the dry fractionation technique, in general, has a lower separation efficiency compared to that of detergent and solvent fractionation method due to the liquid sticking onto crystals or being trapped in agglomerates, also known as liquid entrapment, whereby traces of liquid fractions remain amongst the separated solid fractions. Lately, Sebben et al. (2019) performed a dry, thermal fractionation with subsequent solvent washing to isolate the high melting fraction crystals of milk fat. Liquid milk fat entrapped in the fractionated hard milk fat was undesirable for crystal analysis and was successfully removed via solvent washing with acetone followed by vacuum filtration. Multi-step dry fractionation has been applied to PO and produced softer and harder fractions that can be used in a variety of fat foods (Danthine et al. 2017). A two-step process of dry fractionation with subsequent enzymatic (Lipozyme RM IM) acidolysis process with free fatty acids from high oleic SFO was successfully employed in the production of OPO-rich structured lipids from basa catfish oil (Zou et al. 2015).

6.4.2.1.2 Solvent Fractionation

Solvent fractionation has been introduced to overcome challenges with bulk crystallization, such as slow heat transfer and high viscosity which limits nuclei movement. Fat is first dissolved in the solvent and then cooled to initiate crystallization of TAG with highest melting point, followed by filtration to separate the crystals and solvent evaporation for the recovery of targeted fractions. When solvent is added to the oil sample, the viscosity of the system is reduced drastically. In that way, the TAG molecules can diffuse and attach to the growing crystals' surface and reduce the tendency for co-crystal formation and dislocation in the crystal lattice, leading to the formation of favorable large crystals (Bootello et al. 2015; Illingworth 2002).

Solvent fractionation has the advantages of high separation efficiency, high yield of targeted fractions, ease of filtration, efficient in reducing liquid oil entrapment, and is suitable for obtaining high-value fractions. High selectivity and crystal stability attributed to the solvent-induced β-crystallization of TAG has enabled the enrichment of di-saturated TAG species in a single step (Gibon 2006; Salas et al. 2011). Although the solvent fractionation method has a high energy consumption rate which is not economically feasible for normal olein-stearin fractionation, as well as the risk of fire and issues with residual solvents, the high separation efficiency is still required for the production of fat fractions with a sharp melting profile and high purity that have specific applications, such as the production of specialty fats. Recently, the process disadvantage of high energy consumption has been addressed by Perederic et al. (2020) by applying lipid thermodynamic models and exemplified through a case study for shea butter fractionation using acetone. The use of heat integration concepts and implementation of a side draw column were found to reduce the operating costs and showed significant improvements in process sustainability (i.e., 40% lower global warming potential). Future research into the modelling of the lipid crystallization process was proposed, as this could offer a great advantage to the solvent selection process by reducing the oil-solvent ratio and ultimately, lowering the process energy requirement and environmental impact. This enables the solvent fractionation process to be more sustainable and cost-effective. The choice of solvent is vital as it influences the separation of polar and non-polar lipids, as well as the crystallization rate, fractionation temperature, and the properties of the fractions. Commonly, acetone or hexane is used as the solvent for fractionation. Acetone has been reported to be more selective for separating TAG, whereas hexane concentrates polar compounds in the stearin phase, such as free fatty acids, monoacylglycerols and diacylglycerols (Timms 2012). Bootello et al. (2015) reported that acetone was a better fractionation solvent than hexane for high oleic-high stearic SFO, allowing the oil to be fractionated at higher temperatures and at lower supercooling degrees, leading to a faster crystallization and higher stearin yield. Other than acetone and hexane, the use of other types of solvents for fractionation has been reported. For instance, the utilization of 2-methylpentane-based isohexane which has higher polarity than that of industrial hexane for the selective fractionation of SOS-rich fats from mango kernel fat via a three-stage fractionation process (Jin et al. 2017b).

6.4.2.1.3 Detergent Fractionation

Detergent fractionation, also known as the Lanza or Lipofrac method, is developed to improve the separation of the crystallized phase from the remaining liquid by adding an aqueous detergent solution (wetting agent: sodium lauryl sulfate, electrolyte: magnesium sulfate) to the crystallized oil. Stearin crystals will be wetted by the detergent, releasing the olein occluded in the crystals. The water phase is then separated from the remaining liquid oil via centrifugation followed by heating to recover melted stearin from a second centrifugation step. This method has a high

separation efficiency, producing a higher olein yield and stearin fraction with a minimum amount of olein (Gupta 2017). However, this method has the drawback of high operating costs and the possible contamination of end products due to traces of detergent. Hence, this method is probably the least employed and studied. Recently, Sahu et al. (2019) fractionated RBO fatty acid distillate into fatty acid concentrates as low-melting fatty acid fraction (olein) and high-melting fatty acid fraction (stearin) by using sodium lauryl sulfate and magnesium sulfate. Subsequently, the fractions were converted into neutral glycerides via autocatalytic esterification process for use as a novel antioxidant-rich fatty product in the making of spreads, margarine, and bakery shortenings.

6.4.2.2 Supercritical Fractionation

Up to date, there are much interest in the application of supercritical fluid fractionation (SFF) in oils and fats. SFF was introduced in the late 1970s along with the application of supercritical fluid extraction (SFE). The SFF process utilizes the properties of supercritical fluid (SFC) for selective recovery of targeted components, showing great resemblances with the SFE. Unlike SFE, which applies high extraction pressure to maximize the extraction yield, high pressure is not being employed in the SFF process. The SFC has a high solvating power at high pressure, which enhances the solubility of different components from the matrix (reduction of the density difference between the components) into the SFC and subsequently impedes the anticipated physical separation. Thus, SFF is commonly performed using milder operating pressures and is often paired with SFE, allowing an effective improvement in the selectivity of the SFE process. For example, a $scCO_2$ extraction of dry ginger was coupled with an online fractionation process to obtain gingerols enriched oleoresin and volatile oil (Shukla et al. 2019). The SFE conditions were optimized for the highest extract yield containing the maximum amount of volatile oil and gingerols (6, 8 and 10-gingerols), followed by fractionation in two separators operated at different pressures; higher pressure at the first separator for the recovery of heavy compounds and lower pressure at separator 2 for the recovery of lighter volatile components. This configuration resulted in the high oleoresin and volatile oil recovery (96% and 95%, respectively), containing 51% of gingerols and 6-shogaol. The authors also suggested that the SFE and simultaneous fractionation have great potential for commercial isolation of different bioactive compounds such as vitamins and essential fatty acids.

The SFF process has been used for the separation and enrichment of a specific component in oils, such as the separation of different types of ginsenosides from the roots of Panax ginseng (Yang et al. 2017), gingerols from dry ginger (Shukla et al. 2019), piperitenone and 1,8-cineol from peppermint oil (Gañán et al. 2015), vanillin from vanilla oleoresin (Romero De La Vega et al. 2016), tocols and γ-oryzanol from RBO (Yoon et al. 2014). Separation of valuable compounds from products with lower values or by-products using SFF has also been reported. For instance, Lopes et al. (2012) attempted to fractionate freshwater fish oil with a lower omega-3 fatty

acid content to obtain fractions richer in EPA and DHA, separation and concentration of tocopherols from methyl esterified oil deodorizer distillate (Fang et al. 2007), extraction and fractionation of fish oil from tuna by-products for DHA, omega-3, and omega-6 fatty acids (Ferdosh et al. 2016), and fractionation of palm fatty acid distillate for the recovery of squalene (Al-Darmaki et al. 2012). The application of SFF for the purification of used frying oil has also been reported. Selective separation of oil components based on their polarity and molecular weight was employed to purify used PNO frying oil via a continuous $scCO_2$ fractionation in a packed column, recovering TAG (52%) from the frying oil with similar properties as fresh oil (Osséo et al. 2004).

SFF has the advantages of mild fractionation temperature, ideal for thermolabile components, the solvent can be easily separated from the end product and most importantly, the $scCO_2$ is non-toxic and traces of residual CO_2 do not pose health hazards. However, $scCO_2$ shows limitations compared to organic solvents, i.e., a low affinity for polar and high molecular weight components. Co-solvent can be incorporated to address this issue. A small volume of co-solvent (ranging from 0 to 20 mol %) can be added to increase the polarity of $scCO_2$, increase the solvating power, and reduce the analyte-matrix interactions, which then enhances their quantitative extraction (Machmudah et al. 2019). Organic solvents (e.g., methanol, ethanol, hexane) are commonly used as co-solvents while vegetable oils also show potential as co-extractants that can be used to enhance mass transfer of the targeted compounds within the solid matrix while having desirable properties in the end product (Lee et al. 2019). Additionally, SFF process can be modified by employing a low-cost sorbent (silica gel, amino-propyl bonded silica, neutral alumina, and diol-modified silica) material in place of the sample matrix used in traditional analytical SFE to optimize SFE prior to SFF (Nautiyal Omprakash 2016). Nevertheless, due to the high operational cost of SFF, this fractionation technique is usually used to separate and purify high-value compounds that could not be done via conventional means.

6.4.2.3 Emulsion Fractionation

In comparison to the abovementioned techniques, emulsion fractionation has been introduced as a relatively novel strategy for fractional crystallization. Chaleepa and Ulrich (2011) developed a model emulsion consisting of CNO, water, and sucrose ester laurate. After emulsion fractionation, a higher yield of high-quality coconut stearin was acquired than using a conventional dry fractionation process (in the presence of sucrose ester laurate). The stearin fractions obtained from emulsion fractionation had a higher melting point and solid fat content. This fractionation method is carried out based on the crystallization of low-viscous emulsion in which water and emulsifier are employed to reduce the viscosity of the oil system. The prepared emulsion was fractionated according to the layer-melt crystallization method using a cold finger apparatus under optimized conditions (Chaleepa et al. 2010). The cold finger temperature, temperature of melt, agitation speed, and cooling rate are all important parameters that need to be optimized for fractionation efficacy.

Besides, the choice of emulsifier is crucial for promoting crystallization, enhancing the morphological modification of the fat crystals, and possibly of GRAS grade. Emulsion fractionation shows great potential as a green alternative lipid fractionation technology because the process requires only water and a food emulsifier, as well as the capacity to address the oil entrainment issue from the dry fractionation. However, up to date, studies on emulsion fractionation are still scarce.

6.5 Interesterification

Interesterification (IE) is another key lipid modification technology which was introduced in the 1920s in search of butter alternatives before being commercialized in the 1950s (Kellens and Calliauw 2013). During an IE reaction, the fatty acid moieties are moved or exchanged within a TAG (between the sn-1, 2 and 3) or between one or more TAG molecules. The overall fatty acid composition, the degree of unsaturation or the isomeric state of the lipid system are not altered. IE process can be achieved via chemical- or enzyme-catalyzed methods, leading to either a randomized or directed esterification (regiospecific).

Throughout the years, research on IE has been progressive for this process modifies the lipid properties, allowing for the vast application in the food industry. Most importantly, the dreaded TFA is not produced. Extensive reviews on the IE research progress, reaction mechanisms, application in the food industry, and effect on health parameters are available (Berry et al. 2019; Dayton 2014; Guo et al. 2020; Mensink et al. 2016). In most IE reactions, IE hard stock are produced from palmitic acid-based fats such as the combinations of PO fractions (PKO, PS). The hard stock is then blended in different ratios with other vegetable oils to achieve the required solid fat content for the intended final application (Mills et al. 2017). Later, the IE has progressed into the exploration of new oil sources such as mango kernel fat-third stearin (Jin et al. 2018), Buriti oil (Speranza et al. 2018), coix seed oil, and the medium chain-fatty acid-rich *Cinnamomum camphora* seed oil (Xu et al. 2018), *Schizochytrium limacinum* microalgal oil which is a good source for long-chain PUFAs (Bogevik et al. 2018), lauric acid-rich *Irvingia gabonensis* seed fat (Yamoneka et al. 2018), perilla oil (Huang et al. 2020), and many more. After IE, the TAG structure of the oil is modified, which in turn increases the utilization efficiency and addresses technical challenges and limitations in the application of these oils. Two major catalytic IE methods are being practiced, i.e., chemical interesterification (CIE) and enzymatic interesterification (EIE). Both CIE and EIE can alter the physical and chemical properties of fat, but they differ in their catalytic pathways, whereby CIE is a randomized IE process while EIE can be a directed IE (Fig. 6.2) that produces TAG with targeted molecular species.

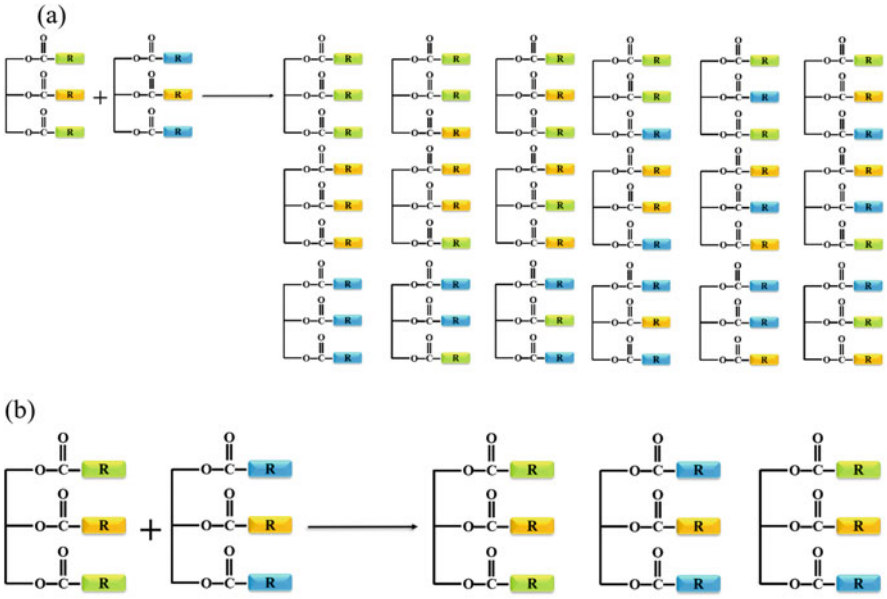

Fig. 6.2 Simplified diagram for (**a**) random interesterification that generally represents chemical interesterification and (**b**) directed interesterification which represents enzymatic interesterification. R, acyl group

6.5.1 Chemical Interesterification (CIE)

CIE employs chemical catalysts such as sodium methoxide to drive the IE in a randomized manner, shifting the acyl group to unspecified locations, resulting in different possible TAG species as shown in Fig. 6.2a. Although CIE is a randomized reaction, it can be regioselective as reported in the early years by Konishi et al. (1993, 1995) during the CIE of SBO and methyl stearate catalyzed using sodium methoxide in hexane at 30 °C. Nonetheless, there are no recent updates on the regioselectivity of CIE. There are two possible CIE reaction mechanisms; carbonyl addition and Claisen condensation. The details for these two types of reaction mechanisms are described in a book chapter by Rousseau and Marangoni (2008). Under alkaline conditions, the nucleophilic catalyst attacks the carbonyl carbon at one of the three fatty acid-glycerol ester bonds, forming a tetrahedral intermediate that releases fatty acid methyl ester and a glycerylate anion. The newly produced glycerylate anion then acts as a nucleophile for the following carbonyl attack, which proceeds until a thermodynamic equilibrium is achieved. In Claisen condensation, a strong nucleophile, carbanion, is produced. A β-keto ester intermediate and a glycerylate are formed when a carbonyl group is attacked by a carbanion, and the free glycerylate then attacks another carbonyl carbon, leading to the exchange of esters intra- and intermolecularly.

6.5.1.1 CIE Reaction Parameters

Prior to CIE, water present in the oil must be removed. This can be done by subjecting the sample to heat treatment (90–115 °C) under vacuum for 15–60 min. The presence of water can cause negative effects because the catalyst tends to react with the FFA and water, causing the formation of soaps which reduces the catalyst's catalytic effectiveness, thereby resulting in low conversion and yield (Lazdovica and Kampars 2020). After the drying process, CIE is then carried out under the following conditions: catalyst (0.2–1.0%, sodium methoxide), temperature (70–105 °C), time (30–60 min), stirring speed (200–400 rpm) and under vacuum. To evaluate the ending time for the CIE reaction, slip melting point (SMP) of fat is monitored until a constant value is achieved (Farajzadeh Alan et al. 2019) or by calculating the CIE degree, which is determined based on the difference between the TAG content before and after the reaction (Zhang et al. 2019a). Upon achieving equilibrium, the common practice to cease CIE process is to add aqueous citric acid to neutralize the catalyst. In some reports, distilled water or hot monosodium phosphate can be added to terminate the CIE process. The bleaching earth is then added to the interesterified fat to remove the excess citric acid and absorb the produced soap, followed by filtration steps. In certain practices, the bleaching process is repeated twice to obtain interesterified fat with the least amount of residual soap. The end product can be dried over sodium sulfate to remove any residual water. Table 6.3 shows some of the more recent examples of CIE of various oil blends under different reaction parameters.

6.5.2 Enzymatic Interesterification (EIE)

EIE, on the other hand, is gaining more consideration as an alternative lipid modification technique. The lipase-catalyzed IE reaction has several advantages over the CIE method, including the specificity and reusability of enzymes, as well as the mild EIE reaction temperature. The type of interesterified lipid and the yield are affected by the reaction parameters such as temperature, time, type of lipase (regioselective or non-selective enzyme), enzyme load, type of reactor (packed bed, stirred tank reactor, shaking water bath, fluidized-bed reactor), composition of substrate, and surface active agent. The optimal reacting parameters are dependent on the targeted outcome and may differ as affected by the enzymatic activity and the substrate's initial fatty acid compositions. A review of the recent research trends regarding the enzymatic synthesis of structured lipids was published by Kim and Akoh (2015).

EIE fats can be used to formulate healthier products while addressing the critical industrial demand for trans-free base-stocks formulation for spreads, margarine, and confectionery fats. EIE fats have been utilized in the preparation of various fat products. For example, preparation of trans-free margarine fat from EIE of

Table 6.3 Chemical interesterification of various substrates under different reaction conditions

Substrate	Conditions[a]	Findings	Reference
CT, PSO	C = 0.6%; T = 90 °C; t = 120 min	After CIE, the polar fraction content and an acid value of the interesterified blends increased. TAG content decreased while the MAG, DAG, and FFA content increased. Emulsions prepared using the interesterified CT/PSO (1:3) as the fatty base and xanthan gum or carboxymethylcellulose as a thickener were stable and can be applied as food, cosmetic, and pharmaceutical emulsions.	(Kowalska et al. 2020)
PS, SFO	C = 0.5%; T = 90 °C; t = 60 min; P = 0.8 bar; S = 300 rpm	PS/SFO blends had TRA of <0.36%. Post CIE, the TAG compositions were altered; S3-, U3-, and S2U-TAGs decreased while U2S-TAG increased. FFA and soap content increased while the PV and oxidative stability index (at 110 °C) decreased. The SMP, SFC, and plastic range decreased, thereby improving the fat blend's melting properties and plastic range. Interesterified PS/SFO blends are suitable for producing TRA-free products, a good alternative to hydrogenated fats.	(Farajzadeh Alan et al. 2019)
PS, CO	C = 0.3%; T = 90 °C; t = 60 min; P = 0.8 bar; S = 300 rpm	To model the rheological and textural properties of chemically interesterified PS/CO blends as a function of SFA, SFC, and temperature by describing and predicting the viscoelastic properties and firmness of the blends change with SFA content. The models can be used to limit the need for instrumental methods. Post CIE, the content of S3, S2U, and U3 decreased significantly while U2S increased dramatically. SFA of the blends increased with the amount of PS, leading to the increment of the structural strength of the samples, showing higher elasticity, higher firmness value, and a solid-like nature.	(Saghafi et al. 2019)
PS, AO	C = 0.5%; T = 70 °C; t = 60 min	To evaluate the effects of CIE on the physicochemical properties of bakery shortening produced from a blend of PS/AO in different ratios. CIE alters the TAG content (S2U and SU2 increased, S3-TAGs decreased), which caused the reduction in the SFC and SMP of interesterified blends. Insignificant change in the chemical properties such as peroxide value, iodine value, free fatty acids, and acid value of interesterified fat blends. Gompertz model can predict the alteration in the physical properties (SCF and SMP) as	(Tourchi Rudsari et al. 2019)

(continued)

Table 6.3 (continued)

Substrate	Conditions[a]	Findings	Reference
		a function of SFA and temperature effective for time, cost, raw material savings. Interesterified PS/AO blend showed improved physical properties (SFC and melting point) and functional properties, can be used to produce trans-free bakery. Shortening for all-purpose shortening, liquid bread shortening, and pie crust shortening.	
BT, POO, PMF, SBO, PS, PKO, PO	C = 0.3%; T = 105 °C; t = 30 min; P = 0.02 bar; S = 200 rpm	To determine the effect of CIE on the physicochemical and crystallization kinetics of oil blends consisting of different types of oils at different ratios. CIE process modified the TAG compositions, crystallization kinetics, growth mechanisms, and polymorphisms of the oil blends. After CIE, POO/BT showed increased crystallization rate, dominant in β' crystals; A significant increase in SU2-TAGs in oil blends consisting of SBO; blends with only vegetable oil as base showed a gradual change in crystallization rate and was dominant in β crystals; blends consisting of PKO showed reduction of SU2-TAGs; blends with SBO and PKO had stronger ability to stabilize the change in SU2-TAGs with accelerated crystallization rate and formation of β' crystals. The addition of a sufficient amount of PMF could improve the high-temperature solid fat content and expand the plasticity range.	(Zhang et al. 2019a)
SB, FPSO	C = 0.3%; T = 85 °C; t = 50 min	To evaluate the effect of CIE on the physicochemical properties and fatty acid profile of SB/FPSO-based bakery shortening. There was no significant change in the peroxide value, iodine value, and refractive index of interesterified blends. The acid value, free fatty acids, slip melting point, density, and solid fat content were significantly reduced after IE. The various ratio of SB/FPSO interesterified blends had different SFC profiles, suitable for the preparation of a wide range of shortenings; SB/FPSO (3:7) all-purpose shortening and frying fat while SB/FPSO of 4:6 and 5:5 had SFC profile suitable for the preparation of bakery fats, icing-shortening, filler fats and all-purpose shortenings.	(Bariwere Samuel et al. 2018)

(continued)

Table 6.3 (continued)

Substrate	Conditions[a]	Findings	Reference
SL, CAO, CAE	C = 0.2, 0.5%; T = 60 °C; t = 60 min; P = 0.95 bar; S = 100 rpm	To obtain and characterize structured lipids by CIE of SL/CAO/CAE. In blends consisting of CAO, the change of FA% in the sn-1,3 and sn-2 positions was more pronounced after CIE. Interesterified SL/CAO present SFC profiles suitable for application in baking products, while SL/CAE interesterified blend showed potential for frying and condiment.	(Engelmann et al. 2018)

C catalyst, *T* temperature, *t* time, *P* pressure, *S* stirring speed, *CT* calf tallow, *PSO* pumpkin seed oil, *PS* palm stearin, *SFO* sunflower seed oil, *CO* canola oil, *AO* Ardeh oil (*Sesamum indicum*), *BT* beef tallow, *POO* palm olein, *PMF* palm mid fraction, *SBO* soybean oil, *PKO* palm kernel oil, *PO* palm oil, *SB* shea butter, *FPSO* fluted pumpkin seed oil, *SL* swine lard, *CAO* carp oil, *CAE* carp esters, *S3* tri-saturated, *U3* tri-unsaturated, *S2U* di-saturated–monounsaturated, *U2S* di-unsaturated–monosaturated

[a]Sodium methoxide is used as catalyst for all the reported literature in this table

RBO-hard PS oil blends (Ornla-ied et al. 2016) and trans-fat free shortening from rice bran stearin-fully hydrogenated SBO-CNO oil blends (Shi et al. 2015). However, low TFA has been detected in EIE fats from tallow-CRO blends (0.1–1.3%), probably attributed to the original trans-fat content (0.6–0.7%) in the physical blends (Aktas et al. 2020). This interesterified fat is suitable for the production of low-trans shortenings, margarine, and frying fats. A healthier margarine fat analog to beef tallow using EIE from SBO-fully hydrogenated PO oil blends was prepared (Li et al. 2018). The EIE fat was reported to contain a lower TFA content of 0.67% than that of the physical blend (1.18%) and beef tallow (4.15%).

6.5.2.1 Enzymatic Catalysis and Physicochemical Characterization

Simple steps are involved in the EIE process. The substrates are first blended, followed by the addition of lipases (5–10%), and the mixture are subsequently subjected to react under different reaction temperatures (50–75 °C) and times (3–48 h). Lipase can be conditioned or activated prior to EIE to achieve full enzymatic activity, prevent hydrolysis of the substrate and excessive free fatty acid production. Moreira et al. (2020) conditioned Lipozyme TL IM by deaerating and dehydrating the lipase by adding the lipase to preheated SBO at 70 °C and sheared at 300 rpm for 30 min. These steps were repeated three times before filtering the enzyme for further use. Lipozyme RM IM was activated in SBO for 30 min at 65 °C (Teh et al. 2016). EIE reaction can be terminated by denaturing the enzyme via heating or adding acetic acid (0.25%). FFA (1.5–2.5%) present in the EIE sample can be removed by (1) Adding phenolphthalein solution and 0.2 M NaOH followed by washing the mixture with hot water (50–60 °C) until the pink color disappear. The washed sample is then filtered through filter paper containing anhydrous sodium

sulfate to remove the moisture and enzyme (Ribeiro et al. 2017); (2) Using short-path distillation (Ifeduba et al. 2016); (3) Liquid-liquid extraction using solvent (e.g., ethanol, acetone, or a mixture thereof) followed by solvent evaporation step (Pang et al. 2019).

The effects of EIE on the physical and chemical properties of fats are evident. In general, slip melting point (SMP) and solid fat content (SFC) of interesterified fats are lower than their physical blends attributed to the alteration in the TAG structure and the reduced concentration of the high-melting and medium-chain TAGs. The FFA% increases significantly after IE, which often leads to the requirement of a neutralization step. EIE fats have lower TAG% while the MAG% and DAG% increased, owing to an enzymatic reaction at the sn-1,3 positions of TAG that results in the formation of MAG and DAG. Nonetheless, these properties can be modified by controlling the reaction parameters. For instance, in a work on EIE of beef tallow-CRO reported by Aktas et al. (2020), the physical and chemical properties showed an opposing effect when a long reaction time was employed (12 h compared to that of 6 h). After 12 h of EIE, a sharp increase in SFC was recorded while the FFA% and TAG% fluctuated and the MAG% and DAG% decreased. Reaction time longer than 6 h had a trend-changing effect on several characteristics of the EIE fats, which was suggested to be influenced by the enzymatic activity.

Post EIE, the TAG compositions are altered; The UUU- and SSS-TAG content decreases while the SUU-, SUS-, and SSU-TAG content increases. The rearrangement of fatty acid in TAG after EIE can lead to the production of new TAG species. For instance, the formation of OLO/LOO after EIE of beef tallow-PS-CMO oil blends (Pang et al. 2019). Change in the TAG compositions has a direct influence on the crystal polymorphism, morphology, and crystallization profile. EIE fats are dominated with β′-crystals, and there are fewer β-crystals attributed to the reduction in SSS-TAG and the increase of SUU-TAG. The increase in the middle melting point-TAG and reduction of high melting point-TAG causes the fat blend to form β′-crystals more easily. β′-crystals are tightly packed small spherical crystals with very dense crystal networks that are ideal for providing fats with plasticity, resulting in better product performances.

Often, EIE fats have lower oxidative stability compared to that of physical blends or substrates. Oxidative stability of the interesterified fats are mainly influenced by the source of the substrate (initial oxidation status and concentration of antioxidant) and the reaction conditions. In the EIE of beef tallow–CRO blends, Aktas et al. (2020) suggested that the TAG structure including the fatty acid composition and positional distribution on the glycerol backbone, as well as the interaction of these factors, influenced the oxidative stability of the EIE fats. The formation of by-products that act as prooxidants, such as FFA and partial acylglycerol, induces autoxidation in the interesterified products. In a study conducted by Sivakanthan et al. (2019), the use of aqueous lipase derived from *R. miehei* during the EIE of CNO with SSO contributed to a substantially high FFA (14%) and suggested that using an immobilized enzyme instead of an aqueous enzyme could minimize the FFA%. These by-products should be omitted via post-processing operations to

improve the oxidative stability and the organoleptic properties when applied in food products.

6.5.2.2 Specificity and Health Attributes

Specific lipase can be utilized for better specificity and control of the EIE reaction to produce interesterified fats with targeted physical and nutritional qualities. The specificity of the IE reaction is crucial as the stereospecificity and chain length of the fatty acid within the TAG molecule govern the IE fats' physicochemical properties and their metabolic fate during lipid digestion and biological processes. Lipase hydrolyzes fatty acid only at sn-1 and sn-3 positions, hence the fatty acid at the sn-2 position is metabolically different (Lopes et al. 2016; Pang et al. 2019). It has been proposed that high UFA at the sn-2 position is preferable since dietary fats with high saturation at sn-2 can be cholesterolemic and atherogenic. TAG with long-chain fatty acids at sn-1,3 positions and UFA at sn-2 was found to show an obesity-reducing effect in C57BL/6 mice, while SFA of different chain lengths at sn-1,3 positions significantly influenced the fat deposition (Gouk et al. 2014).

Depending on the type of lipase employed, it is possible to incorporate a specific fatty acyl group into a targeted position on the TAG molecular backbone, especially when regioselective lipases are used. Regioselective (positional specific) enzymes can distinguish the positions of the acyl group at sn-1,3 from that of sn-2; lipases with sn-1,3-specificity can only act on fatty acyl group at sn-1 and 3 positions while preserving the fatty acid at sn-2. Examples of 1,3-regiospecific biocatalysts that are commonly used are lipases from the strain of *Rhizomucor miehei* (Lipozyme RM IM), *Candida antarctica* (Lipozyme 435), *Thermomyces lanuginosa* (Lipozyme TL IM), *Aspergillus oryzae* (NS 40086), and *Rhizopus oryzae* (Lipase DF-15). True sn-2 regioselective lipases are very rare, but some reports on lipases show preferences for sn-2 fatty acids. For instance, the well-known *Candida antarctica* lipase A (Domínguez De María et al. 2005), *Geotrichum sp*. FO401B lipase C (Ota et al. 2000), and immobilized *Pseudomonas fluorescens* and *Burkholderia cepacia* lipase, which showed high activity in the sn-2 esterification of 1,3-dicaprin with palmitic acid (Sánchez et al. 2017).

The enzyme's specificity characteristic have been utilized to synthesize desired TAG molecular species by incorporating the selected fatty acid into a specific position on the glycerol backbone. Synthesis of Symmetrical TAG (Fig. 6.2b) has various applications in the food and nutraceutical industries. For example, the preparation of TAG in the form of 1,3-arachidonoyl-2-palmitoyl-glycerol by incorporating high value-added functional arachidonic acid onto the sn-1,3 positions (Liu et al. 2017), incorporation of docosahexaenoic acid at sn-1,3 to obtain 1,3-docosahexaenoyl-2-palmitoyl-sn-glycerol (Liu et al. 2015), synthesis of low calorie structured lipids by incorporating behenic acid into the sn-1,3 positions (Kok et al. 2018), incorporation of nervonic acid (fatty acid with beneficial effects for mental health) at sn-2 position for conducive metabolism and absorption (Hu et al. 2017), incorporation of palmitic acid or stearic acid into the end positions

of SBO to produce symmetrical TAG without changing the fatty acid at sn-2 position (Teh et al. 2016), incorporation of caprylic acid and stearic acid into menhaden oil (Willett et al. 2019), incorporation of oleic acid into tripalmitin (Tecelão et al. 2019), increasing the ω-3 PUFA (i.e. EPA and DHA) at the sn-2 position from different mixtures of high oleic SFO and sardine oil (Marín-Suárez et al. 2017).

These EIE fats are reported to be health beneficial. Enzymatically synthesized structured lipids from OO-SBO-fully hydrogenated crambe oil blend containing behenic acid at the sn-1,3 positions and UFA at the sn-2 position was reported to have anti-obesity potential and as a source of essential fatty acids (Moreira et al. 2017). Later, da Silva et al. (2019) discovered that the EIE fats containing the same vegetable oil as above could induce postprandial inflammation in mice. Medium-long chain TAG synthesized via EIE from blends of CMO and *Cinnamomum camphora* seed oil showed reduced body fat deposition in C57BL/6J mice, probably through the modulation of enzymes related to lipid mobilization (Hu et al. 2018). Structured lipids containing CLA and CLnA (*cis*9, *trans*11, *cis*13–18:3, punicic acid) was reported to reduce liver lipid weight and TAG in Wistar rats (Shagholian et al. 2019).

6.5.2.3 Acyl Migration

Principally, 1,3-regiospecific biocatalyst catalyzes IE reaction only at the sn-1,3 positions given the steric hindrance of the sn-2 position, and the fatty acid at sn-2 should remain unaltered. However, changes in the composition at sn-2 are accredited to the occurrence of acyl migration, which demonstrates the evolution of an ideal 1,3-specific IE into a randomized reaction. Lopes et al. (2016) reported that the EIE catalyzed using sn-1,3-specific Lipozyme TL IM was only partially regiospecific due to the spontaneous acyl migration from sn-2 to sn-1,3. Various techniques have been established for evaluating and monitoring the IE and acyl migration degree, which includes measuring the change in SMP, solid fat content, TAG with equivalent carbon number, and sn-2 fatty acid composition of the end product (Zhang et al. 2020a). Several factors can affect the degree of acyl migration, i.e., reaction conditions (time, temperature, enzyme load, water content, or water activity), the type of lipase and support material used for enzyme immobilization.

A prolonged reaction time tends to promote acyl migration. According to Kok et al. (2018), the first 3–5 h was directed by 1,3-specific IE reaction and was thereafter surpassed by randomization under extended reaction. Hence, migration of the acyl group at sn-2 was detected. Acyl migration can be undesirable this case where the newly synthesized low-calorie structured lipid in the form of SUS and SUU was subjected to successive acyl migration and enzymatic hydrolysis under prolonged reaction, leading to the increase in undesirable saturated species (SSS) from 2.3% (0.5 h) to 26.9% (24 h). Wang et al. (2012) described the synthesis of a structured lipid containing medium-chain fatty acid (C8:0) at sn-1,3 and long-chain fatty acid at sn-2. Low acyl migration of C8:0 to the sn-2 position is required to obtain the MLM-type of TAG. Acyl migration was determined to be more prominent

in the reaction catalyzed by Novozyme 435, followed by Lipozyme TL IM and, lastly Lipozyme RM IM (lowest C8:0 level at sn-2 position). The stronger ability of Lipozyme TL IM to promote acyl migration than that of Lipozyme RM IM was also confirmed by Pacheco et al. (2015) during the EIE of SBO and fully hydrogenated SBO blends. The promotional effect of acyl migration was observed when a higher load of enzyme was used, whereas a reduction in the migration occurred when hexane was added due to the decrease in reaction temperature. The type of support material used to immobilize enzyme can have an impact on the occurrence of acyl migration. Lipase from *Thermomyces lanuginosus* immobilized on granulated silica showed complete randomization in the fatty acid distribution within 24 h during the EIE of TAG (LLL and POP). The same lipase supported on polypropylene, on the other hand, caused a moderate rate of fatty acid exchange in the sn-2 position (Cao et al. 2016). EIE performed in a low water activity condition was found to increase the rate of acyl migration; with a water activity of 0.07 resulting in a higher rate of acyl migration of 10.86% compared to 5.07% without water addition (Peng et al. 2020). Acyl migration is deemed to be favorable for the formation of 1,3-oleic-2-medium chain-rich TAG from CNO and high oleic RSO. Hence, the synthesis of TAG can be tailor-made by adjusting the reaction parameters and choosing the right type of lipase.

6.5.2.4 Enzymatically Interesterified Fats for Food Application

EIE fats have been applied to produce ingredients for chocolate and bakery products such as CBA (cocoa butter substitute, CBS and CBE), margarine, shortenings, baking fats, and HMFS. Rohm et al. (2018) has published a concise review on the IE synthesis reaction and the application of EIE fats in various products. In most EIE, the reaction is done on a small scale. More recently, the utilization of a pilot-scale packed bed reactor for the EIE of fats (POO, fully hydrogenated PO and PKO) combined with PKO intended as CBS was reported (Zhang et al. 2020b). Performing EIE on a pilot-scale reactor is vital for building a solid theoretical foundation for the industrialization process. This is because during the up-scaling process, modification of the properties of IE fats can be different from that of bench-scale operations. Palm-based CB alternatives are very common (Mohd Hassim et al. 2018), and the search for new oil sources for the synthesis of value-added CBS from *Cinnamomum camphora* seed oil (Ma et al. 2019) and from *Irvingia gabonensis* seed fat (Yamoneka et al. 2018) was carried out. The evaluation of different new oil sources for synthesizing CBE from illipe butter (Bahari and Akoh 2018) and CB improver from mango kernel fat stearin (Jin et al. 2018) has also been reported.

EIE fat contains minimal or is trans-fat free. Hence, it is an excellent source for making shortenings, margarine, and baking fats. Rohm et al. (2018) reviewed the synthesis of IE fats, application and evaluation of the performances in different products such as in dough, puff pastry, biscuit, and frying fats. Some of the more recent applications of EIE fats include the EIE fats from blends of fully hydrogenated expanded-pressed SBO and cold- pressed CRO in the preparation of margarine

stock high in natural bioactives (phospholipids, tocopherols, and phytosterols) (Yu et al. 2018). Interestingly, the EIE was performed in a supercritical CO_2 system (2 h, 70 °C, 8 MPa, 300 rpm). Synthesis of new functional trans-fat free lipid with low calories for potential application in shortening was carried out using EIE fat from blends of coix seed oil, *Cinnamomum camphora* seed, and fully hydrogenated PO (Xu et al. 2018). EIE of blends consisting of milk fat and POO was used to prepare probiotic table spread, which resulted in an end product with improved melting behavior and spreadability compared to commercial butter (dos Santos et al. 2020).

Human milk fat (HMF) contains high levels of medium- and long-chain TAG (MLCT) with high concentration of C16:0 esterified at sn-2 (>60%) and UFAs (oleic and linoleic acids) predominantly esterified at sn-1,3. Vegetable oils and cow milk fat are presently used as HMFS or analog in infant formulas. Nonetheless, the TAG structures of these fats are completely different from those of HMF, whereby the UFAs are esterified at sn-2 and SFAs at sn-1,3. Consumption of vegetable oil- or cow milk-based infant formulas may impair the digestion, absorption, and metabolization of the fatty acids, as well as the mineral absorption in infants. Structured lipids enriched with medium and long-chain TAGs for HMFS were synthesized from EIE of catfish oil and CNO, producing MLCT and MLL of 62% and 39%, respectively, with 46% of C16:0 at sn-2, suggesting its potential application in infant formulas (Yuan et al. 2020). Synthesis of TAG with a similar molecular structure as HMF can be achieved via regiospecific EIE reaction. USU-type TAG is abundant in HMF, predominantly the OPO, and of more recently, 1-Oleoyl-2-palmitoyl-3-linoleoylglycerol (OPL) has been identified as another vital TAG structure in HMF (Gao et al. 2020). Tecelão et al. (2019) reviewed the synthesis of HMFS via EIE reaction catalyzed by TL IM and RM IM from blends of tripalmitin with methyl oleate or ethyl oleate using hexane as solvent. The authors then introduced the use of a non-commercial recombinant lipase/acyltransferase from *Candida parapsilosis* immobilized in Accurel MP1000 as biocatalyst for the EIE of tripalmitin with ethyl oleate in a solvent-free medium. The enzyme showed higher activity than most of the commercial immobilized lipases (faster reaction in solvent-free media, low enzyme load, and low molar ratio of substrates needed), incorporating 32–51 mol% of C18:1 into the TAG and resulting in OPO-rich blends suitable for use as HMFS in infant formulas. The preparation of OPL was done via enzymatic acidolysis and fermentation (Wang et al. 2020b; Zhang et al. 2020), while the EIE process is still lacking. HMFS enriched with DHA, EPA, and ARA was also produced (Ghosh et al. 2016; Sproston and Akoh 2016).

Other applications of EIE fats include the preparation of fast-frozen special fats from PS- RSO oil blend (Zhu et al. 2019), edible film for sports nutrition products from blends of CNO and high-oleic SFO (Moore and Akoh 2017), improving the quality and oxidative stability of meat batters from blends of lard and RSO (Wirkowska-Wojdyła et al. 2019), and preparation of potential natural wax substitutes from EIE of rice bran wax and POO (Zhang et al. 2019b).

6.5.2.5 Comparison of CIE and EIE

Up to date, IE via chemical or enzymatic way is still being studied. Compared to EIE, the CIE process is more economical for the catalysts are efficient, low-cost, and easy to control. Besides, the reaction time is shorter in CIE compared to that of EIE. CIE took place in the first 10 min of the reaction, while EIE only took place after 4 h during the IE process to produce oils rich in long-chain fatty acids (Berčíková et al. 2020). To overcome the long reaction time, the innovative use of microwave radiation enabled one-hour enzymatic synthesis of structured lipids enriched in unsaturated fatty acids from silkworm pupae oil (Wang et al. 2020c). Although the CIE reaction consumes less time compared to EIE, this process suffers from several drawbacks. For instance, the process is energy and solvent intensive, low IE product yield, high oil loss, high purification cost (removal of catalysts), lacks of specificity, has low oxidative stability, destruction of UFAs and bioactive compounds in the end products, and the formation of a high amount of by-products (Vafaei et al. 2020).

6.6 Conclusion

Oil blending, hydrogenation, fractionation, and interesterification, are all viable lipid modification techniques. Physical and chemical profiles of fats and oils can be modified physically by blending or fractionation process, and chemically via hydrogenation and interesterification processes. The quality, stability, nutrition, and technological application features of the fats and oils can therefore be enhanced. Oils with different fatty acid compositions are blended to attain balanced saturated and unsaturated compositions, resulting in better thermo-oxidative stability and nutritional qualities. Hydrogenation is carried out to reduce the degree of oil unsaturation so that they can be used in fat products. However, the application of partial hydrogenation process can be limited due to the formation of TFA. This issue can be addressed by the progressing research and development in finding new alternative catalysts, support materials, and innovative hydrogenation processes. Fractionation is used for separating and enriching targeted fractions (TAGs, bioactive compounds, or a specific type of fatty acid). Interesterification causes the re-distribution of fatty acyl group in the TAG molecular backbone, thereby altering the physicochemical and nutritional properties while retaining the overall fatty acid compositions. Research and development on new technologies, improving the existing methods, and integrating different lipid modification technologies are progressively carried out by the lipid industry to meet the new quality standards.

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Chapter 7
Ionic Liquid as a Green Solvent for Lipid Processing

Ling-Zhi Cheong, Bolin Mou, Wei Wei, Yang Hongli, Zhang Hai, Zhao Gege, and Xu Xuebing

Abstract Ionic liquids (ILs) are regarded as "green" alternatives to conventional organic solvents due to their tailorable physicochemical properties and can be innovatively used in many fields related to lipid processing. ILs can be used to replace traditional organic solvents as reaction media or catalysts in the lipid biocatalytic production process, which, significantly improve the lipid conversion rate. In addition, ILs can be used either by itself or in combination with other solvent for lipid extraction, enrichment and separation processes. It is also worth mentioning that ILs can be combined with other conventional extraction and separation techniques to achieve better extraction and separation. Although it has not been widely applied yet, ILs can also be used in detection and separation of contaminants in lipids. We believe that this chapter can justify the benefits of ILs as compared to conventional solvents in lipid processing in order to decrease the gap between applications of ILs in academia and industrial biotechnology.

Keywords Ionic liquid · Biocatalysis · Extraction · Recovery · Separation

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7.1 Introduction

Ionic liquids (ILs) are defined as compounds completely composed of ions with melting point below 100 °C. As they can be synthesized from various combinations of cations, anions and substituents, ILs possessed tailorable physicochemical properties ranging from "nonvolatile, non-flammable, and air and water stable" to "volatile, flammable, and unstable". Their melting point, density, viscosity, polarity, hydrophilicity/lipophilicity, solvation power, etc. can be easily tailored to suit specific requirements (Lei et al. 2017; Xu et al. 2016). Due to their "tailorable" properties, ILs became more popular solvents, catalysts and/or reagents as compared to conventional volatile solvents in many physical and chemical processes. However, up till now, there exist very few industrial applications of ILs (DiphasolTM process (Maase and Massonne 2005) and BASILTM process (Wasserscheid 2002) due to their high cost and difficult to synthesis at high purity (Durand et al. 2013).

Lipids are a family of organic compounds comprising of glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides, sterol lipids, and prenol lipids. They are usually insoluble in water but soluble in other organic solvents such as hexane, acetone, chloroform and ether (Han and Zhou 2015). Due to its tailorability, ILs is widely considered as a "greener" alternative to conventional organic solvents and has been innovatively applied in many areas related to lipid processing. For example, ILs can be used for biocatalysis and biotransformation of lipids (Bauer et al. 2017; Katsoura et al. 2006; Lozano et al. 2020), extraction, enrichment and recovery of lipids in a multiphase system (Krishnan et al. 2020a, b; Motlagh et al. 2020; Pajewska-Szmyt et al. 2020), removal of toxicants and contaminants in lipids (Kakaei et al. 2020; Sun and Shi 2012; Zhang et al. 2015a, b), separation, identification and analysis of lipid materials (Calvano et al. 2012; Desai et al. 2019; Fan et al. 2020a, b), etc. Present chapter aims to introduce and elucidate the various types of ILs used in lipid processing; and justify the benefits of ILs as compared to conventional solvents in lipid processing in order to decrease the gap between applications of ILs in academia and industrial biotechnology.

7.2 Ionic Liquid in Biocatalysis of Lipids

Biocatalysis is defined as the use of natural substances that include enzymes from biological sources or whole cells to speed up chemical reactions (Dev et al. 2018). The use of enzymes specifically lipases as catalytic tools to modify fats and oils begins in the mid-eighties to produce structured lipids with specific functional and nutritional properties [monoacylglycerols (MAGs), diacylglycerols (DAGs), margarine, cocoa butter substitutes, human milk fat substitutes etc.] either by hydrolysis, esterification, interesterification or a combination of the above processes. The aforementioned lipases-catalyzed reactions are now well understood and successfully commercialized. Researches are now mainly conducted to explore new lipases

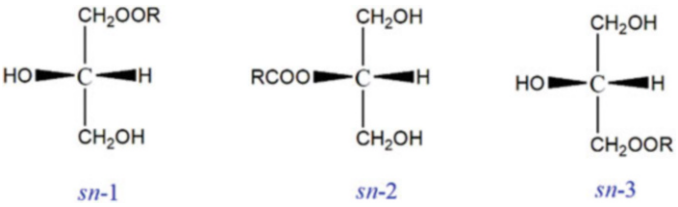

Fig. 7.1 Molecular structures of monoacylglycerols. R, fatty acid chain

and novel reaction medium such as ILs to enhance lipases performance in the industrial applications.

MAGs; a type of food-grade additives, are esters of glycerol with one of their hydroxyl group esterified with fatty acid (Fig. 7.1). MAG can be enzymatically synthesized through esterification of fatty acids with glycerol, interesterification of glycerol and fatty acid esters, glycerolysis and hydrolysis of fats and oils by using 1,3-regiospecific lipases (Bornscheuer 1995). Some of the attractive features of the aforementioned enzymatic synthesis of MAGs include lower reaction temperatures, higher purity and quality of the MAGs products (sn-1 MAG or sn-2 MAG) (Kahveci et al. 2016). Due to the significant differences of substrates polarity (polar glycerol and non-polar fatty acids), selection of suitable solvents that are able to dissolve both substrates while being compatible with lipases are utmost important. Only few conventional solvents (e.g. tert-butanol) are found to meet the above requirements (Ganske and Bornscheuer 2005). It is also worth noting that most lipases-synthesized of MAGs are conducted at low-temperature. Thus, room temperature ILs with tunable solvation power (good at dissolving compounds with varying polarity) are deemed suitable alternatives to conventional solvents in lipase-catalyzed synthesis of MAGs.

The structures of ILs have been reported to have significant effects on kinetics and purity of MAGs produced from enzymatic glycerolysis of fats and oils. Presence of long chain hydrophobic substituents and hydrophilic ethoxyl or hydroxyl moieties in ILs such as cocosalkyl pentaethoxi methyl ammonium methylsulfate (Ammoeng 100), tetraalkyl ammonium sulfate (Ammoeng 102) and cocosalkyl pentaethoxi methyl ammonium methosulfate [CPMA][MS] is beneficial for dissolving glycerolysis substrates and shifting reaction equilibrium towards higher formation of MAGs (Kahveci et al. 2016; Guo et al. 2006; Chen et al. 2008). For example, Novozym 435 (*Candida antarctica* lipase) has been used catalyse glycerolysis of TAG to produce high yield of MAGs (>90%) in [CPMA][MS] (Guo and Xu 2005). Water activity of the ionic liquid reaction system was found to affect the kinetic properties of glycerolysis in the ionic liquid system (Guo and Xu 2006).

In addition, ionic liquid also showed great potential as reaction medium for synthesis of monoesters of fatty acid and other sugar. *Candida antarctica* lipase B was used to synthesize glucose fatty acid ester synthesis in [BMIM][BF4] and [BMIM][PF6] (>30% conversion) (Ganske and Bornscheuer 2005). A mixture of

ionic liquid and tert-butanol can be used to further enhanced the conversions up to 90% and isolated yields up to 89% (Ganske and Bornscheuer 2005).

Diacylglycerol (DAG) are esters of glycerol with two of their hydroxyl groups esterified with fatty acids. DAGs are amphiphilic lipids which are commonly used as emulsifiers in the food industry. ILs has been used as reaction medium to produce DAGs (Kahveci et al. 2016; Kahveci et al. 2009; Kahveci et al. 2010). For example, lipase-catalysed reactions in [BMIM][BF4], [BMIM][PF6], and trioctylmethylammoniumbis (trifluo-romethylsulfonyl)imide [TOMA][Tf2N] has resulted in a conversion rate of 30–40%. These aforementioned ILs also showed higher selectivity towards 1,3 DAG as compared to 1,2 DAG (Kahveci et al. 2009). Ammoeng series (Ammoeng 100, Ammoeng 102, and Ammoeng 120) were also reported to be good reaction medium for high conversion of DAG (up to 44%). The effects of mixture ILs system on DAG conversion were also investigated. Novozym 435-catalyzed glycerolysis in binary ionic liquids ([TOMA][Tf2N]/Ammoeng 102) were reported to produce higher DAG yield (70 wt%) (Kahveci et al. 2010).

Besides acting as reaction medium, ILs can also be used as immobilization matrix to increase enzymatic activity for high production of DAG. Zhong and colleagues employed imidazole based ionic liquids modified mesoporous silica (SBA-15) for immobilization of *Candida antarctica* lipase B. The immobilized lipase demonstrated an improvement of enzymatic activity (from 1855 to 5044 U/g) and DAG content (from 53.6 to 67.2%) (Zhong et al. 2018).

Biodiesel is commercially produced by transesterification of TAG (usually vegetable oil/animal fat) with methanol in the presence of alkaline catalysts (sodium or potassium hydroxide) (Akoh et al. 2007). The aforementioned process is energy intensive and not cost effective. ILs are recyclable and less hazardous can be used as alternative to traditional catalysts for production of biodiesel. Ishak and colleagues have systematically reviewed the used of ionic liquids as catalysts for transesterification reactions of biodiesel production (Ishak et al. 2017).

Zhang and colleagues employed Brønsted acidic ionic liquid 1-(propyl-3-sulfonate)-3-(3-trimethoxysilylpropyl) imidazolium hydrogen sulfate supported on Fe-incorporated SBA-15 (Fe-SBA-15) as catalyst for esterification of oleic acid with methanol reactant to reach 87.7% of conversion (Zhang et al. 2012). In another study, Brønsted acidic ionic liquids, 1-(4-sulfonic acid) butylpyridinium was investigated as catalysts for transesterification of cottonseed oil with methanol to produce high yield of fatty acids methyl esters (up to 92%) (Wu et al. 2017). The produced biodiesel can be easily separated by simple decantation process. Brønsted acidic ionic liquids based on 1-benzyl-1H-benzimidazole as catalysts for transesterification of canola oil with methanol resulted in more than 95% of methyl esters in 5 h (Ghiaci et al. 2011). Nowicki et al. have investigated alkylguanidinium and alkylamidinium hydroxides as catalysts for synthesis of biodiesel. A high biodiesel conversion of 83% can be obtained for trimethylolpropane ester of oleic acid and ionic liquid [cyclic guanidine-Bu]OH (Nowicki et al. 2014).

In addition to catalysts, ionic liquids are also well documented as solvent (Muhammad et al. 2015) and co-solvent (Ruzich and Bassi 2010) for biosynthesis of biodiesel. ILs are also a suitable medium for separation of biodiesel and removal

of glycerol by-product (Prabhavathi Devi et al. 2016). The different roles of ionic liquids in biodiesel production have previously reviewed (Ishak et al. 2017; Muhammad et al. 2015; Liu et al. 2012; Zhao and Baker 2013; Troter et al. 2016). In addition, ionic liquid (N,N,N-trimethyl-N-propanesulfonic acid ammonium hydrogen sulfate [TMPSA]HSO4) also showed unique property as a dual solvent–catalyst for the protection of carbonyls via formation of acetals or ketals (Fang et al. 2007).

In addition to MAG, DAG and biodiesel, ionic liquid has also found in-creasing application as new reaction media for oleochemicals synthesis (Prabhavathi Devi et al. 2016). Ionic liquids have been used for Diels-Alder reaction of oleochemicals. Scandium triflate has been used as a catalyst for reaction of conjugated ethyl linoleate with methyl vinyl ketone in a neat ionic liquid (1,3-dialkylimidazolium) to produce long-chain fatty acid esters with 46% conversion rate (Behr et al. 2010). ILs were also studied as reaction medium for lipase-catalyzed alkyl dihydrocaffeates (Gholivand et al. 2017) and caffeic acid phenethyl ester (Rajapriya et al. 2018). Very recently, the binary ionic liquid-solvent system was reported for effective enzymatic esterification of naturally occurring phenolic glycosides (Pedersen et al. 2019).

7.3 Ionic Liquids in Extraction, Enrichment and Recovery of Lipids

Lipids extracted from various sources such as high lipid bearing unicellular microorganisms (Vermaak et al. 2011), plants (Severa et al. 2013), waste from animal or fish processing plants and etc. are widely used in food, pharmaceutical, and cosmetic industries (Fan et al. 2020a, b). Conventionally, lipid is extracted using organic solvents, which are usually volatile organic compounds (VOCs) (de Jesus and Filho 2020; de Jesus et al. 2019). For example, chloroform-based lipid extraction is effective in lab setting, but an alternative organic solvent, like hexane, which is easier to handle and more cost-effective, is usually used in industrial setting (Halim et al. 2013). Solvent treatment is generally accelerated or enhanced by application of high pressures (accelerated solvent extraction or supercritical solvent extraction) and/or temperatures (Soxhlet digestion) that promotes solvent penetration through the internal barriers (Cooney et al. 2009). The aforementioned systems allows manipulation of solvents physical properties which facilitate selective extraction. However, these systems are high energy and require the use of complex and costly capital equipment. It is of interest to find alternative to VOCs solvents which is more environmentally friendly as the high amount of solvent used in extraction process constitutes a real problem for the environment (de Jesus and Filho 2020).

ILs with the advantages of low melting point, non-volatility, wide range of liquid range, good thermal stability, strong solubility, adjustable properties, non-flammability, and wide electrochemical window have the potential to replace

traditional organic solvents in the extraction of lipids (Lu et al. 2017). Choi and colleagues investigated the effects of solvents on lipid yield extracted from *Chlorella vulgaris*. They compared lipid yield obtained using ILs as extraction solvent with lipid yield obtained using combination of organic solvents and ionic liquid mixtures (Choi et al. 2014). Among the 12 ILs, [EMIM]OAc (1-ethyl-3-methyl imid-azolium acetate), [EMIM]DEP (1-ethyl-3-methyl imidazolium dieth-ylphosphate), [EMIM] AlCl4 (1-ethyl-3-methyl imidazolium tetrafluoroborate), and [EMIM]Cl (1-ethyl-3-methyl imidazolium chloride) showed high (>200.0 mg/g cell) lipid extraction yields. This shows ILs can be used to improve lipid extraction yield from *Chlorella vulgaris*.

Cheong and colleagues investigated the use of ILs containing aromatic rings (N, N′-dialkylimidazolium and N-alkylpyridinium) for rapid (<30 min), selective extraction and enrichment of n-3 polyunsaturated compounds. The extraction process can be enhanced by increasing volume ratio of IL to solvent, addition of silver tetrafluoroborate, and usage of double bond containing solvents (1-hexene) as stripping solvent (Cheong et al. 2011). At optimal conditions, 1-butyl-3-methylimidazolium hexafluorophosphate [bmim]PF6 and 1-butyl-3-methylpyridinium dicyanamide [BuMePyr]DCN had increased extraction capabilities of n-3 polyunsaturated fatty acids (n-3 PUFA) (16.19%) and n-3 polyunsaturated fatty acid ethyl esters (PUFAEE) (144.54%), respectively. In addition, n-3 PUFAs can be further enriched using multiple-step reverse extraction up to three times. Following this reverse extraction operation, the purity of n-3 PUFA and n-3 PUFAEE in the IL phase increased from the initial 15.88 to 38% (step 1) and further to 45% (step 3) and from the initial 72.56 to 82% (step 1) and further to 89% (step 3), respectively.

Due to high cost and high viscosity of ILs, industrial use of ILs is still limited. Traditional solvents were used in combination with ILs for mediating extraction of lipids. This can reduce the overall solvent costs and add additional function at the molecular lever to the extraction system. Traditional ILs co-solvent systems were composed of ILs and tradition organic solvent. Young et al. studied the applicability of IL-polar covalent molecule as co-solvent to extract bio-oils from biomass. In their approach, IL-polar covalent molecule (imidazole ILs) was mainly used to disrupt the cell wall and improve the efficiency of lipid extraction from the biomass (Young et al. 2010). The most important factor to consider in co-solvent system is the mutual solubility of organic solvent and IL, which can be expressed by difference in the dielectric constant of the solvent in the IL. The mutual solubility of imidazole ILs and alcohols has enhanced their usage in preparation of biodiesel. The binding affinity of anions to alcohols is as followed: $[(CN)_2N]^- > [CF_3SO_3]^- > [(CF_3SO_2)_2N]^- > [BF_4]^- > [PF_6]^-$.

In some cases, mechanical force such as ultrasound, high pressure homogenization, and microwave pretreatment are required to disrupt and liberate lipids from cellular matrix into solvent extraction medium (Halim et al. 2012). Mechanical-assisted extraction of lipids in ILs is receiving more and more attention. Pan et al. reported microwave-assisted extraction of lipid from three algal species in [BMIM][HSO4] (1-butyl-3-methylimidazolium hydrogen sulfate). The experimental results

indicated that microwave irradiation increased extraction rate over 15 times for *Chlorella sorokiniana*, several hundred percent for Nannochloropsis salina and over 10 times for *Galdieria sulphuraria* when compared with conventional solvent extraction method (Pan et al. 2016). Krishnan and colleagues reported that addition of IL during high pressure microwave enhances lipid extraction from *Chlorella vulgaris* (Krishnan et al. 2020a, b).

Supercritical fluid extraction (SFE) technology is considered one of the oldest green technologies applied in extraction of lipids. Supercritical CO_2 fluids and ILs have attracted much attention as green media, and combination of the two has also attracted great attention especially in chemical industry (Zhou et al. 2005, 2006). High-pressure CO_2 can be dissolved in ionic liquids. As ions are insoluble in high-pressure CO_2, supercritical CO_2 can be used to recover dissolved organics in ionic liquids without cross-contamination. Supercritical CO_2 has high volatility and low polarity; meanwhile, ILs has non-volatility and considerable polarity. The combination of the two will produce an interesting two-phase system. Literature has not reported the use of a combination of supercritical-CO_2 and ILs for extraction of lipids. But this approach could be developed in future for lipid extraction.

Due to the negligible vapor pressure of ILs, volatile substances dissolved in ILs could be easily removed through decompression, and the ILs can be easily recycled. As a liquid salt in room temperature, IL has a "salt effect" similar to that of solid salt. This property can significantly change the relative volatility of certain components, and even directly destroy the azeotropic composition. Thus, functional-ILs have been used as stationary phase for chromatographic analysis. IL cation functionalized with long alkyl group substituents has been demonstrated to improve the separation of aliphatic hydrocarbons such as tripalmitin and cholesteryl stearate using comprehensive two-dimensional gas chromatography (Hantao et al. 2014; Zhang et al. 2015a, b; Zhang et al. 2016). A total of eleven lipidic ILs were applied as stationary phases in two-dimensional gas chromatography separation. Lipidic ILs possessing long alkyl chains as well as low melting points have the potential to provide unique selectivity as well as wide operating ranges when used as stationary phases in GC.

The versatility of ILs is extremely advantageous for extraction, enrichment and separation process of lipids. IL can be better combined with solvent extraction and separation technology to develop more stable and reliable extraction and separation technology.

7.4 Ionic Liquids in Removal and Analysis of Contaminants in Lipids

Lipids can be easily contaminated by many risk substances, such as 3-monochloropropane-1,2-diol esters (3-MCPDE), glycidyl esters (GE), polycyclic aromatic hydrocarbon (PAH), phthalate ester (PAE), bisphenol A (BPA), nonylphenol (NP), heavy metals and etc. Conventionally, these contaminants are

separated by steam distillation, physical absorption and liquid-liquid extraction. However, these processes are energy-consuming or environmentally hazardous. Therefore, development of safe and eco-friendly separation processes play an important role for clean and green removal of contaminants in lipids. ILs can provide new possibilities for removal of contaminants in lipids.

Both 3-MCPDE and GE are the contaminants resulted from processing of edible oil. Many important factors caused formation of these compounds in oils include long exposure of lipids to high temperatures during deodorization and presence of chlorinated species in the bleached oils before deodorization (Destaillats et al. 2012). Mitigation methods have been proposed based on the refining process (Oey et al. 2019).

ILs was used for the first time to mitigate 3-MCPDE and GE in edible oils (Fedor et al. 2016). IL treatment allowed mild conditions in degumming, bleaching and deodorization of edible oils. The treatment of palm oil with basic ionic liquid choline bicarbonate resulted in significant decrease of 3-MCPDE and GE contents. In IL treated crude palm oil, a reduction of deodorization temperature <230 °C was possible. The amount of GE and 3-MCPDE was significantly mitigated in comparison to the conventional refined palm oil (RBDPO). The basic IL was also considered as a reactive agent for extraction of free fatty acids; similar to the chemical refining with sodium hydroxide, but could be regenerated. The quality of the crude palm oil was also significantly improved after the basic IL treatment, where the contents of both phosphorus and iron were reduced and the free fatty acids were almost completed eliminated. Interestingly, the basic IL also presented the ability to reduce the already formed 3-MCPDE and GE, by about 80% and 70%, respectively.

Among numerous approaches to extract PAHs from different kind of matrix in analytical chemistry, liquid extraction is a preferred method which has lots of successful examples. Magnetic ILs were applied to the magnetic solid phase extraction (MSPE) of seven heavy molecular weight PAHs from coffee and tea samples (Shi et al. 2016). The nanoadsorbent (Fe_3O_4@MPS@IL NPs) was highly efficient with an enrichment factor ranged from 106.3 to 123.8, reusable and environmentally friendly. This approach was similar to that used to extract sixteen PAHs in vegetable oils by Zhang et al. (2017). The novel three-dimensional ILs functionalized magnetic graphene oxide nanocomposite (3D-IL@mGO), functionalized by IL, also exhibited high adsorption towards PAHs, which was reusable, eco-friendly and easily separated from the sample solution. Compared with molecularly imprinted solid phase extraction (MISPE), the MSPE method based on 3D-IL@mGO consumed less solvent and was more efficient to light PAHs in quantitative analysis.

Like PAHs, ILs technology has been widely used in PAEs extraction and analysis. Compared with enrichment of PAEs from water or alcoholic samples, PAEs in lipid is more difficult to be extracted and separated. A novel analytical approach based on QuEChERS- DLLME (Quick, Easy, Cheap, Effective, Rugged and Safe—Dispersive Liquid–Liquid Microextraction) was successfully developed for extraction, cleanup and pre-concentration of diethyl phthalate (DEP), diisobutyl phthalate(DIBP), dibutyl phthalate(DBP), and benzylnbutyl phthalate(BBP) in edible oils by Xie et al. (2014). The pre-concentration of the analytes was performed by

IL-DLLME with the cleaned-up extracts as disperser solvent and [C6MIM][PF6] as extraction solvent. Compared with conventional solid phase extraction and gel permeation chromatography cleanup, the main advantages of this method were its simplicity, shorter analysis time, lower consumption of toxic organic solvent. Therefore, it was suggested that ILs could be used as extraction solvent to selectively determine trace amounts of PAEs in oil matrices when IL-DLLME was combined with QuEChERS.

Compared with the environmental water matrix, the strong interaction between vegetable oil and alkylphenols has complicated its analysis. Since bisphenol A and nonylphenol had medium polarity (log *P* values of 4.15 for bisphenol A and 5.47 for nonylphenol), a longer chain was not beneficial to the extraction efficiency. Zhu et al. (2017) developed a novel method for the determination of bisphenol A and nonylphenol in vegetable oil by using a magnetic IL as the microextraction solvent in DLLME. [C6MIM][FeCl4] was selected as the magnetic ILs in this study. It was showed that ILs had good capacity to separate alkylphenols from lipids.

Toxic metal ions represent a hazardous category of food contaminants which can cause serious problems to human health and environmental safety. It has been demonstrated by Bai et al. (2010) that hydrophobic ILs can be adopted as DLLME extraction solvent for enrichment of heavy metals in water sample. Recently, it was shown that ILs could be used to separate or detect the heavy metals in lipids. Baldo et al. (2017) presented an electrochemical approach to prepare standard solutions of metal ions in [P14,6,6,6][NTf2], which was highly hydrophobic and could be mixed well in certain ratios with oils, resulting in decrease of the electrical resistivity of the solution and also furnishing the required ions that acted as supporting electrolyte. It was a good potential approach for direct analysis of the heavy metal content in a variety of lipophilic samples, including fatty foods such as milk, butter and other biological matrices. Yao et al. (2018) developed a novel ultrasound-assisted surfactant-enhanced emulsification microextraction (UASEME) using a magnetic ILs coupled with micro-solid phase extraction for pre-concentration of cadmium and lead in edible vegetable oils prior to analysis. For the first time, the magnetic IL was used as microextraction solvent in UASEME for organic matrix samples. This was an excellent alternative for preparation of oil samples since it was simple, fast and sensitive.

7.5 Conclusion

ILs have many favorable chemo-physical properties, generally considered as green alternative to the conventional solvent. It can be used for lipid processing from structural modification to extraction, enrichment, recovery of lipids. In addition, ILs can also be applied in the removal and analysis of contaminants in lipids.

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Chapter 8
Diacylglycerol Oil: Health Benefits, Synthesis and Applications

Oi-Ming Lai, Yee-Ying Lee, Eng-Tong Phuah, Teck-Kim Tang, Yong Wang, Ling-Zhi Cheong, and Chin-Ping Tan

Abstract Diacylglycerol (DAG) is a type of structured lipid that is made up of glycerol and two fatty acids. It is present in majority of vegetable oils in minute amounts. On a separate note, DAG can also be enzymatically or chemically synthesised through structural modification of conventional fats and oils *via* several possible mechanistic routes like esterification, glycerolysis, partial hydrolysis, and interesterification. Studies demonstrate that consumption of DAG significantly

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reduces body fat accumulation and lowers serum triglyceride level as DAG exhibits different metabolic pathway as compared to conventional fats and oils. As a result, DAG can be utilised as low-calorie fat substitute in many food applications. The performance of DAG as frying shortening, oil-in-water or water-in-oil emulsion was extensively studied so far. In fact, DAG can be found commercially available in Japan and United States market to be sold as healthy cooking oil. Nevertheless, the sale of DAG was put to a halt recently due to the presence of probable carcinogen which is 3-MCPDs and GEs in refined vegetable oil. This chapter aims to summarise the information on the production, process developments, applications, health benefits and safety concern of DAG oil.

Keywords Diacylglycerol · Obesity · Esterification · Partial hydrolysis · Glycerolysis · Transesterification

8.1 Introduction

Dietary fats and oils are essential life nutrients as they provide energy to sustain physical activities, carry fat-soluble nutrients, protect internal organs, maintain body temperature as well as serve as important building blocks for plasma membrane. Moreover, its addition into food enhances the sensory and textural experience of consumers. Unfortunately, there is strong evidence that suggests that consumption of high-fat foods associated with sedentary lifestyle is the major cause to increasing incidences of obesity (Golay and Bobbioni 1997; Astrup 2005).

According to World Health Organization (WHO) (2014), 39% of adults aged 18 years and above are overweight while 13% are obese. Previous literatures demonstrate that obesity is strongly correlated with various adverse health conditions including heart disease, cancer, diabetes mellitus, hypertension and others (Lavie et al. 2009; Artham et al. 2011; Louie et al. 2013). As a consequence, it may lead to early retirement, potentially reduce the life expectancy and increase the healthcare cost that burdens the government and slows down the economic growth. Although WHO advises a diet with fat content not exceeding 30% of the total energy intake for better human health, this recommendation may sacrifice the sensory pleasantness of fat-based food.

With increased awareness on health, the advent of diacylglycerol (DAG)-oil in the early 1990s, has drawn tremendous interest among researchers and food manufacturers to replace the conventional edible oil or triacylglycerol (TAG)-oil. Studies indicate that DAG-oil enhances the breakdown of fatty acids (β-oxidation) and reduces the blood serum TAG, thereby preventing the accumulation of visceral fat (Flickinger and Matsuo 2003; Teramoto et al. 2004). Besides, the exposed hydrophilic group on the DAG structure makes it a good food emulsifier (Shimada and Ohashi 2003; Masui et al. 2001; Nakajima 2004). With the increasing demand for DAG-oil, Kao Corporation (Japan) started to launch and commercialise the

functional edible oil called "Healthy Econa Cooking Oil" in the late 90's. In Japan, this healthy functional oil accounted for 80% of premium oil as well as 14% of the total Japanese edible oil market at that time (Sakaguchi 2001). Unfortunately, the sale of the DAG-oil was halted in 2010 when probable carcinogenic compounds were detected in the product (Masukawa et al. 2010).

8.2 Diacylglycerol: Properties

Conventional edible oil, also known as TAG-oil has a molecular structure of glycerol backbone bonded with three fatty acids while DAG-oil shows a glycerol backbone esterified with only two FAs. Therefore, DAG can exhibit three distinct stereoisomeric forms such as sn-1,2-DAG, sn-1,3-DAG, and sn-2,3-DAG, depending on the esterification position of the two FAs (Lo et al. 2008). The molecular structures of TAG and various DAG isomers are illustrated as in Fig. 8.1.

DAG-oil normally exists as a mixture with 30%–40% of 1,2-(2,3-) DAG and 60%–70% of 1,3-DAG under equilibrium conditions. The abundance of 1,3-DAG isoform in the DAG-oil is attributed to the steric effect of the molecule, rendering it to be stable thermodynamically. Previous studies indicate that acyl migration of DAG isomers can be initiated by the addition of an acid, alkali or heat (Serdarevich 1967; Takano and Itabashi 2002).

DAG is also found naturally in various vegetable oils. Generally, levels up to about 10% (w/w) of DAG is found in these oils, with the relative content depending on the origin of the oil (Table 8.1). The DAG content depends on several factors namely the variety of oil plant, processing methods as well as storage conditions. Other than the plant-based DAG, the human body can also synthesise DAG as intermediate product endogenously in TAG metabolism.

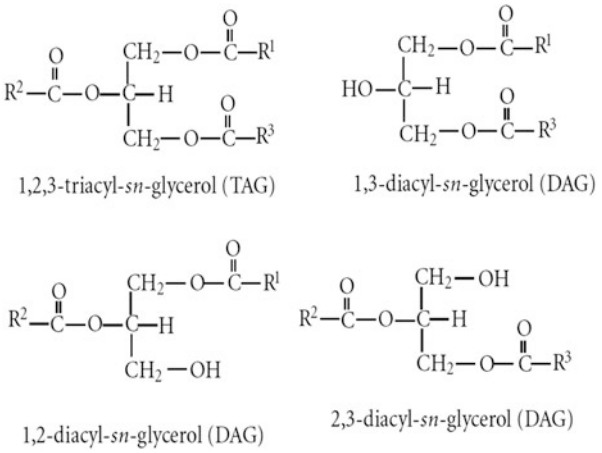

Fig. 8.1 Structures of triacylglycerol (TAG) and various diacylglycerol (DAG) isomers wherein R1, R2 and R3 are hydrocarbon chain fatty acids esterified on the glycerol backbone. (Source: Madawala et al. 2011)

Table 8.1 Relative contribution of acylgycerides in chosen edible oils

Edible oils	MAG	DAG	TAG	Others
Soybean	–	1.0	97.9	1.1
Cottonseed	0.2	9.5	87.0	3.3
Palm	–	5.8	93.1	1.1
Corn	–	2.8	95.8	1.4
Safflower	–	2.1	96.0	1.9
Olive	0.2	5.5	93.3	2.3
Rapeseed	0.1	0.8	96.8	2.3
Lard	–	1.3	97.9	0.8

Source: Flickinger and Matsuo (2003)

DAG has different physicochemical properties when compared to TAG and this is due to the occurrence of the exposed hydroxyl group in the DAG molecule. The hydrophilic nature of this function group provides DAG with excellent emulsifying and stabilising properties. Therefore, DAG is commonly employed together with monoacylglycerol (MAG) at different degrees of purity in both food and pharmaceutical industries (Nakajima 2004). Besides, DAG as starting precursor for the synthesis of various organic products including phospholipids, glycolipids, lipoprotein, pro-drugs such as 1,3-DAG conjugated chlorambucil for treatment of lymphoma and 1,2-DAG conjugated (S)-(3,4-dihydroxyphenyl)alanine (L-Dopa) for treatment of Parkinson's disease are well documented (Garzon-Aburbeh et al. 1983, 1986; Giacometti et al. 2001; Gonçalves et al. 1989). Moreover, DAG can also modify the crystalline behaviour of TAGs in fat-containing food (Yamane et al. 1994).

DAG is an interesting structured lipid with numerous health effects, particularly suppressing the postprandial serum TAG and body fat accumulation in which these benefits are not observed in conventional TAG-oil (Maki et al. 2002; Nagao et al. 2000; Takase et al. 2005). Most of the pre-clinical and clinical studies demonstrated that DAG-oil can serve as adjunct suppressive therapy for the management of obesity *via* reducing the postprandial TAG and glucose level, thereby preventing body fat accumulation and weight gain, especially among the high body mass index population (Maki et al. 2002). However, these benefits of DAG are only observed when taken at concentrations of at least 40% (w/w) and these levels are not available naturally in our edible oils.

In 1999, Kao Corporation in Japan patented a cooking oil with more than 80% DAG where the ratio of 1,3-DAG to 1,2 (2,3)-DAG stands at 7:3. The remaining composition includes approximately 20% TAG, 5% MAG and minute amount of antioxidants and emulsifier. Enzymatic esterification coupled with refining process are generally employed by KAO Corporation to produce refined DAG-oil. The esterification approach involves the reaction between fatty acids derived from vegetable oil and glycerol with the aid of immobilised 1,3-specific lipase. The commercialisation of DAG-oil had been a great success for KAO Corporation. The product has received wide acceptance among the consumers in the Japan and US market under the brand name of Econa™ and Enova™, respectively, achieving

revenue of about ¥10 billion in Japan market alone in the early 2000s (Flickinger and Matsuo 2003). In Japan, DAG-oil has been certified as "Food for Specified Health Uses" ("FOSHU") while in the United States, Food and Drug Administration (FDA) has given DAG-oil product the GRAS (Generally Recognized as Safe) status.

There is also no significant difference found in the energy values between DAG (38.9 kJ/g) and TAG (39.6 kJ/g) (Taguchi et al. 2001). In terms of digestibility of DAG, digestibility of rats fed with DAG and TAG are reported to be the same at 96.3 ± 0.3% and 96.3 ± 0.4%, respectively (Taguchi et al. 2001).

8.3 Synthesis of Diacylglycerol Oil

Generally, techniques to synthesise DAG includes direct esterification (Lo et al. 2004a, b, 2007), glycerolysis (Yang et al. 2004) partial hydrolysis (Cheong et al. 2007; Phuah et al. 2012, 2016a), interesterification (Weber and Mukherjee 2004) or a combination of partial hydrolysis and esterification reactions (Yamada et al. 2001). The complete synthesis methodology for the preparation of DAG has been reviewed and discussed extensively by Phuah et al. (2015) and Lee et al. (2020). These reactions can be conducted in the presence of inorganic alkaline catalysts such as alkali and alkaline earth metals or biodegradable enzyme lipase to accelerate the rates of reaction. Both approaches have their advantages and disadvantages. Chemical method is relatively cheaper but extreme operating conditions (temperatures ~220 °C–260 °C) may sacrifice the product quality, lowering DAG yield and purity. There can also be some degradation/oxidation of the heat sensitive fatty acids. Therefore, extensive purification steps are required to remove undesired by-products and improve the product quality. In contrast, enzymatic method is more reaction specific, environmental-friendly and require only mild reaction conditions.

Studies on esterification of fatty acids or its derivatives to glycerol to produce DAG have been done quite extensively. The process can be quite expensive due to the costly free fatty acid (FFA) used as raw material. Besides, elimination of water molecules formed during esterification is crucial to ensure reactions shift towards product (DAG) formation. Back in the early 1990s, Mazur and George (1992) patented a process for preparing DAG *via* esterification of fatty acid anhydride and glycerol in organic solvents assisted by 1,3-specific immobilised lipase. Since then, the application of enzyme technology for lipid modification has gained attention over the few past decades owing to its high catalytic activity, mild reaction conditions and reduced reaction time. For instance, Yoon et al. (2004) successfully produce high-purity DAG (>85%) through enzymatic esterification of MAG with conjugated linoleic acids. In addition, Lo et al. (2007) disclosed the production of DAG by esterifying glycerol with oleic acid, palmitic and oleic acid mixture as well as stearic and oleic acid mixture in the packed bed reactor. Approximately 50% DAG was reported under optimised operating conditions and additional purification step could be carried out to improve the DAG purity up to 90%. The study showed

that several specific DAG species such as 1,3(2)-diolein, 1-palmitoyl-3(2)-oleoyl glycerol and 1-stearoyl-3(2)-oleoyl glycerol can be synthesised using the method. The authors also found that the Lipozyme RMIM lipase favours the production of oleic acid-enriched DAG as compared to palmitic acid- and stearic acid-enriched DAG. In another study, fatty acid deodoriser distillate of corn oil and soybean oil were used as starting raw materials for DAG production *via* enzymatic esterification with the objective of reducing the overall manufacturing cost (Lo et al. 2004a, b). The use of the cheaper deodorizer distillates may be a cause of concern when the DAG is meant for edible purposes. In 2016, Lu et al. produced DAG enriched rice bran oil in a special packed bed column that is made up of two sets of enzyme packed bed columns and a third middle column that consists of molecular sieves to remove the water produced from the reaction.

Other than esterification, transesterification reaction between MAG and TAG for DAG synthesis has also been reported (Toshinori et al. 2000; Chen et al. 2017). Nevertheless, this production method may not be feasible at industrial scale owing to the expensive MAG as the raw material. In 2017, Chen and co-workers utilised distilled saturated MAG and refined soybean oil to produce their DAG. Besides that, Yamada et al. (2001) demonstrated a 2-step procedure, combining both hydrolysis and esterification reactions for DAG synthesis. The process involves the release of FFAs from TAG *via* hydrolysis at the first stage, followed by the esterification of these FFAs with glycerol for DAG formation.

Enzymatic partial hydrolysis is another route to produce DAG from TAG. This method was explored and patented by Lai et al. (2005a, b, 2006a, b, c, d) who filed 5 patents in Malaysia (PI 20066218), European (EP1803819), United States (US2007/0148745), Japan (2007–175,049) and PCT International (WO2007/075079) for this method. This invention involves reacting TAG with water and enzyme to form a mixture of DAG, MAG, and FFAs which is further purified to obtain high purity of DAG. The advantage of this process lies in the single-step hydrolytic reaction. Cheong et al. (2007) and Phuah et al. (2012) used the partial hydrolysis process catalysed by Lipozyme RMIM lipase to produce palm-based DAG. Cheong et al. (2007) obtained around 32-wt% of palm-based DAG which was later purified to 90% following short path distillation using 10-wt% enzyme load and 50-wt% water content at 65 °C.

Another interesting method involves the use of a molecular distillation technique to produce DAG-enriched oil from conventional edible oil (TAG-oil) at temperature of 300 °C under vacuum level of not exceeding 0.01 Torr (Choo et al. 2009). Furthermore, DAG can also be produced through glycerolysis reaction by reacting the TAG with glycerol. Tang et al. (2013) successfully produced approximately 44% palm kernel-based DAG (PKDG) using glycerolysis process in a packed bed reactor. They noticed an inverse correlation between the PKDG and TAG content during the glycerolysis reaction. PKDG content was found to increase from 1.6% to 43.5% while the TAG composition reduced from 98.4% to 11.5% after 9 h. of reaction as illustrated in Fig. 8.2. The purification step was then conducted using molecular distillator to remove undesired by-products such as FFA, MAG and TAG in the crude mixture, yielding DAG of purity around 90%. Similarly, Yeoh et al. (2014)

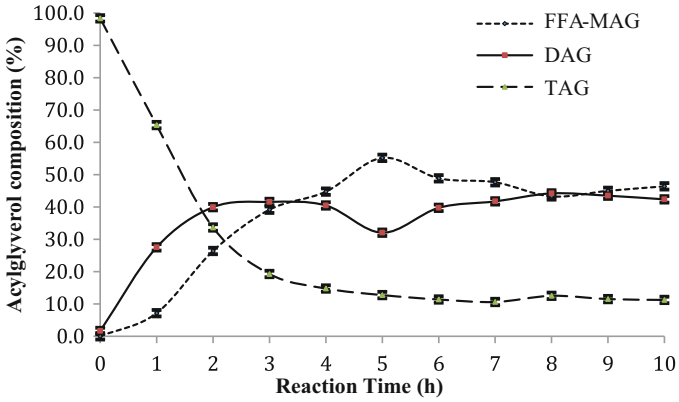

Fig. 8.2 Acylglycerol composition during lipase-mediated glycerolysis reaction in pilot-scale packed bed reactor. (*FFA-MAG* free fatty acid and monoacylglycerol, *DAG* diacylglycerol, *TAG* triacylglycerol). (Source Tang 2013)

achieved a high purity DAG-oil (~90%) after performing glycerolysis of palm olein, followed by 2-step distillation process. In another study, Zhao et al. (2018) used ultrasonic pre-treatment to produce DAG from lard. His study showed that the yield of DAG doubled compared to the control. However, increasing the intensity of the ultrasonic power reduced the reaction rate due to inactivation of the lipases. Besides ultrasonic treatment, Huang et al. (2015) used 15% of ionic liquid, 1-butyl-3-methylimidazolium imidazole to produce 60% DAG. Their yield is much higher compared to the conventional enzymatic method.

Besides enzymatic catalysis, DAG can also be produced using chemical catalysts or combination of both methods. The use of chemical catalysts confers several benefits. The catalyst is cheaper than enzymes, easily separated, and for some, can be reused for many cycles. Jacobs et al. (2003) used potassium acetate to catalyse the glycerolysis process between TAG and glycerol to obtain DAG. The method is very energy intensive and costly due to the extremely high operating temperature of 190 °C to 240 °C. In another approach, Lo et al. (2007) produced 1,3(2)-diolein-enriched, 1-palmitoyl-3(2)-oleoyl glycerol- and 1-stearoyl-3(2)-oleoyl glycerol-enriched DAG by esterifying glycerol with palm-based fatty acids catalysed by macroporous ion exchange resin. The reaction was carried out for 1.5 h at 110 °C using 25% resin. The reaction yielded around 40-wt% DAG. In this scenario, the chemical catalyst was found to display specificity towards the synthesis of oleic acid-enriched DAG as compared to Lipozyme RMIM lipase-catalysed esterification mentioned earlier (Lo et al. 2007). Despite a slightly lower DAG yield (by ~10%) reported for resin catalyst compared to enzyme lipase, the resin catalyst can offer the advantage of lower amount of TAG being produced during the esterification, thereby making the purification stage later much easier as both DAG and TAG exhibit close vapour pressure or boiling point. A patent was filed for the DAG production using heterogenous catalyst consisting of ion-exchange resin (Lai et al. 2006e).

8.4 Health Benefits of Diacylglycerol Oil

To date, the beneficial health properties of DAG-oil have been well studied and documented. DAG-oil is claimed to be capable of suppressing abdominal and visceral fat accumulation despite similar energy value and digestibility compared to conventional TAG-oil, making DAG-oil an interesting alternative to replace native fats and oils for obesity management (Flickinger and Matsuo 2003). The anti-obesity characteristic of DAG-oil arises because of its unique molecular structure, particularly the 1,3-isoform DAG, thereby resulting in the distinct metabolism pathway of DAG-oil from TAG-oil (Hideto 2007). Figure 8.3 shows the metabolic pathway of DAG and TAG.

Upon entering the gastrointestinal tract, digestion of acylglycerols (TAG and DAG) will be initiated by the intestinal lipase enzyme which shows sn-1,3-positional specificity. Hydrolysis of TAG will typically release FFA and 2-MAG into the small intestine. These hydrolysed products will be absorbed by the intestinal epithelial cells before being re-esterified into TAG through 2-MAG pathway and glycerol-3-phosphate pathway (Friedman and Nylund 1980). Subsequently, the re-synthesised TAG will enter the intestinal lymph and circulate throughout the blood circulation system as chylomicron. In the circulation, TAG carried in chylomicron will de metabolised, releasing fatty acids that tend to capillary vessels in the heart, muscle, and adipose tissues. On the contrary, DAG particularly 1,3-DAG exhibits different metabolism routes as compared to conventional TAG. Digestion of 1,3-DAG forms intermediate 1(3)-MAG instead of 2-MAG in the small intestine. Consequently, reformation of TAG from the 1(3)-MAG *via* the metabolism pathways abovementioned will be slow as both DAG acyltransferase and MAG acyltransferase enzymes show low affinity towards 1(3)-MAG, rendering the release of chylomicron into intestinal lymph. Thus, formation of clots at the capillary vessels and fat deposits in the adipose tissues will be reduced (Matsuo 2004). As a result, ingestion of DAG-enriched products especially 1,3-DAG lowers the TAG-rich lipoprotein, serum TAG, and the postprandial hyperlipidaemia (Flickinger and Matsuo 2003; Rudkowska et al. 2005; Tada 2004; Taguchi et al. 2002; Yanai et al. 2007).

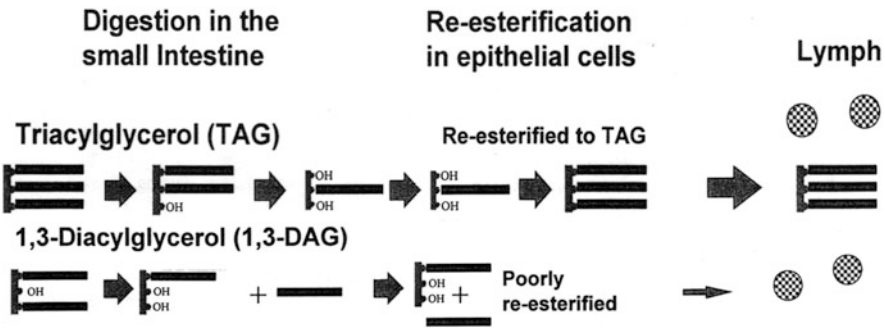

Fig. 8.3 Metabolic pathway of DAG and TAG. (Source: Matsuo 2004)

In another perspective, consumption of DAG-oil enhances the rate of β-oxidation which is associated with the reduction of visceral abdominal fat and body weight (Kamphuis et al. 2003). Previous study revealed that diet enriched with 10% DAG stimulated the β-oxidation activity accompanied by the downregulation of the enzymatic activity related to fatty acid synthesis in rats' liver (Murase et al. 2001). Another study also showed that diets containing high DAG level enhanced the hepatic acyl-CoA oxidase and medium-chain acyl-CoA dehydrogenase activity in rats which are responsible the fat oxidation in hepatic cell (Murase et al. 2005). Besides, previous literatures also reported that dietary DAG upregulates β-oxidation activity and lipid metabolism-related gene expression in C57BL/6 J mice. For instance, acyl-CoA synthase and uncoupling protein (UPC)-2 mRNA were increased in the small intestine of mice (Murase et al. 2001, 2002, 2005). Elevation in the UPCs expression increases the thermogenesis effects, thereby boosting the energy expenditure (Murase et al. 2005).

Several clinical trials had also been carried out to evaluate the correlation between DAG-oil consumption and obesity-related indices. Nagao et al. (2000) reported significant reductions in body weight (-2.6 ± 0.3 kg), body fat (-22 ± 3.0 cm^2) and waist circumference (-4.4 ± 0.6 cm) on 38 healthy Japanese men consuming DAG-oil and TAG-oil for 16 weeks. The authors also observed decreases in total fat, visceral fat area and subcutaneous fat on subjects under the DAG group as compared to TAG group (Nagao et al. 2000). In another study, the Chicago Centre for Clinical Research (CCRC) had conducted a randomised, double-blind, and parallel trial, involving 127 overweight or obese adults consuming DAG- or TAG-diets for 6 months. The study found that DAG-diet significantly enhances loss in body weight and fat (Matsuo and Tokimitsu 2001). A similar observation was also reported by Maki et al. (2002) who examined the anti-obesity effects of DAG-enriched diet among 131 overweight subjects in Japan. After that, in 2005, Tada and others conducted a randomised crossover study and they reported that the administration of emulsified DAG-oil reduced the postprandial serum TAG and lipids in moderately diabetic subjects as compared to TAG-oil.

Most of the clinical and pre-clinical studies emphasise on DAG made of long-chain unsaturated fatty acids particularly linolenic, linoleic or oleic acid (Eom et al. 2010; Murase et al. 2001, 2002, 2005; Nagao et al. 2000; Taguchi et al. 2002; Saito et al. 2017). As a matter of fact, fatty acids with different chain length and degree of unsaturation in the DAG itself may exert distinct health benefits. For example, Tang et al. (2013) reported that the ingestion of both PKDG (high in medium-chain fatty acids) and the soy canola-based DAG (SCDG) resulted in a significant decline in the fat accumulation in the epididymal and retroperitoneal region in C57BL/6N mice as compared to palm kernel-based TAG. Besides that, several obesity-related indicators such as serum glucose, cholesterol, leptin, and insulin level were found to be lower in mice consuming PKDG and SCDG diet. Also, the study also demonstrated that both PKDG and SCDG could possibly bring down the low-density lipoprotein level in the body as indicated by the reduced expression of apolipoprotein B mRNA in mice subject. Meanwhile, the authors also pointed out that PKDG promoted the expression of acyl-CoA synthase long chain (ACSL) and acyl-CoA synthase

medium chain mRNA in the small intestine whereas SCDG induced the high expression of ACSL in liver and small intestine, indicating that the types of fatty acid within DAG molecule may potentially induce β-oxidation in different organs in mice subjects.

8.5 Applications of Diacylglycerol Oil

In 2006, DAG was approved as novel food ingredient and could be used in a variety of food products including cooking oil, fat spreads, salad dressing, mayonnaise, beverages, and bakery products in Japan. Together with the many positive health benefits of DAG such as body weight management (Flickinger and Matsuo 2003; Nagao et al. 2000; Maki et al. 2002; Teramoto et al. 2004), reduction of visceral fat, postprandial and fasting TAG (Taguchi et al. 2001; Tada et al. 2005), many food manufacturers have started to develop DAG-based food products.

DAG was also incorporated into fermented sausages (Mora-Gallego et al. 2013) and meat emulsion substitutes (Miklos et al. 2011). The DAG-enriched sausages were found to have better chewiness, lower crumbliness and held the sliced shape better. This could be due to the higher solid fat content (SFC) in DAG compared to sunflower oil. Similarly, the meat emulsion substitutes that contained DAG were found to be more elastic, solid and had better hydration and water binding ability as compared to lard-derived meat emulsions.

Mayonnaise is another product where attempts have been made to substitute the oil in it with DAG. In 2016b, Phuah et al. substituted soybean oil in mayonnaise with 10% PKDG. The study found that the incorporation of up to 10% PKDG produced mayonnaise with similar rheological and textural properties with the control (100% soybean oil). In another study, Kawai (2004) prepared the mayonnaise using the DAG-oil derived from rapeseed oil and soybean oil. The author found that DAG-based mayonnaise shows comparable taste, flavour and colour compared to conventional TAG-based mayonnaise, but the former exhibits a more viscous texture.

DAG-oil was first sold as a cooking oil in Japan. Studies by Mori et al. (2000) and Kudo et al. (2005) have shown that potatoes, chicken, and doughnuts fried in DAG-oil had lesser water content that made them more crisp and had pleasant texture, taste, and higher shelf life, respectively. However, DAG-oil have lower smoke points than TAG-oil due to its lower molecular weight despite having similar fatty acid compositions. This made it undesirable for deep fat frying. Even so, the frying stability of the DAG-oil could be enhanced with the incorporation of antioxidative compounds such as L-ascorbic ester, catechin, rosemary, sage or turmeric extracts as reported by Sakai et al. (2005). Another study also revealed that the addition of organic carboxylic acids (citric acid, tartaric acid or malic acid) and their derivatives up to 70 ppm or more to DAG can increase the thermal oxidation of the oil during prolonged heating or storage, as well as reduce smoking (Sakai et al. 2003).

Ice-cream coatings are typically made with TAG consisting predominantly of medium-chain fatty acids, such as lauric acid. In a recent study, Cain et al. (1999) examined the possibility of utilising 50–90% DAG of vegetable origin to make ice-cream coating fat. According to this invention, the ice cream coating fat had resulted in a product that is softer and less brittle but had quicker and smoother meltdown, than cocoa butter-based coating fats.

DAG-enriched shortening was produced by Latip et al. (2013) using stearin fraction of palm-based DAG blended with palm mid fraction in the ratio of 50:50 and 60:40 (w/w) while Cheong et al. (2011) used purified palm-based DAG (PDG) with palm stearin for application as bakery shortenings. Subsequently, the PDG-enriched shortenings were used to bake Madeira cakes. The cakes were found to have higher specific volumes compared to its control and this may be attributed to the emulsifying properties of PDG. PDG could lead to the incorporation of more air bubbles and stabilised the gas bubbles in the batter, resulting in higher volume and softer texture in cakes.

DAG-oil was also used in preparation of bakery margarine by blending PDG and palm olein as reported by Cheong et al. (2010). The mixture had melting and SFC profile close to the commercial palm-based bakery margarine. Nevertheless, these fat systems are costly which hinder the widespread application of these blends to produce bakery margarine. Therefore, palm stearin could be incorporated into the mixture to lower the production costs. These ternary fat systems exhibited plastic-like texture at room temperature and showed low percentages of solids at body temperature. The author also reported that the presence of PDG could slow down the undesired polymorphic transformation from β' to β crystals in the fat system (Cheong et al. 2010).

Additionally, several studies had also been conducted to produce shelf-stable margarine and soft tub margarine from palm-based DAG (Saberi et al. 2011, 2012). Saberi and his teammates (2011) reported that the DAG-based shelf-stable margarine with similar physicochemical properties to that of commercial product could be produced by blending palm olein: palm kernel oil: palm-based DAG in the ratio of 42.5:42.5:15 (w/w). In the subsequent work, Saberi et al. (2012) prepared DAG-enriched soft tube margarine from a fat mixture containing sunflower oil: palm kernel olein: palm-based DAG in the ratio of 35:15:50 (w/w). The product formulated showed similar SFC profile and SMP as compared to the commercial soft tub margarine (Saberi et al. 2012).

Besides that, PDG-enriched margarine comprised of PDG, palm olein and palm stearin at different ratios could also be applied to make cookies. Study showed that cookies prepared using different margarines (PDG-enriched and commercial margarine) were fairly accepted among the panellists. However, margarine prepared with PDG-enriched margarine had softer texture, thereby leading to lower preference in terms of texture. The phenomenon could be attributed to water-retaining ability of the polar DAG molecules, thereby promoting the development of gluten, and changing the texture of cookie from snap type to soft-batch type (Sikorski 2004).

Besides food applications, DAG can also be used to measure the quality of fresh olive oil (Caponio et al. 2013) where higher 1,3-DAG in the olive oil indicates lower quality oils.

8.6 Conclusion

In the early 1990s, work on DAG was fuelled by the willingness of consumers to try new structured lipids such as DAG. Many rushed to file patents with newer production and processing methods. Safety evaluations on DAG on animals and humans for short- and long-term studies indicated that DAG is safe for consumption under normal use and the labelling requirement was revised in several countries. Food containing DAG was labelled to inform the consumer of its health benefits. Unfortunately, in September 2009, Kao Corporation halted all sales of DAG following the concerns expressed by the German Federal Institute for Risk Assessment over the occurrence of glycidol esters (GE) found in DAG oils. GE is a process contaminant found in refined edible oils and was found to be a probable carcinogen that can result in development of tumour. There were also studies indicating a correlation between DAG content in oils with the formation of GE. The safety of DAG is still inconclusive as of today and this issue has shifted the research direction of many to mitigation strategies to modify the refining/deodorization process to reduce the process contaminants in DAG. Some of these mitigation strategies are already able to reduce the process contaminants but are yet to be implemented at industrial scale due to cost concerns. Thus, the advent of mitigating strategies that are economical at industrial scale will determine if the DAG-oil will be placed back on the shelves in the near future.

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Chapter 9
Medium-and Long-Chain Triacylglycerol: Production, Health Effects and Applications

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Abstract Medium-and long-chain triacylglycerol (MLCT) is a type of structured lipid where the glycerol backbone is made up of medium chain fatty acid (MCFA)

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(C6-12) and long chain fatty acid (C14-C22). It is produced through structural modification process. Studies demonstrated that MLCT has the ability in managing obesity which is attributed to the unique metabolic pathway of MCFA. MCFAs are rapidly metabolised in the body due to its small size and hyrophilicity. Therefore, it can prevent the accumulation of fat. Meanwhile the presence of LCFAs allow the delivery of essential fatty acid to our bodies. Various modification pathways can be utilised to synthesise MLCT from conventional fats and oils including esterification, interesterification, and glycerolysis catalysed either by enzyme or chemical catalyst. This chapter aims to highlight the overview of MLCT which includes metabolism, production, health benefits, and its applications in the various food systems.

Keywords Medium-long chain triacylglyceride · Obesity · β-oxidation · Medium chain fatty acid · Interesterification · Esterification

9.1 Introduction

World Health Organisation (WHO) estimated that there were around 1.9 billion adults who were overweight. Out of this approximately 650 million of them were clinically obese as in year 2016. The epidemic of overweight and obesity is a challenging issue as it is not only strongly linked with the development of several life-threatening metabolic diseases such as cardiovascular disease, stroke, diabetes, osteoarthritis, and certain types of cancer but also possesses a major economic burden to the country (Must et al. 1999). The fundamental cause of obesity is mainly attributed to the imbalance between energy intake and energy expenditure in which energy intake exceeds the energy expenditure, resulting in the excessive accumulation of fat in the body. However, obesity is preventable. Among the various interventions, changes in dietary patterns and increased physical activity are believed to be the safest approach in managing the prevalence rise of obesity.

Today, with the aid of enzyme lipase or chemical catalyst, vegetable oils and animal fats can be structurally modified *via* esterification, interesterification and glycerolysis process to change the composition or the position of fatty acids that are attached to the glycerol backbone to improve their healthful functional properties. Such structural alteration also indirectly changes the physicochemical characteristics of the conventional fats and oils such as the melting and crystallisation properties. Typically, modification is aimed to not only to improve the nutritional value, provide low or zero calories attributes but also for the development of fat substitutes. For instance, structured lipids, such as diacylglycerol (DAG), medium- and long-chain triacylglycerol (MLCT), medium chain triglyceride (MCT) along with short and long-chain triacylglycerol (SLCT) are developed to manage obesity.

On the other hand, those like cocoa butter substitute, or human milk fat substitute are meant for the replacement of the expensive and limited supply of cocoa butter and human breast milk, respectively. The aforementioned structured lipid are not new. They are commercially sold in the market and widely distributed across different countries.

MLCT is a type of structured lipid that is made up of medium chain fatty acid (MCFA) (C6-C12) and long chain fatty acid (LCFA) (C14-C22). It demonstrated to have the capability in managing obesity by suppressing visceral fat accumulation due to the presence of MCFA (Hu et al. 2018; Matsuo and Takeuchi 2004; Ogawa et al. 2007). MCFA is unique as it is small and hydrophilic. Hence, it can be delivered directly to the liver to undergo β-oxidation process instead of re-synthesized to form the new triacylglycerol (Papamandjaris et al. 1998). The development of MLCT was initially prompted by the initiative of lipid scientists to overcome several setbacks of the MCT that tends to foams easily when used as a frying medium and deprive of the essential fatty acid necessary to maintain the physiological function of the body. Nisshin Ollio Ltd. was the first company to commercialise the MLCT in the Japanese market. A patented was filed by Nisshin Oilio Ltd. for the synthesis of MLCT in 2004 *via* chemical and enzymatic routes. Approximately 60% of MLCT managed to be synthesised from these approaches. With strong evidence, MLCT was sold widely as healthy cooking oil under the brand name of "Resetta" to modulate obesity. It bears with it the claim of functional oil that "refrains fat accumulation in the body". In this chapter, we will highlight the processes used for MLCT production, focusing mainly on the enzymatic routes; the unique metabolism of MLCT; its touted health benefits and recent applications in the various food systems.

9.2 Structure of MLCT

MLCT is made up of MCFA (C6 to C12) and LCFA (C14 to C20) attached to the glycerol backbone. It gives rise to six different types of configurations which are MLM, LLM, MML, LML, MLL and LML, depending on the position of these fatty acids that are bound to the glycerol backbone (M = medium chain fatty acid, L = long chain fatty acid). Figure 9.1 shows the structure of MLCT and the six different configurations of the MLCT species. In Resetta, the MLCT species is mainly composed of 2L1M followed by 1L2M. Among them, MLM configuration is preferred for its anti-obesity effect.

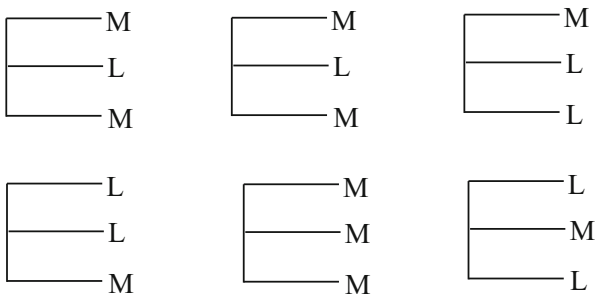

Fig. 9.1 Structure of six different configurations of MLCT species. *M* MCFA, *L* LCFA

9.3 Metabolism

In the normal metabolism of fats and oils, lingual lipase in the mouth and pancreatic lipase in the stomach are responsible for the digestion and breakdown of triacylglycerol. Due to the *sn*-1,3-regiospecific property of the lipase, the triacylglycerol is hydrolysed at the *sn-1* and *sn-3* position. It results in the formation of glycerol, free fatty acid (FFA) and 2-monacylglycerol (2-MAG). Subsequently, the 2-MAG is utilised for the re-synthesis of a new triacylglycerol molecule. The newly synthesised triacylglycerol is then circulated to the lymphatic system and distributed throughout the whole body as chylomicron. Ultimately, the newly formed triacylglycerol is deposited as fatty tissue in the body.

Digestion and breakdown of MLCT is similar to most of the vegetable oils and animal fats. However, as compared to conventional fats and oils, MLCT has a unique metabolism. It acquires both the metabolism of MCFA and LCFA. When broken down by the endogenous lipase, MLCT produces 2-MAG and FFA (MCFA or LCFA). Since MCFA is shorter in chain length, smaller in size and more hydrophilic than LCFA, it can be rapidly metabolised in the body (Papamandjaris et al. 1998). MCFAs are transported directly into the liver *via* the hepatic portal vein to go through β-oxidation, producing ketones bodies, such as acetoacetate, acetone, β-hydroxybutyrate, which bypasses the carnitine transport system within the mitochondria matrix. Ketones bodies will then enter the Kerbs cycle and be converted into Acetyl-CoA that can provide a rapid source of energy. Thus, MCFA can be easily burned off without being used to form a new triacylglycerol molecule that potentiates to be accumulated as adipose tissue in the body. In contrast, 2-MAG are metabolised to produce new triacylglycerol molecules that serve to deliver the essential fatty acid to the body. Hence, MCLT is able to be metabolised rapidly as well as to provide the LCFA needed by our body.

When taken into consideration such metabolism of MLCT, it was postulated the MLM is more preferred over the other types of MLCT species in managing obesity since it will release two MCFAs upon digestion to give a better weight-reducing effect than other MLCT isomers. This, however, remains to be investigated as no studies so far were conducted to assess and compare the health benefits of all the individual MLCT species albeit its structural differences.

In short, the metabolism of fats and oils varies depending on the chain length of the fatty acid. Majority of the vegetable oils and animal fats follow the conventional metabolism pathway. There are only a few exceptional cases whereby the fats and oils undergo a different mode of metabolism, particularly, those that are made up of MCFA.

9.4 Source of Medium Chain Fatty Acid

The health attributes of MLCT are mainly contributed by the presence of MCFA viz. caproic (C6), caprylic (C8), capric (C10) and lauric (C12) acid. As compared to vegetable oils that is make up of LCFA, fats and oils with high MCFA content are limited in the market. Table 9.1 shows the MCFA content in animal fats and vegetable oils. Tropical oils like coconut oil and palm kernel oil are the only few examples of vegetable oils that contain high amount of MCFA (around 50%). Hence, tropical oils it is considered to be more suitable for the synthesis of MLCT. Of the 50% of MCFA in coconut oil and palm kernel oil, there are 40% of them that are bound to glycerol backbone together with LCFA. Thus, MLCT are said to be present naturally in the coconut and palm kernel oil. Apart from that, MCFA can also be found in milk fat at around 20% which is significantly lower than the MCFA present in tropical oils making it insignificant to be utilised for MLCT production.

Table 9.1 MCFA in animal fats and vegetable oils

	Fatty acid			
Common name	Carbon number	Milk fat	Coconut oil	Palm kernel oil
Butyric	C4	11.8	N/A	N/A
Caproic	C6	4.6	0.4	0.3
Caprylic	C8	1.9	7.3	4.2
Capric	C10	3.7	6.6	3.7
Lauric	C12	3.9	47.8	48.7
Myristic	C14	11.2	18.1	15.6
Palmitic	C16	23.9	8.9	7.5
Stearic	C18	7	2.7	1.8
Oleic	C18:1	24	6.4	14.8
Linoleic	C18:2	2.5	1.6	2.6
Others	Others	2.6	0.1	0.1

Adapted from Lubary et al. (2011) and Pantzaris and Ahmad (2001)

9.5 Production of MLCT

Synthesis of MLCT involves the modification of fats and oils *via* a few routes such as esterification (FFA and glycerol), interesterification (triacylglycerol with triacylglycerol) and acidolysis (triacylglycerol and free fatty acid) catalysed by enzyme lipase or chemical catalyst like sodium methoxide (Xu et al. 2016). Unlike other approaches in producing structured lipid, one of the prerequisites to synthesise MLCT is the use of the vegetable oil or animal fats containing MCFA. Even though MLCT produced through chemical reaction is fast and economical, it has several drawbacks. Chemical catalyst is harsh in the reaction which may cause the degradation of the thermal sensitive essential fatty acids. It may also lead to instrument corrosion and the formation of dark coloured products. Furthermore, the reaction catalysed by the chemical catalyst is random and often resulted in the formation of nonspecific MLCT species. On the other hand, enzyme-catalysed reaction provides a promising alternative to overcome the setbacks of the chemical catalysed reaction. Enzyme lipase is specific in its reaction and requires only a mild condition to react. Thereby, it can help to protect the degradation of thermo sensitive fatty acid. Enzyme is usually dosed at around 1–10% (depending enzyme activity) for the synthesis MLCT. Table 9.2 shows the list of the available enzyme used for the production of MLCT which is manufactured and distributed by Novozymes. However, the disadvantage of utilising enzyme lipase is that it can be slightly expensive and may incur additional production costs. However, with immobilization technology, the operating cost of the enzymatically catalysed reaction could be reduced as the enzyme immobilisation allows multiple reuses of the enzyme without sacrificing its activity. As such, it was more environmentally friendly compared to chemical catalysts. Under certain circumstances, the enzyme-catalysed reactions are performed in the presence of a chemical solvent such as hexane. Chemical solvent is used for the purpose to increase the miscibility between the reactants and further reducing the mass transfer of reaction. Nevertheless, most of the reactions nowadays are conducted in a solvent-free system due to sustainability and safety issues. Furthermore, organic solvents were toxic to enzymes, resulting in the reduction of their activity. In some studies, various type of reactors such as bubble column reactor or pack bed reactor is used to increase the mass transfer reaction apart from the used of organic solvent. Generally, the production of structured lipid is always followed suit by purification process to remove the impurities formed during the reaction such as FFA, MAG, diacylglycerol and non-targeted TG. These secondary compounds were

Table 9.2 List of commercially available lipase for the synthesis of MLCT

No	Commercial available lipase
1	Novozyme 400,086
2	Lipozyme IM
3	*Thermomyces lanuginosa*
4	Lipozyme RMIM
5	Lipozyme TLIM
6	Novozyme 435

produced due to acyl migration, a secondary reaction promoted by the enzymes, which involves the movement of a fatty acid chain from the *sn*-2 to *sn*-1,3 position (Utama et al. 2019). Purification is often carried out *via* molecular distillation or deodorisation to remove these impurities and often resulted in around 80–90% of the MLCT.

9.5.1 Esterification

The production of MLCT *via* esterification process involves the esterification between free fatty acid in the form of MCFA and LCFA with glycerol. Water is generated or released as the by-products during the reaction. Usually, the free fatty acid to glycerol is supplied in the mass ratio of 3:1 (w/w) and 3.5:1 (w/w) and water is eliminated to push forward the reaction to form triacylglycerol. Studies demonstrated the use of MCFA sources like capric acid and caprylic acid and LCFA sources such as oleic acid for the synthesis of MLCT *via* the esterification process. Today, only four studies utilised this approach for the production of MLCT (Arifin et al. 2012; Kamariah et al. 2012; Koh et al. 2010; Yang et al. 2014). Few works were conducted utilising this approach because the esterification process took a long duration to complete which is more than 12 h. Furthermore, extra equipment such as a vacuum pump is needed to push forward the esterification process. Otherwise, it will lead to the formation of MAG and DAG. Apart from that, the FFA used particularly, MCFA is relatively costly compared to vegetable oil.

9.5.2 Interesterification

Interesterification takes place *via* the exchange of fatty acid between two triacylglycerol molecules. Table 9.3 shows the interesterification reaction used for the production of MLCT for the past 5 years. The feedstock commonly used comes from the two different types of vegetable oils, each contributed to MCFA and LCFA, respectively. It is much easier to produce MLCT using the interesterification process than esterification due to the formation of lesser amount of by-products. The source of MCFA is usually contributed by vegetable oil like MCT, palm kernel, coconut oil while the LCFA is provided by soybean oil, sunflower oil, palm oil, camellia oil and single cell oil (Korma et al. 2018; Lu et al. 2017; Zhang et al. 2020; Zou et al. 2014). Recently, ethyl linoleate was utilised to synthesis MLCT given that the by-products formed are much easier to be removed when the feedstock is in ester form (Lian et al. 2019). Usually, a higher substrate molar ratio of the vegetable oil that is rich in MCFA is preferred to increase the production yield of MLCT. Lee et al. (2015) showed that MLCT yield increased with the increase in the ratio of palm kernel oil to palm oil where the substrate ratio of 80:20 (w/w) of palm kernel oil: palm oil resulted

Table 9.3 Interesterification process used for production of MLCT for the past 5 years

Enzyme	Catalyst	Substrate		Substrate ratio	Enzyme (%)	Time (h)	Temp (°C)	Yield (%)	References
		MCFA	LCFA						
Interesterification	Thermomyces lanuginosus	MCT	Soybean oil	45:55 (MCT: SBO)	783 g	16 min	75	76.61	Zhang et al. (2020)
	Talaromyces thermophilus lipase	Tricaprylin	Ethyl linoleate	Ethyl linoleate: tricaprylin (6:1)	6	6	60	97 (LML)	Lian et al. (2019)
	Lipozyme 435	MCT	Single cell oil	1:1	8	3	90	53.75	Korma et al. (2018)
	Lipozyme 435	MCT	Soybean oil	40:60 Soybean oil: MCT	8	6	90	74.9	Lu et al. (2017)
	Thermomyces lanuginosus	PKO	Palm oil	PKO:PO (90:10 w/w)	5	7.26	50	60	Lee et al. (2015)
	Lipozyme RMIM	*Cinnamomum camphora* seed oil	Camellia oil	1:1.5	NA	3	60	55.81	Hu et al. (2018)

in 60% of MLCT as compared to the 20:80 (w/w) of palm kernel oil: palm oil that gives around 40% of MLCT (Lee et al. 2015). Also, interesterification achieves equilibrium at a shorter period. Around 8 h of reaction time is usually needed for interesterification to reach the equilibrium state as compared to esterification that usually took more than 12 h.

9.5.3 Acidolysis

Acidolysis is the most commonly used approach for the synthesis of MLCT among other methods. Table 9.4 shows the acidolysis process used for the production of MLCT for the past 5 years. Acidolysis involves the reaction between FFA in the form of MCFA (capric acid, caprylic acid and lauric acid) and vegetable oil (corn oil, mango kernel fat, pumpkin seed oil, avocado oil, canola oil and olive oil) that provides LCFA (Sneha and Jeyarani 2018; Sousa et al. 2018; Yue et al. 2019; Zhang et al. 2020). On the other note, acidolysis between FFA of LCFA like oleic acid and MCFA containing vegetable oil such as *Cinnamomum camphora* was currently be used to produce MLCT meant for the production of human milk fat substitute (Zou et al. 2014). Typically, the substrate ratio of the FFA to the triacylglycerol used for acidolysis is in the range of 2.5:1 (w/w) to 5:1 (w/w).

9.5.4 Two-Step Reactions

As the yield of structure lipids produced can be considerably low, it was believed that a two-step reaction can help to promote the synthesize MLCT with a high purity. Two-step reactions had various types of strategies. For instance, 2-MAG could be obtained and purified from a pure LCT through alcoholysis. The concentrated 2-MAG was then esterified with caprylic/capric acid to form MLCT (Adamczak 2004). However, an alternative procedure was conducted by Morales-Medina et al. (2017) to produce PUFA rich MLCT. The authors they investigated and compared the efficiency of a one-step esterification reaction, which involves a direct esterification between glyceride, PUFA and MCFA, and a two-step esterification process, where DAG was firstly prepared between glyceride and MCFA and subsequently reacted with PUFA to form MLCT. It was discovered that the later resulted in a higher MLM content, with a 72% increase in the fatty acid regiodistribution, compared to the former. While two-step reactions had yet to be researched on extensively, they a great potential to improve the yield of MLCT compared to the conventional means.

Table 9.4 Production of MLCT *via* acidolysis process for the past 5 years

	Enzyme	Substrate		Substrate ratio	Enzyme (%)	Time (h)	Temp (°C)	Yield (%)	References
		MCFA	LCFA	FFA:TAG					
Acidolysis	Novozyme 400,086	Caprylic acid	Corn oil	8:1	12	6	40	45.5 MCFA incorporated	Yue et al. (2019)
	Lipozyme IM	Capric acid	Mango kernel fat	5:1	10	24	55	49.2	Sneha and Jeyarani (2018)
	Thermomyces lanuginosa	Capric acid	Pumpkin seed	2:1	5	31	45	34	Sousa et al. (2018)
	Lipozyme RMIM	Caprylic acid	Microbial oil (rich in DHA)	3:1	8	6	55	95	Zou et al. (2020)
	Thermomyces lanuginosus	MCT Caprylic and capric	Soybean oil	45:55 (MCT: SBO)	783 g	16 min	75	76.61	Zhang et al. (2020)

9.6 Characterisation of MLCT

One of the most important analyses to determine the success of interesterification, acidolysis and esterification reaction is by quantifying the changes in the acylglycerols content or by observing the transformation of the acylglycerol profile *via* liquid chromatography or gas chromatography before and after interesterification. Lee et al. (2015) observed that MLCT triacylglycerol species increases after interesterification reaction (Lee et al. 2015). Some new peaks started to form while other existing peaks decrease after the reaction which is an indication the modification of the triacylglycerol structure. For instance, the triacylglycerol species present prior to the enzymatic reaction were LaLaLa, LaLaM, LaLaP/LaMM, LaLaO while major triacylglycerol species after reaction include LaLaLa, LaLaM, LaLaO, LaLaP/LaMM, LaMO, LaMP, MMO. Similar study also found that the major triacylglycerol species for MLCT were LaCC/CLaC, LaCO/LCL, CCO/LaCL, COO/OCO after interesterification (Hu et al. 2018).

Apart from that, interesterification can also be assessed *via* the changes of the thermal profile using differential scanning calorimeter. Melting and crystallisation behaviours of the fats and oils mainly depend on the mixture of the triacylglycerol species. Thus, changes in the triacylglycerol profile will definitely influence the melting or crystallisation profile. Nusantoro et al. (2016), in their study on the interesterification of lauric fat blends, found that there was a significant reduction in the melting point of the lauric-vegetable oil mixture along with a decrease in crystallization temperature after interesterification due to the loss of the high-melting TAG fractions upon MLCT formation. Also, analysis from the solid fat content can also provide an alternative to evaluate the changes of the acylglycerol composition after the reaction. Sneha and Jeyarani (2018) showed that the solid fat content profile of the interesterified fat produced through the acidolysis reaction between mango kernel fat and capric acid was lower than the non interesterified fat when analysed by the differential scanning calorimeter (Sneha and Jeyarani 2018). Analogously, a lower solid fat content was attributed to the reduction of high melting triacylglycerol after interesterification. However, monitoring only the changes in the melting and crystallisation profile of fats and oils has its downside where it is not able to distinguish the types of triacylglycerol formed or diminished during the reaction but merely an indirect indication of the changes in the triacylglycerol profile.

9.7 Health Implications of MCLT

9.7.1 Anti-Obesity Effect of MLCT

MLCT was developed by Nisshin Oillio Ltd. right after the successful launching of the DAG oil by Kao Corporation. DAG is a type of anti-obesity functional oil that shows a promising effect in tackling the rising incidence of obesity. Like DAG,

MLCT is able to manage obesity. Weight management of MLCT is touted to be contributed greatly by the rapid metabolism of MCFA that reduces the formation of newly synthesized triacylglycerol that subsequently lower the tendency of the triacylglycerol to be accumulated as fatty tissue in the body. Lee et al. (2018) demonstrated that mice fed with palm-based MLCT have a lower body weight gain, particularly in the perirenal, epididymal, retroperitoneal and mesenteric regions (Lee et al. 2014, 2018). A similar study also demonstrated that MLCT reduces body weight, body fat, waist circumferences, and hip circumferences in Chinese hypertriglyceridemic subjects (Xue et al. 2009; Zhang et al. 2010). However, the weight reduction is more pronounced in overweight hypertriglyceridemic subject than the normal or obese hypertriacylglyceridemic subjects. This inconsistency was caused by the difference in metabolic characterization between the individuals, resulting in variations in fat oxidation and energy expenditure. Apart from the patient's clinical background, it was shown that the weight reduction of MLCT depends on the amount of MCFA. Mice fed with MLCT having 30% of MCFA have a lesser body weight gain as compared to the mice fed with MLCT having 10% and 20% of MCFA, further indicating the dose-dependent effect of MLCT (Matsuo and Takeuchi 2004; Zhou et al. 2017). In a review by Lee et al. (2012), it was determined that at least 12% of MCFA must present in MLCT to exert its anti-obesity properties. It was then postulated that the reduction in weight gain was attributed to the β-oxidation of MCFA in the liver. This was further confirmed where a study revealed that the consumption of a liquid meal containing MLCT with 12% of MCFA increases the diet induced thermogenesis where it elevated the resting energy expenditure (Ogawa et al. 2007). A fundamental study showed that the activity of the enzyme that involves in lipid mobilisation such as cyclic adenosine monophosphate, protein kinase A, hormone-sensitive lipase, adipose triglyceride lipase was elevated whilst enzyme that responsible for *de novo* fatty acid biosynthesis like fatty acid synthase was reduced following the consumption of MLCT (Hu et al. 2018). Furthermore, there was evidence that the weight reduction effect of the MLCT could be attributed to the microbiota that controls the body weight. The ratio of *Firmicutes* to *Bacteroidete*s as well as the abundance of the *Proteobacteria* that are responsible for weight loss was lesser in mice subject that consumed MLCT (Zhou et al. 2017). Apart from reducing body fat mass, MLCT is also beneficial in improving blood profile whereby the plasma triacylglycerol and total cholesterol level, as well as hepatic triacylglycerol and total cholesterol level, was significantly lower in the subject that consumed MLCT (Hu et al. 2018). However, in the study carried out by Lee et al. (2018), consumption of MLCT demonstrated to increase the cholesterol level mainly because of the present of mytristic acid that showed to be hypercholesterolemic (Lee et al. 2018). Nevertheless, the rule of thumb to obtain both weight reduction effect without elevating the cholesterol level is to have a proper selection of fats and oils used for modification. Lastly, in another study, consumption of MLCT showed to have remarkably reduced the inflammatory markers such as tumour necrosis factor-α, interleukin-6 in mice subjects (Du et al. 2020). This would eventually aid in preventing chronic metabolic disorders, such as type 2 diabetes and coronary diseases.

9.8 Applications

The first commercially available MLCT was sold in the Japanese market by Nisshin Oillio Ltd. as a healthy version cooking oil. It is the one of the few cooking oils in the world that demonstrates to suppress visceral fat deposition besides DAG oil which was also sold in Japan. However, DAG oil has been banned from sales for the past few years due to the presence of probable carcinogenic processing contaminants, glycidol esters. Hence, the only healthy functional cooking oil left in the market that shows to exert weight suppression effect nowadays was MLCT. As a result of the increasing demand of consumer towards healthier choice product and the beneficial health attributes of MLCT, many studies were conducted to evaluate the suitability and possibility of incorporating MLCT into various food system such as deep-frying medium, shortening, and salad dressing for the preparation of reduced calorie and trans fat free food products. Recently, MLCT had shown to be suitable human milk fat substitute as a cheaper alternative to human breast milk. Thus far, it is challenging to find a commercial food product made from MLCT in the market mainly because of the price of the structured lipid that is relatively higher than the conventional fats and oils. However, with the tremendous clinical and preclinical evidences demonstrating the purported health benefits, it is wise to rethink the way forward of using MLCT as the healthier version of edible fats and oils in the future.

9.8.1 Deep Frying Medium

The initial intention to develop MLCT was to overcome the short-comings of MCT. MCT tends to foam and smoke easily when used as deep frying medium due to the high concentration of MCFA. Incorporation of LCFA managed to increase the smoke point and reduce foaming, thereby rendering it suitable for deep frying purposes. Deep fat frying is the most popular method used for food preparation. As medium of deep frying, fats and oils act to provide the fried foods with the desirable organoleptic properties (crunchiness, aroma, flavour) that is loved by everyone. Koh et al. (2011b) studied the deep-frying performance of palm-based MLCT with the addition of synthetic and natural antioxidant for 5 consecutive days. It was found that the when palm-based MLCT was added with 200 ppm of TBHQ and 1000 ppm of oleoresin sage extract, it possessed a better oxidative and thermal stability than refined bleached deodorised palm olein despite the presence of MCFA (Koh et al. 2011a, b). MLCT shows to have a lower FFA content and anisidine value as well as high induction period. Nevertheless, MLCT resulted in a higher total polar compound after frying. A high total polar compound produced is mainly attributed to the presence of MCFA. They also discovered that the potato chips fried with palm-based MLCT or palm olein show no significant different in terms of odour, taste, crispiness and overall acceptability when evaluated by the sensory panellists. Besides that, blending of MLCT oil with other vegetable oils also provide another

alternative to enhance the deep-frying performance of the MLCT. Blending increases the the smoke point of the MLCT. Furthermore, blending also serves the purpose to reduce the production cost of MLCT. Koh et al. (2008) blended the MLCT with palm olein and soybean oil prior to further evaluating their deep-frying performance. It was found that MLCT blended with soybean oil has a better frying stability than those blended with palm olein (Koh et al. 2009). It was revealed that soybean oil contains more LCFA such as oleic, linoleic acid and linolenic acid than palm olein which contributed to the higher thermal and oxidative stability.

9.8.2 Human Milk Fat Substitute

Breast milk is recognised as the optimal choice of nutrition for preterm infant during the first year of life. It provides infant with balanced nutrients and bioactive compounds that are essential for their proper growth and development. Breast milk contains around 3–5% of lipid. Approximately 50% of the infant's dietary energy is supplied by the fat component in breast milk. The breast milk fat is predominantly made from 98% of triacylglycerol with 20–25% of palmitic acid (C16:0) (Zou et al. 2016). Majority of the palmitic acid occupied the sn-2 position of the milk fat, thus giving rise to OPO configuration. It was only recently that MLCT gain tremendous interest to be used as substitute for human milk fats, particularly, the MLL species, following a study conducted to evaluate the fatty acid composition and triacylglycerol composition of the human breast milk as compared to infant formula (Yuan et al. 2019). It was found that the human breast milk fat is mainly composed of MLCT that exists in the form of MLL which is typically different from the desired MLM species that is well sought after for its weight management attributes. The types of MCFA present in human breast milk range from C8-C14 with C10 and C12 being the major fatty acid. It was also revealed that the content of MCFA increases with lactation whereby the MCFA content increases from colostrum to mature milk. Nonetheless, a thorough study by the author found that most of the infant formula available in the market are mainly made up of the MCT instead of MLCT. MCT is undesirable for infant as the sn-1, sn-2 and sn-3 position of MCT are composed entirely of MCFA. As a result, it has a higher rate of metabolism as compared to MLCT. In addition, MCT is undesirable as it will not increase the body mass of infant. This was also the concern of Łós-Rycharska et al. (2016), who reviewed that there was no explicit evidence on the improvement in the infant's body mass upon MCT consumption. It was also noted that when MCT was consumed in large doses (>400 mOsm/kg), the infant was inflicted with diarrhoea and essential fatty acid deficiencies. Thus, replacement of MCT with MLCT was needed to render the consumers with more nutritional benefits. With that in mind, several studies were conducted in China to use MLCT as a source of human milk fat substitute. In one study, catfish oil was interesterified with the coconut oil to produce MLCT with MLL species for use as human milk fat substitute. Around 39.85% of the MLL was synthesised under the optimized operating condition (Yuan et al. 2020). In another

study, acidolysis of *Cinnamomum camphora* seed oil with oleic acid was used to produce MLL structured lipid catalysed by RMIM (Zou et al. 2014). MLCT received a lot of attention to be used as human milk fat substitute recently mainly because of the food regulation in China which specifies that MLCT is needed to be claimed as human milk fat substitute. Under the Chinese national standard, the fat emulsion must consist of MLCT, MCT, and LCT content within 70–80%, 5–15% and 1–20%, respectively.

9.8.3 Shortening

Shortening is widely used for baking purposes to give flaky and crispy texture. It is commonly made up of 100% of fats and oils. Conventionally, shortening is mainly made from hydrogenated fat as hard stock. However, the use of hydrogenated fat as shortening was not in favour today because of the presence of trans fatty acid. Structured lipid turns out to be a better alternative to replace the hydrogenated fat. Apart from that, interesterified fat is able to provide the desired melting and crystallization properties for shortening that is not possible to be achieved by simple blending of the hydrogenated fat with other vegetable oils. It was found that interesterified fat enhanced the formation of the β′ and small crystal as compared to the dense crystal that is formed during physical blending. A sophisticated study was furthermore conducted to evaluate the blending of MLCT with hardstock such as palm stearin or partially hydrogenated soybean oil in order to increase the solid fat content for the preparation of shortening. Arifin et al. (2011) showed that the shortening with solid fat content of 15% to 25% at 25 °C was obtained by blending MLCT with 10–30% of palm stearin or a mixture containing 30–40% of palm stearin and 10–20% of palm olein. Madeira cake produced from the aforementioned shortening was well accepted by the sensory panellists (Arifin et al. 2011). Similar study also demonstrated that blending MLCT with 30% of palm stearin is able to produce fat with β′ crystal polymorphism which is required to produce shortening with a smooth texture. In another study, MLCT-based shortening was prepared through the blending of coconut oil with MLCT (Adhikari et al. 2012; Zhang et al. 2014). Adhikari et al. (2012) used MLCT produced through the blending of coconut oil and interesterified fat (rice bran oil and fully hydrogenated soybean oil) to prepare shortening. The MLCT blend showed to have softer texture and a lower solid fat content besides inducing the formation of β′ crystal which is desired to prevent post hardening as compared to the shortening prepared from physical blend of the rice bran and coconut oil. Nevertheless, the shortening produced through interesterified fat demonstrated to have a lower oxidative stability as most of the tocopherols and phytosterols were destroyed during the refining process that is meant to eliminate the FFA formed during interesterification. Similar study was conducted by Shi et al. (2015) to prepare a blend from interesterified fat (rice bran stearin and fully hydrogenated soybean oil) and coconut oil that is suitable for shortening production.

9.8.4 Margarine

Margarine is developed as a cheaper substitute for butter that is usually used as bakery fat. It is a type of oil-in-water emulsion that constitutes of up to at least 80% of fat. Similar to shortening, hydrogenated fat is commonly used to provide the solid like texture to margarine. However, the use of hydrogenated fat started to phase out and being replaced with trans-fat free structured lipid following the mandatory labelling of trans fatty acid in food products. Interestingly, it was revealed that the use of structured lipid has the advantages of not only able to reduce the trans-fat content but also promote the development of β′ fat crystal in margarine. Arifin et al. (2010) utilised MLCT for margarine production. MLCT prepared was blended with palm olein and palm stearin in ratio of 60:30:10 (w/w/w) and 70:20:10 (w/w/w) prior to incorporating it into cookies (Arifin et al. 2010). The cookies incorporated with MLCT-based margarine had a higher value for all the texture properties but lower sensory score than the cookies made from commercial margarine. Further improvement in the formulation of the margarine is needed to produce cookies with a better organoleptic attribute.

9.8.5 Salad Dressing

Salad dressing is an emulsified fat that is typically pourable and stabilised by egg yolk as primary emulsifier or starch as secondary stabiliser. The most common example of salad dressing is mayonnaise which has around 75% of fat. As it is considered as a high fat product, consumption of salad dressing may increase the risk of obesity. Koh et al. (2008) evaluated the feasibility of using MLCT as salad dressing. It was found that the salad dressing prepared from MLCT has similar rheological properties as those prepared from soybean oil. In order to extend the shelf life of the mayonnaise, the authors further incorporated natural and synthetic antioxidants to prevent fat hydrolysis caused by the presence of water in the salad dressing (Koh et al. 2008). Study revealed that addition of either synthetic or natural antioxidant managed to retard the degradation rate of MLCT in the salad dressing. Nevertheless, the incorporation of the natural antioxidant which is oleoresin sage extract tends to result in a stronger colour and odour than the synthetic antioxidant when evaluated by the trained panellists *via* the quantitative descriptive analysis.

9.8.6 Parenteral Nutrition

Structolipid 20% is well known intravenous lipid emulsion containing structured lipid MLCT. It was made from interestefication process between 36% soybean oil and 64% MCT. This combination allows adequate consumption of essential fatty

acids while exhibiting less inflammatory properties (Raman et al. 2017). Its clinical safety was proven by Rubin et al. (2000), who studied the differences in blood lipid profile of the patients administrated with Structolipid 20% and Intralipid 20%, a pure LCT emulsion, for 4 weeks. They assessed that there was no significant difference in the plasma TG, FFA, phospholipid and free cholesterols between the two group of subjects. The study also noted that Structolipid (20%) had the potential to treat liver dysfunction as it successfully treated 2 patients who had this disorder after the infusion of Intralipid 20%. Furthermore, a short-term study by Piper et al. (2006) showed that patients treated with Structolipid 20% had less plasma triglyceride levels compared to those treated with Lipofundin 20%, a physical emulsion blend of MCT and LCT, after 5 days of research. Hence, the structured triglyceride emulsion led to a more significant effect compared to the MCT/LCT physical blend.

9.9 Conclusion

Since the banning of the sales of DAG oil by Kao Corporation due to the presence of probable carcinogen-glycidol ester, MLCT is the only healthy cooking oil with anti-obesity effect that is sold commercially in the Japanese market currently. With the use of lipase, the production of MLCT through modification of conventional vegetable oil *via* esterification, interesterification and acidolysis route has become feasible. Study showed that MLCT offered a significant reduction in body weight gain and suppressed body fat accumulation due to the rapid metabolism of the MCFA. Nevertheless, only a few vegetable oils can contribute MCFA such as coconut oil and palm kernel oil whilst MCFA from other sources are typically low in amount, rendering the production of MLCT in large scale a challenging task. As the tropical oils contain certain amount of the myristic acid that are hypercholesterolemic, it is important to relook into the synthesis of MLCT using these vegetable oils. More works can be conducted to further improve the nutritional profile of the MLCT oil that works best not only to reduce body weight gain but also blood lipid profile.

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Chapter 10
Enzymatic Synthesis of Human Milk Fat Substitutes

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Abstract Human milk fat (HMF) is a natural complex lipid possessing a unique fatty acids composition and distribution. In order to mimic HMF, human milk fat substitutes (HMFSs) have been produced via the enzymatic or chemical modifications of natural lipids. HMFSs have attracted increasing attention as functional lipids owing to their beneficial effects on infant's growth and development. It is very difficult to prepare HMFSs that will exactly match HMF in all lipid compositions. However, it is possible to produce HMFSs by enzymatic reactions that closely resemble HMF. There are two approaches for HMFSs synthesis. The first approach is performed in one step by acidolysis of one triacylglycerol with free fatty acids or interesterification between two triacylglycerols or between one triacylglycerol and fatty acids methyl or ethyl esters. The second approach can be accomplished in two steps through hydrolysis and re-esterification with the acyl groups to be incorporated.

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10.1 Introduction (Definition, Brief History, Applications)

Human milk possessed certain features of great significance for infants. The composition of human milk displays all the nutrients in quality and quantity required, as well as provides protection against several types of allergies and infections, and stimulates the immune system. Consequently, the fatty acids composition and distribution in the triacylglycerols (TAGs) molecules are targets of studies on infant formulas. The TAGs of human milk fat (HMF) should serve as a model for the lipid constituents. This chapter aims to report the different methodologies applied for the enzymatic synthesis of structured lipids (SLs) as human milk fat substitutes (HMFSs).

HMF is considered to be one of the main constituents of breast milk for newborn, term, and preterm infants feeding. Accordingly, it delivers the highest portion of an infant's necessary dietary nutrients and energy (Long et al. 2013). In some uncommon circumstances, infants cannot be fed with natural breast milk. Hence, the significance of HMFSs synthesis arises. The profile of HMF should be simulated in order to produce HMFSs for better and improved digestion. The fatty acids steric structure is determined by the length of carbon chain and the degree of unsaturation (Dong et al. 2015), while polyunsaturated fatty acids (PUFAs) possess higher steric impediment that may prevent them from binding to the active site of lipases, especially when located at the sn-2 position. The PUFAs potential as feedstock for lipase-catalyzed HMF synthesis was not clear. Human milk is categorized by the ascendancy of TAGs (more than 98% of HMF), which contain palmitic acid (C16:0, 20–40% of total fatty acids) at the sn-2 position (70% of all palmitic acid) and unsaturated fatty acids at the sn-1,3 positions. Consequently, the previous studies on the synthesis of desirable structured TAGs focused on the production of TAGs rich in particular fatty acids at the sn-2 position. Qin et al. (2012) studied the incorporation of diverse fatty acids (C8:0-C18:2) into tripalmitin (PPP)-enriched TAGs for the production of HMFS via lipase-catalyzed reactions. The incorporation degree of the different fatty acids into the PPP-enriched TAGs through the acidolysis reaction was reported. Essential fatty acids, for instance α-linolenic acid (C18:3, ω-3) may be used as the starting material to prepare C18:3-C16:0-C18:3-style HMFS for infant formulas. A possible approach would be to mix this product with 1,3-dioleoyl-2-palmitoylglycerol (OPO) enriched fats and minor lipids based on the HMF chemical composition.

Several authors tried to produce HMFSs by using several substrates (Zou et al. 2011; Teichert and Akoh 2011; Turan et al. 2012; Li et al. 2014). The majority of these studies adopted the enzymatic reactions because of its moderate reaction conditions in order to protect the PUFAs from deterioration throughout the reaction, and to utilize the enzymes specificity as well. Zou et al. (Zou et al. 2012a, b) produced HMFSs from palm stearin through the combination of physical and enzymatic methods. In these studies, HMFSs were synthesized by two-step process specifically, acidolysis of interesterified high-melting palm stearin with rapeseed oil

Table 10.1 Fatty acids composition (wt%) of OPO-rich TAGs, HMFSs, and HMF

	OPO-rich TAGs		HMFS 1		HMFS 2		HMFS 3		HMF	
	Total	%sn-2	Total	%sn-2	Total	%sn-2	Total	%sn-2	Total	%sn-2
C8:0	–	–	1.24	3.20	1.02	3.20	1.24	3.20	–	–
C10:0	0.06	44.23	1.02	16.07	0.84	16.07	1.02	16.09	0.52–1.64	NP
C12:0	0.07	51.94	8.37	54.95	6.89	54.98	8.37	55.04	1.23–6.41	20.10–25.31
C14:0	1.33	66.62	3.31	16.58	2.73	16.55	3.31	16.62	1.20–5.29	47.08–82.32
C16:0	24.58	83.21	22.21	62.88	23.64	65.46	24.39	66.46	17.02–24.39	>70
C16:1n-7	1.67	69.11	0.04	0.00	0.04	0.00	0.03	0.00	1.12–3.47	NP
C18:0	5.68	25.47	2.17	3.19	2.00	3.14	1.82	3.30	3.89–7.37	5.70–10.71
C18:1n-9	51.47	13.08	36.73	10.56	39.06	9.64	39.48	8.79	28.50–42.37	13.00–16.80
C18:2n-6	11.79	15.16	21.72	42.67	20.67	42.49	17.47	42.70	16.58–27.29	20.09–25.31
C18:3n-3		–	2.04	31.19	2.01	31.20	1.86	31.23	0.46–2.04	16.01–32.75

HMFSs human milk fat substitutes, *HMF* human milk fat, HMFS 1, HMFS 2, and HMFS 3 were prepared by blending of OPO (100% purity), coconut oil, soybean oil, flaxseed oil, and sunflower seed oil at mass ratios of 0.4291:0.1779:0.3537:0:0.0394, 0.4600:0.1779:0.3541:0:0.008, and 0.5000:0.1780:0.3220:0.0000:0.0000, respectively; –, not detectable, *NP* not provided; *% sn-2* represents relative percentage of corresponding fatty acid located at the *sn*-2 position of the TAG and it was calculated as *%sn-2%* content of a fatty acid at the sn-2 position/(content of the fatty acid in the total fatty acid × 3) × 100. (Qin et al. 2014)

fatty acids using Lipozyme RM IM and mixing the enzymatic product with the selected oils. Table 10.1 shows the fatty acids composition of OPO-rich TAGs, HMFSs and HMF. The fatty acids content of HMFSs was in the ranges of those of the referenced HMF and a high amount (70.09%) of palmitic acid was located at the sn-2 position.

HMFSs are SLs with enhanced functional and/or pharmaceutical characteristics mimicking the composition and positional distribution of HMF fatty acids. Numerous infant formulas comprising HMFSs have been presented to the markets by certain large-scale foreign enterprises which manufacture infant foods (Jensen 1998). Abbott Laboratories in the United States in 2007 commercialized an infant formula containing HMFSs in which palm oil was not involved. The fat in "Smile" infant formula manufactured by Meiji in Japan was obtained from a HMFS produced through fractionation of lard (60% or more of the palmitic acids located at the sn-2 position) rather than palm oil, and the company stated that the product was beneficial to minerals absorption and energy in the newborns. Advanced Lipids AB in Israel prepared a HMFS which was used as the major fat source in infant formulas by

numerous Chinese dairy enterprises such as Wondersun in Heilongjiang Province (Yadong and Guicheng 2011). The structure of the fat used in the infant formulas was shown to be significantly associated with nutrients absorption in infants. Several researches on HMFSs have been conducted.

It was shown that symmetrical TAGs with long-chain PUFAs at the sn-2 position have numerous benefits in clinical nutrition and health (Ghazali et al. 1995). A symmetrical TAG was prepared in a two-step reaction. Firstly, microbial oil rich in arachidonic acid (ARA; 48.8%) was used to prepare 2-monoacylglycerol with ARA at the sn-2 position through the ethanolysis reaction with Novozym 435 as the catalyst. Then, a symmetrical TAG was prepared through the enzymatic transesterification of 2-monoacylglycerol with vinyl palmitate as the acyl donor rather than palmitic acid (Tang et al. 2015).

10.2 Enzymes Used in the Synthesis of Human Milk Fat Substitutes

There are two reaction approaches for HMFSs synthesis in general (Fig. 10.1). The first approach is performed in one step, i.e., the acidolysis of one TAG with free fatty acids either in a single or in a blend form, or the interesterification between two

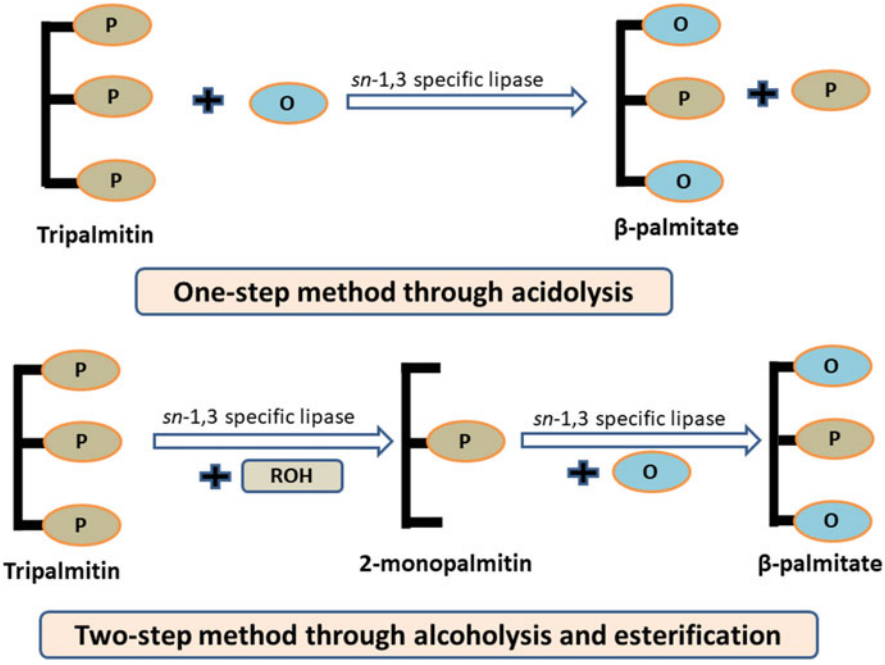

Fig. 10.1 Reaction approaches of HMFs production. *O* oleic acid, *P* palmitic acid, *ROH* (e.g. alcohol) (Zou et al. 2016a)

TAGs or between one TAG and fatty acids methyl or ethyl esters. The one-step route is simple and can be achieved without further hydrolysis processes. Betapol®, the first produced HMFS, was manufactured through the acidolysis reaction of tripalmitin-rich palm fraction with unsaturated fatty acids using sn-1,3-specific lipase as a catalyst (King and Padley 1989). HMFSs were also produced by using multiple lipases in one stage (Pande et al. 2013). It was found that in spite of the fact that enzymes had a better reusability in the two-stage (successive addition of enzyme) synthesis, one-stage (dual lipase) synthesis was faster and resulted in higher acyl groups' incorporation. The second approach can be accomplished in two steps, i.e., hydrolysis and re-esterification with the acyl groups to be incorporated. This can lead to high yield and high purity products. The ideal two-step method mainly includes the alcoholysis of one TAG with the sn-1,3-specific lipase in order to first produce 2-monoacylglycerol, followed by the esterification of the purified 2-monoacylglycerol with unsaturated fatty acids in the next step (Schmid et al. 1999). Moreover, additional factors, such as substrate type, lipase activity, lipase load and specificity, reaction time, temperature, water content, and reactor type should be taken into consideration when choosing suitable approaches for a low-cost production of high purity HMFSs (Soumanou et al. 2013; Zou et al. 2014).

Currently, lipases (TAG acyl-hydrolase; EC 3.1.1.3) have been extensively employed in the chemical restructuring of lipids aimed at improved physicochemical and/or nutritional characteristics. By using lipases, the production of 'tailor-made' SLs for particular applications in several food systems can be achieved (Mu et al. 1998; Xu 2000). Lipases are known by their ability to naturally catalyze the hydrolysis of acylglycerols groups located at the oil/water interface. They are also considered efficient facilitators for the esterification and interesterification reactions under low-water activity in non-aqueous media. Lipases have demonstrated certain advantages as compared to the conventional chemical catalysts by working under moderate conditions of atmospheric pressure and temperatures lower than 70 °C, in addition to displaying a higher selectivity (substrate, regio-, stereo-, and typo-selectivity), with the formation of smaller byproducts quantities. Furthermore, lipases are enzymes that do not require cofactors. The most vital parameters that may be taken into consideration to accomplish a lipase-catalyzed reaction for HMFSs production mainly include biocatalyst, type of reaction, composition of reaction medium, and the mode of reaction operation (Ferreira-Dias and Tecelão 2014).

Table 10.2 shows examples of lipases frequently used in HMFSs synthesis. The most commonly used enzymes for HMFSs synthesis are the commercial immobilized specific lipases, Lipozyme TL IM and Lipozyme RM IM (Irimescu et al. 2000; Qin et al. 2011; Liu et al. 2015a). Lipozyme TL IM is classified as one of the sn-1,3-specific enzymes from *Thermomyces lanuginosus* and immobilized on coarse silica by ionic adsorption. Lipozyme TL IM has been designated for use in the food industries including the synthesis of medium- and long-chain TAGs, HMFSs, trans-free or low-trans plastic fats, cocoa butter analogues, monoacylglycerols, and diacylglycerols (Zhao et al. 2014; Fernandez-Lafuente 2010; Rodrigues et al. 2013). Using a faced central composite design, the enzymatic interesterification reaction

Table 10.2 Examples of a variety of HMFSs prepared through lipases-catalyzed syntheses

Product name	Substrates	Enzymes	Reaction type	Reaction conditions	References
HMFS containing omega-3 fatty acids	Tripalmitin, hazelnut oil fatty acids, and omega-3 fatty acids concentrate	Lipozyme RM IM	Acidolysis	Substrate molar ratio, 12.4 mol/mol; enzyme (10 wt%), temperature 55 °C, shaking water bath at 200 rpm; reaction time 24 h	Sahín et al. (2006)
HMFS containing 4 types of omega-3 fatty acids	Microalgae oils from Nannochloropsis oculata and Isochrysis galbana	Novozym 435 from Candida antarctica, Lipozyme 435 from C. antarctica, Lipozyme TL IM from T. lanuginosus, and Lipozyme RM IM from R. miehei	Acidolysis	Free fatty acids/ triacylglycerols molar ratio, 3:1; temperature, 60 °C (Novozym 435 and Lipozyme TL-IM) and 50 °C (Lipozyme 435 and RM-IM); enzymes loading, 10%; reaction time 24 h	He et al. (2017)
HMFS containing stearidonic acid	Tripalmitin and free fatty acids from hazelnut oil and commercial oil mixture containing Echium oil.	Lipozyme TL IM	Acidolysis	Substrate molar ratio, 4 mol/mol; enzyme load, 10 wt% of total substrates; temperature, 60 °C; reaction time, 8 h	Yüksel and Yeşilçubuk (2012)
HMFS rich in palmitic and DHA at sn-2 position and oleic acid at sn-1,3 positions	Tuna oil and commercial oleic acid	Novozym 435 from and lipase DF from Rhizopus oryzae	Acidolysis	Free fatty acids/TAG molar ratio, 6:1; oleic acid purity, 90 mol/100 mol; intensity of treatment, 0.4 g lipase x h/g TAG (reaction time of 1 h and 0.4 g lipase/g TAGs); 10 mL hexane/g TAGs; 37 °C and 200 rpm	Robles et al. (2011)
OPO-rich HMFS	Tripalmitin and oleic acid	Candida lipolytica lipase	Acidolysis	2% water, 20 mg/mL enzyme, 1:6 tripalmitin/oleic acid, 50 °C, 2 h	Zheng et al. (2017)

HMFS	Lard and soybean fatty acids	Lipozyme RM IM	Acidolysis	Temperature 61 °C, water content 3.5%, lard: fatty acids 1/2.4 (mol/mol), Lipozyme RM IM load 13.7%, and time 1.0 h	Yang et al. (2003)
HMFs contained palmitic, oleic, stearic and linoleic acids	Tripalmitin, hazelnut oil fatty acids and stearic acids	Lipozyme RM IM	Acidolysis	For oleic acid; 65 °C and 24 h of incubation with the highest substrate molar ratio of 1:12:1.5. For stearic acid; 1:3:0.75 substrate molar ratio at 60 °C and 24 h	Sahin et al. (2005b)
OPO-rich HMFS	Palm stearin and oleic acid	Aspergillus oryzae Lipase	Acidolysis	Temperature 65 °C; 1:8 substrate molar ratio between palm stearin and oleic acid; 8% (w/w) enzyme load; 3.5% water content of the immobilized lipase; and 1.5 h reaction time.	Cai et al. (2015)
HMFS-rich in medium-chain fatty acids	Tripalmitin, hazelnut oil fatty acids and Neobee fatty acids	Lipozyme RM IM	Acidolysis	Substrate molar ratio $\geq$ 4 mol/mol and enzyme content above 19 wt % for C8:0 and C10:0; substrate molar ratio below 2.5 mol/mol for C16:0; temperature 54–56 °C for C8:0 and C10:0; 59.5 °C for C16:0. Shaking at 200 rpm	Ilyasoglu et al. (2011)
OPO-rich TAGs	34 L-leaf lard, coconut oil, soybean oil, flaxseed oil, and sunflower seed oil	Lipozyme RM IM	Acidolysis and physical blending	Evaporation temperature of 180 °C and a pressure of 6.7–7.5 Pa; blending ratio of 0.6700: 0.1638: 0.1627: 0.0035: 0.0000 for OPO-rich	Qin et al. (2014)

(continued)

Table 10.2 (continued)

Product name	Substrates	Enzymes	Reaction type	Reaction conditions	References
				TAGs/coconut oil/soybean oil/flaxseed oil/sunflower seed oil	
HMFS rich in palmitic acid	Lard and fish oil rich in DHA	Novozym 435 and Lipozyme RM IM	Ethanolysis	7 mmol lard, 3 mmol 2-P-MAG, 5.2 mmol oleic acid, 3.5 mmol linoleic acid, 10 mL hexane, 10 wt% enzyme, 550 rpm, 37 °C, 6 h	Kotani et al. (2015)
APA-HMFS	Silkworm (Bombyx mori L.) pupae oil and glycerol-3-palmitate	Lipozyme RM IM and Lipozyme TL IM	Interesterification	10% Lipozyme in n-hexane; substrates mole ratio of 1:12; temperature of 65 °C; reaction time for 48 h	Liu et al. (2015b)
HMFS	Lard and milk thistle oil	Lipozyme RM IM	Interesterification	Lard and milk thistle oil at mass ratio 6:4 and 8:2 were interesterified for 2, 4 and 6 h at the temperature of 60 °C	Bryś et al. (2017)
Caprine milk infant formula analogs	Tripalmitin with vegetable oil blends	Lipozyme RM IM	Interesterification	12 h of incubation at 55 °C with a substrate molar ratio of 1:0.4 of tripalmitin to vegetable oil blend	Maduko et al. (2007a)
HMFS rich in DHA and ARA at sn-1,3 positions and palmitic acid at sn-2 position	Hazelnut oil, DHA-single cell oil, ARA, single cell oil	Lipozyme RM IM and Novozym 435	Interesterification	3:2 ARA oil: DHA oil ratio, and 1:0.1 substrate mole ratio; 10% of total reactants; temperature 60 °C	Turan et al. (2012)
HMFS enriched with capric acid	High melting point palm stearin, high oleic sunflower oil, and tricaprin	Lipozyme TL IM	Interesterification	60 °C, 200 rpm, substrate molar ratios of 2:3, 1:2, 1:3 and 1:4, reaction time 4, 8, 12 and 24 h. Second stage	Álvarez and Akoh (2015)

				molar rations of 2.8:0.5, 2: 0.5, 1.5:0.5 and 1.2:0.5	
OPO-rich HMFS	Tripalmitin, coconut oil, soybean oil, salmon oil	Lipozyme RM IM	Interesterification	Interesterified product with vegetable oils at molar ratios of (58:20:20:2) and (70: 18:10:2); temperature, 50 °C, 200 rpm for 2 h	Djajasoepena et al. (2015)
HMFS	Palm oil, palm kernel oil, olive oil, sunflower oil, and marine oil	Lipozyme TL IM	Interesterification	Mass ratios of 4.0:3.5:1.0: 1.5:0.2; enzyme load (10 wt %); temperature 60 °C; reaction times 2, 4, 6, 8, 12 and 24 h	Karabulut et al. (2007)
HMFS	Palm stearin and fish oil	Lipozyme TL IM	Interesterification	1:1, 1:2, 1:3, 2:1 and 3:1 substrate molar ratio; temperature of 60 °C; enzyme load 10%; reaction time for 6, 12, 18 and 24 h	Ghosh et al. (2016)
HMFS enriched in omega-3 PUFAs	Tripalmitin with oleic acid or omega-3 PUFAs	Lipozyme RM IM, Lipozyme TL IM, Novozym 435, and C. parapsilosis lipase/acyltransferase	Interesterification	3.90 g tripalmitin and 2.76 g oleic acid, 3.90 g tripalmitin and 3.17 g omega-3 PUFAs, 8.9% (w/w tripalmitin) of enzyme, 60 °C, 24 h	Tecelão et al. (2010)
HMFS	Butterfat, rapeseed oil and soybean oil fatty acids	Lipozyme RM IM	Interesterification	Reactor dimension 800 mm in length and 60 mm in diameter, temperature 65 °C, water content in substrate 0.07 wt%, substrate weight ratio between butterfat and fatty acid mixture 1:2, feeding rate 500 mL/h with	Li et al. (2010)

(continued)

Table 10.2 (continued)

Product name	Substrates	Enzymes	Reaction type	Reaction conditions	References
				gradient flow reduction of 10 mL/day	
HMFS	Lard, sunflower oil, canola oil, palm kernel oil, palm oil, ARA oil from Mortierella alpine, DHA oil from Schizochytrium sp.	Lipozyme RM IM	Physical blending and interesterification	Lard: sunflower oil: canola oil: palm kernel oil: palm oil: algal oil: microbial oil = 1.00: 0.10: 0.50: 0.13: 0.12: 0.02: 0.02 residence time, 1.5 h; reaction temperature, 50 °C	Zou et al. (2016b)
SLs	High oleic sunflower oil and fully hydrogenated high oleic	Lipozyme TL IM	Enzymatic and chemical interesterification	Molar ratios 60:40, 50:50, 40:60, and 30:70; 7% (w/w) of the enzyme; reaction time 3 h at 70 °C and 300 rpm	Ribeiro et al. (2018)
SLs rich in DHA and ARA	Anhydrous milk fat, tripalmitin, DHA-single cell oil, ARA-single cell oil	Lipozyme TL IM	Physical blending and acidolysis and interesterification	0.2 (w/w) tripalmitin to anhydrous milk fat; 2:1 ARA/DHA TAGs blend to the obtained SL; enzyme 5% (w/w); water bath at 65 °C for 9 h with shaking at 200 rpm	Sproston and Akoh (2016)

between palm oil and palm kernel oil for medium- and long-chain TAGs synthesis was optimized by using Lipozyme TL IM (Lee et al. 2015). SLs were produced from capric acid and menhaden oil using two different enzymes. The yield of capric acid incorporation with Novozym 435 was higher (28.63 mol%) than that obtained with Lipozyme RM-IM (9.81 mol%) (Willett and Akoh 2018).

The heterologous *Rhizopus oryzae* lipase immobilized on different supports has been successfully used in the acidolysis reaction of oleic acid and tripalmitin at 60 °C in a solvent-free media for OPO synthesis (Tecelão et al. 2012a). Both the activity (30% of oleic acid incorporation after 6 h of reaction time) and the batch operational stability (half lifetime of 202 h) of lipase immobilized in Lewatit VP OC 1600, were comparable or even better than those obtained with commercial immobilized lipases working under similar reaction conditions (Tecelão et al. 2010). The study of Simões et al. (2014) revealed the potential of recombinant *R. oryzae* lipase, immobilized on Accurel MP 1000, as a promising non-commercial biocatalyst for HMFSs rich in docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) preparation. Compared with the frequently used commercial immobilized lipases, similar combination levels of PUFAs in lard TAGs were achieved with *R. oryzae* lipase. In another study, Carica papaya latex has been reported as a cheap promising biocatalyst for HMFSs production in a solvent-free media, offering comparable batch operational stability and activity to the values reported for commercial immobilized lipases (Tecelão et al. 2012b). *Talaromyces thermophilus* lipase was immobilized using five resins and utilized to synthesize medium- and long-chain TAGs through the interesterification of tricaprylin with ethyl linoleate. Under the optimal reaction conditions (including substrates molar ratio of ethyl linoleate to tricaprylin of 6:1, enzyme loading of 6%, temperature of 60 °C, and reaction time of 6 h), TAGs with one medium- and two long-chain fatty acid content as high as 52.86 mol% was achieved (Lian et al. 2019).

Candida lipolytica lipase is distinguished by its low price, availability, and outstanding performance. More notably, its sn-1,3 position selectivity has been demonstrated by Li et al. (2013). It was used as the biocatalyst in the synthesis of OPO-rich TAGs. Nevertheless, the free lipase is not preferred in the industrial sectors since it is easily broken and susceptible to denaturation under severe conditions of organic solvents, temperature, and pH. These shortcomings were circumvented through immobilization on solid supports, which resulted in improved catalytic stability, selectivity and reusability of lipases (Zaidan et al. 2012). The process of immobilization may modify the enzyme performance in various remarkable ways, for instance selective oxidations or hydrolysis, kinetic resolutions of racemic mixtures, or kinetically controlled synthesis (temperature and pH). However, the application of immobilized lipases on the industrial scale may be limited by their easy attrition and high cost (Palla et al. 2012).

Carbon nanotubes have been used as a matrix for lipases immobilization (Feng and Ji 2011; Verma et al. 2013; Ke et al. 2014; Marzuki et al. 2015). In this case, Verma et al. (2013) immobilized *T. lanuginosus* lipase on carbon nanotubes for ester hydrolysis. Also, Ke et al. (2014) immobilized *Burkholderia cepacia* lipase on carbon nanotubes for kinetic resolution of (R, S)-1-phenylethanol. Because of the nanometer character of carbon nanotubes, all of the immobilized lipases described

above have the common disadvantage of being troublesome during separation. To overcome this issue, a magnetic property can be imparted to the carbon nanotubes before the process of immobilization, which have the ability to allow the immobilized lipases to be easily separated from the reaction systems with an external magnetic field, without the need for centrifugation or filtration steps. The covalent linking of lipases onto the magnetic carbon nanotubes has been previously reported (Tan et al. 2012; Fan et al. 2016). However, the preparation procedure was to a certain extent complex, including the surface modification of carbon nanotubes, the preparation of magnetic carbon nanotubes and the covalent interactions between the enzyme and support, which were organic reagents- and time-consuming.

10.3 Synthesis of Various Structured Lipids as Human Milk Fat Substitutes

HMFSs are SLs mimicking the fatty acids profile of HMF. SLs are TAGs that have been enzymatically or chemically modified through the incorporation of novel fatty acids or through the rearrangement of their original fatty acid's positions. These new structures are appropriate for several applications since they demonstrate distinct characteristics, such as their thermal profile, digestion, absorption, and nutritional values. These SLs can be applied in the manufacture of several products, such as margarine, cocoa butter substitutes, modified fish oil products, emulsifiers, and HMFSs (Ratledge and Gunstone 2001). To mimic the HMF, a typical SL for infant formula should contain palmitic acid mainly esterified at the *sn*-2 position and unsaturated fatty acids at the *sn*-1,3 positions. Betapol® (Loders Croklaan, The Netherlands) was produced through the reaction of tripalmitin with oleic acid using an *sn*-1,3-specific lipase. It contained 53.5% of palmitic acid at the *sn*-2 position and 42.1% of total oleic acid (Zock et al. 1996). Following the development of Betapol, several investigations have been carried out during the last decades for the preparation of infant formula fat substitutes through enzymatic acidolysis and/or esterification reactions. Through the enzymatic acidolysis of palm stearin, Zou et al. (2014) synthesized a set of SLs rich in palmitic acid (more than 60%) at the *sn*-2 position using a commercial *sn*-1,3-specific lipase from *Rhizomucor miehei*. Also, Zou et al. (2012b) reported the synthesis of an infant formula fat substitute containing 61.6% of palmitic acid at the *sn*-2 position via lipase-catalyzed interesterification reaction between palm stearin and a mixture of free fatty acids from sunflower, palm kernel, and rapeseed oils in a continuous packed bed reactor. Other studies included the use of either tripalmitin or ethyl palmitate with stearidonic acid soybean oil (Teichert and Akoh 2011), extra virgin olive oil (Pande et al. 2013), hazelnut oil (Turan et al. 2012), and amaranth oil (Pina-Rodriguez and Akoh 2009b). In spite of the fact that higher *sn*-2 palmitic acid levels were successfully attained in Betapol and the other products, all of them exhibited lower reaction yields as a result of the nature of the materials used (ethyl esters and free fatty acids). The yield of SLs

produced for diverse applications through the acidolysis reactions usually ranges between 55 and 70 wt% (Pande et al. 2013; Pina-Rodriguez and Akoh 2009b; Ifeduba and Akoh 2013).

Compared with the different categories of nutrients, vegetable oils and animal fats are considered to be the most energy-rich food constituents, demonstrating higher caloric values (Soumanou et al. 2013). The preparation of structured TAGs using oil and fat as raw materials mainly depends on the enzymatic process for the production of the fat substitute. The synthesis of TAGs and HMFSs through enzymatic catalysis has been reported (Qin et al. 2012; Xu 2003). To change the original natural structure of oils and fats, diverse approaches can be employed, such as interesterification, total hydrogenation, and fractionation. Interesterification is considered one of the major techniques used for the modification of TAGs composition, designed for the production of low-trans or trans-free lipids with desirable texture and crystallization profile (Osborn and Akoh 2002). The formation of desirable acyl residues or esters through specific enzyme catalysis makes the modification of lipids via enzymatic methods more attractive in comparison with the chemical approaches. It was stated in several studies that interesterification via the enzymatic catalysis is more efficient with regard to selectivity and reaction control as compared to chemical catalysis (Ahmadi et al. 2008; Farfán et al. 2015). Enzymatic procedures are friendly to the environment and can be accomplished under moderate conditions, resulting in greater safety of the obtained product (Qin et al. 2012). In order to enhance enzyme efficiency, low water content organic solvents are frequently used (Serdakowski and Dordick 2008), which play an important role in the control of acyl group migration during the synthesis of a desired product (Zhao et al. 2013).

Álvarez and Akoh (2015) reported the synthesis of SLs in two stages of enzymatic interesterification as shown in Fig. 10.2. Firstly, high melting point palm stearin and high oleic sunflower oil were mixed and reacted in the presence of Lipozyme TL IM. The objective of using this enzyme was to increase the incorporation of palmitic acid at the *sn*-2 position, mainly in the form of OPO-type TAG. In the second stage, the intermediate SL previously synthesized in the first stage was mixed and reacted with tricaprin in a second interesterification reaction, aiming to incorporate capric acid into the TAG molecules while maintaining palmitic acid at the *sn*-2 position. The overall reaction yield reached 90 wt%, and the obtained SL contained 20.13 mol% of the total palmitic acid, approximately 40% of which were located at the sn-2 position. The total capric acid content was 21.22 mol%, mainly at *sn*-1,3 positions. The most abundant TAGs in the final SL were oleic-palmitic-oleic, palmitic-oleic-palmitic, and capric-linoleic-capric. The crystallization onset and melting completion temperatures of the synthesized SL were 6.1 and 27.7 °C, respectively. The obtained SL might be totally or partially used in marketable oil mixtures for infant formulas manufacture.

Several investigations have been performed using immobilized lipases as catalysts for the synthesis of HMFSs resembling HMF characteristics. Many of these studies revealed that HMFSs were synthesized through the acidolysis of tripalmitin or lard (with a high palmitic acid content) with free fatty acids from various sources (Table 10.2). Consequently, HMFSs containing palmitic, stearic, oleic, and linoleic

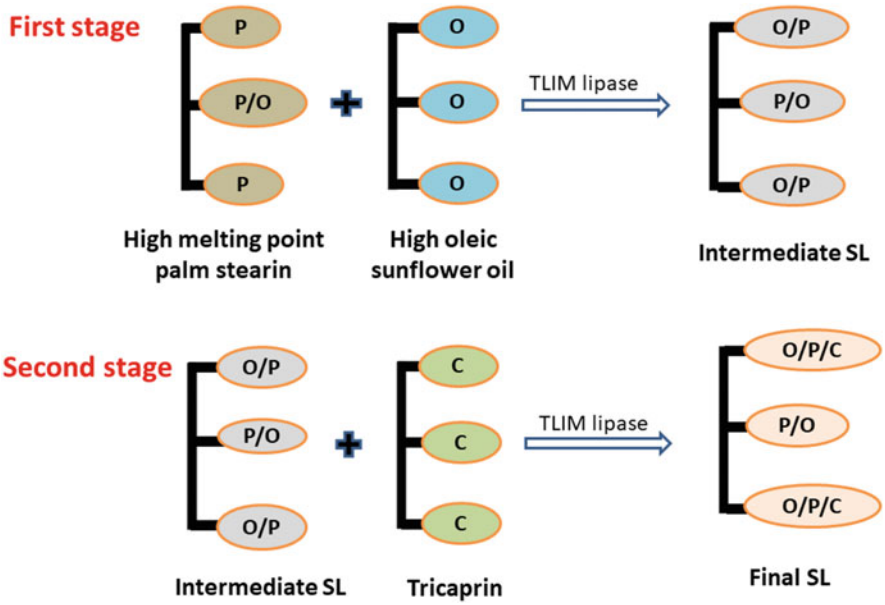

Fig. 10.2 Two-stage reaction scheme for SL synthesis. *P* palmitic acid, *O* oleic acid, *C* capric acid (Álvarez and Akoh 2015)

acids (Sahin et al. 2005b), essential and long-chain polyunsaturated fatty acids (Mukherjee and Kiewitt 1998; Nielsen et al. 2006), ɣ-linolenic acid (Sahin et al. 2005a), and omega-3 polyunsaturated fatty acids (Sahín et al. 2006), were synthesized. The inclusion of omega-3 polyunsaturated fatty acids in infant formulas, in particular DHA, has recognized benefits for nervous system and brain development of infants (Helland et al. 2003; Valenzuela 2009). HMFSs were also produced via the interesterification of tripalmitin and vegetable oils mixtures (Maduko et al. 2007a, b) or the interesterification between soybean oil and lard (Silva et al. 2009).

Using the human milk fatty acids composition as a reference, many studies have carried out the production of TAGs rich in palmitic acid at the sn-2 position or of OPO, looking for novel substrates (Pina-Rodriguez and Akoh 2009a; Sørensen et al. 2010), innovative lipases (Guncheva et al. 2008; Lee et al. 2010; Pfeffer et al. 2007; Srivastava et al. 2006) and working conditions in a solvent-free media (Wang et al. 2010). For example, Pina-Rodriguez and Akoh (2009a) reported the supplementation of amaranth oil with palmitic acid at the sn-2 position. Through the enzymatic interesterification with ethyl palmitate and Novozym 435 as a catalyst, the obtained TAGs could be used in infant formulas after substituting the palmitic acid that existed at sn-1,3 positions. Also, Lee et al. (2010) synthesized structured TAGs containing 81% of palmitic acid at the sn-2 position and 65% of oleic acid at the sn-1,3 positions via enzymatic interesterification between ethyl oleate and a tripalmitin-rich fraction with Lipozyme TL IM obtained from *T. lanuginosus*. Furthermore, in a solvent-free system, Wang et al. (2010) synthesized a HMFS by

Lipozyme RM IM-catalyzed acidolysis reaction between lard and fatty acids obtained from soybean, palm kernel, and tea seed oils. Sahín et al. (2006) produced HMFS by the enzymatic interesterification of tripalmitin, hazelnut oil fatty acids, and omega-3 polyunsaturated fatty acids concentrate from menhaden oil. Additionally, Tecelão et al. (2010) have reported the inclusion of 21.6 mol omega-3 polyunsaturated fatty acids/100 mol total fatty acids to tripalmitin by using Novozym 435.

Owing to the moderate reaction conditions, high specificity, and biocatalysts reusability features, several studies have focused on HMFSs production by the enzymatic acidolysis (Soumanou et al. 2013; Xu 2000; Yang et al. 2003). Wang et al. (2016) used an innovative miniaturized microfluidic reactor to produce HMFS by the enzymatic acidolysis of a commercial tripalmitate and free fatty acids rich in DHA and docosapentaenoic acid from Schizochytrium sp. using Lipozyme RM IM. The production of HMFS rich in stearidonic acid from Echium oil or oleic acid from hazelnut oil have been accomplished via acidolysis reaction using Lipozyme TL IM (Yüksel and Yeşilçubuk 2012). The majority of the available reports used commercial tripalmitate, and animal and vegetable oils, for instance, lard, butterfat, palm stearin, and urushi wax for the large-scale manufacture of HMFSs, due to the high concentration of palmitic acid at the *sn*-2 position (Soumanou et al. 2013; Wang et al. 2016; Yüksel and Yeşilçubuk 2012; Yang et al. 2003; Zou et al. 2016b). Nevertheless, the concentration and region-distribution of palmitic acid in the animal fat are affected by the given source of oil in forage (Lerch et al. 2015), causing unstable HMFSs quality. Furthermore, the complex and energy-intensive procedures (e.g. crystallization and dry fractionation) used for the separation and purification of urushi wax and palm stearin resulted in an expensive production cost (Zaliha et al. 2004). In order to overcome the existing issues in butterfat, lard, and palm stearin, the commercial tripalmitate appears to be an exceptional raw material for HMFSs synthesis (Wang et al. 2016; Yüksel and Yeşilçubuk 2012). Nevertheless, the commercial tripalmitate cost is substantially higher as compared to butterfat, lard, or palm stearin (Xu 2000), resulting in an increased cost. Consequently, other sustainable TAGs sources rich in palmitic acid at *sn*-2 position should be investigated in order to find a substitute for the commercial tripalmitate.

Numerous HMFSs have been developed by the enzymatic interesterification reaction with either vegetable oil or animal fat, such as cow milk fat and lard. Accordingly, the fatty acids composition and distribution in HMFS are to some extent comparable to those in HMF, and it is anticipated that they will be widely used in infant food preparation due to their potential benefits. HMFS rich in palmitic, stearic, oleic, and linoleic acids close to HMF was synthesized via the enzymatic acidolysis reaction between tripalmitin, hazelnut oil fatty acids and stearic acids. The enzymatic acidolysis reaction was catalyzed using a commercially immobilized Lipozyme RM IM obtained from *R. miehei*. It was shown that the highest incorporation of oleic acid (47.1%) and the highest oleic/palmitic ratio were achieved at 65 °C and 24 h of incubation with the highest substrate molar ratio of 1:12:1.5. The highest incorporation of stearic acids was obtained at a 1:3:0.75 substrate molar ratios at 60 °C for 24 h. The incorporation level of both stearic acid and oleic acid

increased with progress in reaction time (Sahin et al. 2005b). Qin et al. (2014) prepared a HMFS by enzymatic synthesis in combination with physical blending methods. The predominant fractions in HMFS, OPO-rich TAGs were synthesized through the Lipozyme RM IM-catalyzed acidolysis reaction of 34 L-leaf lard in one step, and molecular distillation was then applied for the purification. It was revealed that OPO-rich TAGs with a purity of 91.39 wt% were attained at a pressure of 6.7–7.5 Pa and an evaporation temperature of 180 °C.

10.4 Developed and Marketed Human Milk Fat Substitutes Products

The HMFS (also called *sn*-2 palmitate) product was developed in the 1990s by Loders Croklaan, and was later commercialized as Betapol® in 1995 (Happe and Gambelli 2015). The product was synthesized by lipase-catalyzed (*R. miehei*) acidolysis of palm stearin with oleic acid. The commercial production of Betapol® was conducted in a two-stage reaction process (Xu 2000). The product was produced in two packed bed reactors filled with the enzyme to increase the fatty acid conversion (Ferreira and Tonetto 2017). Currently, there are several commercial HMFSs being produced. In 2019, Bunge Loders Croklaan (BLC) published the next generation in Europe, the Betapol® Plus, which offers a "60% OPO or sn-2 palmitate level". In Fat® contains 70–75% of palmitic acid esterified at the *sn*-2 position of the glycerol backbone and is manufactured by Advanced Lipids (Sweden, Karlshamn). In the Chinese market, HMFS products are being synthesized by Wilmar International (Singapore) and Zhejiang Beijia Biotechnology (Hangzhou, China). Other commercial HMFS products include the Bonamil (Wyeth Ayerst), Alsoy (Nestlé), and Cow & Gate Premium (Nutricia). For the detailed information on the commercial HMFS products, the reader is referred to the following valuable reviews and chapters: Xu (2000), Happe and Gambelli (2015), and Ferreira and Tonetto (2017).

10.5 Conclusion and Future Prospects

In recent twenty years, our understanding of HMF and the development of HMFSs have improved. The universal aim to develop infant formula that mimics human milk as much as possible continues to fuel more research and will remain so in the near future. The application of HMFS products in infant formula has been accepted by many manufacturers and is becoming legal in some countries. Infant formulas supplemented with HMFS have significantly higher OPO content compared to typical infant formula (Sun et al. 2018a, b). However, there are still significant differences in TAGs composition of human milk and infant formula prepared with HMFS. In preparing HMFS, the health benefits of PUFAs such as DHA, ARA, and

others should not be overlooked and they need to be included in the TAG and/or phospholipid forms. It is very difficult to produce HMFS that will exactly match HMF in all lipid compositions. However, it is possible to produce HMFS by enzymatic reactions that closely resemble HMF. In some cases, it may be prudent to blend produced HMFS with each other or with other fats and oils to formulate/prepare infant formula. A major challenge in the production of HMFSs is its high price. The exploration of novel oil ingredients, screening for cheaper and stable *sn*-1,3 regioselective lipases and very recent engineering of stereoisomeric structure of seed oil (Van Erp et al. 2019) may be promising technologies in the future study. The HMFS used in food for special medical purposes, for example, of infant with special fat absorption requirements, such as preterm infant formula, short bowel syndrome and atopic disorders should be investigated (Wei et al. 2019). As for all new developments, conclusive evidence regarding the nutritional effects and safety of HMFSs should be obtained from clinical trials prior to introduction in new infant formula products.

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Chapter 11
Cocoa Butter Alternatives for Food Applications

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Abstract Cocoa butter is the main ingredient for chocolate and chocolate related confectionery products. Because of the high price, high demand and limited supply, cocoa butter alternatives are becoming important. Different vegetable fats are used for cocoa butter alternative preparation by using blending, fractionation, hydrogenation and interesterification techniques. Depending on their compatibility with cocoa butter, cocoa butter alternatives have been classified into three categories namely cocoa butter equivalent, cocoa butter replacer and cocoa butter substitute. Fatty acid and triacylglycerol profile are important chemical properties that can impact on various physical properties such as melting and crystallization behavior. Depending on temperatures and storage duration, different fat polymorphic forms are formed. Cocoa butter alternatives have been recommended for various usages like chocolate, confectionery filling, coatings and other confectionery products based on their unique physical and chemical properties.

Keywords Cocoa butter alternative · Cocoa butter equivalent · Cocoa butter replacer · Cocoa butter substitute · Chocolate and confectionery · Fat bloom

11.1 Why Cocoa Butter Alternatives Are Needed?

Cocoa butter (CB) is the main ingredient for chocolate products. This is mentioned as 'Food of God' which is extracted from the fermented cocoa beans (Jahurul et al. 2013). But it is becoming costly and less available gradually. According to International Cocoa Organization (ICCO), the cocoa market review in September 2019 showed that the price for cocoa rose by 18% per tonne in London and 14% per tonne in New York compared to the initial trading of the same month. One of the main reasons of the price shoot up was the spreading of Cocoa Swollen Shoot Virus

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Diseases in Ghana and the weather condition in Côte d'Ivoire being wetter than before. These countries are the two top producers of cocoa globally (International Cocoa Organization 2019). To make the problem worse, ICCO projection for 2018/19 also shows that the production rate will go down by 0.6% in both Asia and America. Although this rate was expected to increase by 1% in Africa (International Cocoa Organization 2019), it will not be enough to mitigate the scarcity. So, cocoa industry will suffer from shortage of supply for the upcoming year due to increasing demand and subsequently raises the price of cocoa. In the face of this troublesome situation, chocolate and confectionery industries are looking for alternatives to have sustainable demand and supply ration (Kadivar et al. 2016b).

As the continuation of this search, different fats and oils have been used as cocoa butter alternatives (CBAs). These have been categorized according to their physical, chemical properties and their compatibilities with CB. Based on the physical and chemical properties, their usage in food application varies.

11.2 Cocoa Butter Alternative

Apart from providing economic benefits, CBA offers some technological advantages in the chocolate and confectionery industry such as low temperature resistance in tropical climates, resistance to fat bloom, tempering issue, reduced cracking etc. (Beckett et al. 2017). These two reasons are prompting the industries in putting ongoing and intensive efforts to look for CBAs in chocolate production. CBAs are produced from various vegetable fats. The compositional difference among CBAs is depicted in Table 11.1.

CBAs are classified as below:

11.2.1 Cocoa Butter Equivalent (CBEs)

CBEs are lauric fats and compatible with CB in higher ratios. The physical and chemical properties are similar with CB. There are two sub-divisions of CBEs.

- Cocoa Butter Extenders (CBXs): These are helpful in extending or diluting CB but not compatible in high ratios.
- Cocoa Butter Improvers (CBIs): It contains higher amount of StOSt (St = stearic acid, O = oleic acid) so the hardness is higher with higher level of solid fat content (SFC). It is useful for the coating of confectionery items.

Table 11.1 Fatty acid composition of CBE, CBR and CBS

Fatty acids (%)	Designation	CB	CBE	CBR	CBS
Caprylic acid and Capric acid	C8:0 and C10:0	NM	NM	NM	5.79 ± 0.06
Lauric acid	C12:0	NM	NM	0.63 ± 0.007	40.06 ± 0.21
Myristic acid	C14:0	0.08	0.45 ± 0.273	1.09 ± 0.007	13.06 ± 0.035
Palmitic acid	C16:0	25.09 ± 0.042	37.21 ± 15.12	52.87 ± 0.084	13.0 ± 0.084
Stearic acid	C18:0	35.36 ± 0.042	25.02 ± 14.81	6.26 ± 0.007	26.68 ± 0.083
Oleic acid	C18:1 trans	NM	0.35 ± 0.55	4.22 ± 0.007	NM
Oleic acid	C18:1	33.96 ± 0.014	33.29 ± 1.15	32.30 ± 0.098	0.44 ± 0.162
Linoleic acid	C18:2 trans	NM	2.63 ± 1.00	0.24 ± 0.014	NM
Linoleic and Linolenic acid	C18:2 and C18:3	3.15 ± 0.008	NM	1.3 ± 0.0035	0.14 ± 0.06
Arachidonic acid	C20:0	1.23 ± 0.0	0.88 ± 0.44	0.52 ± 0.0	0.29 ± 0
Behenic acid and Lignoceric acid	C22:0 and C24:0	0.32 ± 0.001	NM	0.57 ± 0.007	NM

CB Cocoa butter, *CBE* Cocoa butter equivalent, *CBR* Cocoa butter replacer, *CBS* Cocoa butter substitute, *NM* not mentioned; Source: Modified from Lipp et al. (2001) and Quast et al. (2013)

11.2.2 Cocoa Butter Replacers (CBRs)

These are non-lauric fats with similar profile as CB but have different triacylglycerols (TAGs) level than that of CB. CBRs are not compatible at high ratios and if these are produced from hydrogenation then it will produce trans-fat that is bad for health. However, CBR, which are produced by partial hydrogenation and fractionation, has fair mouthfeel, snap and good gloss, flavor and oxidative stability (Hassim and Dian 2017). CBRs are used for coating on ice cream, other frozen desserts, cakes, bakery items etc. and are particularly useful in the making of these kinds of products (Beckett et al. 2017).

11.2.3 Cocoa Butter Substitute (CBS)

These are mainly lauric based fats which have limited compatibility with CB. The physical properties are similar but the chemical properties are different compared to CB (Mokbul 2014).

11.3 Compositional Variation of CB with CBE, CBR and CBS

All CBAs are not fully compatible with CB. This is because of their compositional variation compared to CB. CBAs contain different fatty acids (FA) and TAG profile than CB (Table 11.1).

11.4 Usage of Different Fats as Alternatives

According to EU regulation 2000/36/EC, six vegetable oils have been allowed for usage at 5% replacement as CBE. Other oils are not permitted to be used as alternatives (European Parliament and Council 2000). In different studies, researchers used different oils for preparing CBE, CBR and CBS. In Table 11.2, sources, functionality, FAs and TAGs content of different CBAs are listed.

The six vegetable fats that can be used as CBA are palm, shea, kokum, mango, sal and illipe butter (European Parliament and Council 2000). Palm kernel oil, coconut oil or babassu are the main oils which have been mentioned to be used for CBS production (Limbardo et al. 2017; Naeem et al. 2019). CBS contains high amount of lauric fats, and this does not need to follow the tempering process. There are a few suggested processing techniques for CBS, such as hydrogenation, fractionation and interesterification (Oracz et al. 2015). Blending and interesterification techniques are

Table 11.2 Chemical composition, source and functionality of CBAs

CBA	Source of fats	Functionality	FAs	TAGs
CBE	Palm oil, sal, shea, mango fat, illipe and kokum	Non lauric fat, compatible with CB as physicochemical properties are similar to CB	Palmitic, stearic and oleic acid	POP, POS, SOS
CBR	Soybean oil, palm olein, cotton oil, sunflower oil, rapeseed oil and ground nut oil	Non-lauric fat, similar physical properties with different chemical properties and partially compatible	Elaidic and/or oleic, palmitic, stearic acid	PEE, SEE
CBS	Coconut oil and palm kernel oil,	Lauric fats, possible to substitute 100% fat	Lauric and Myristic acid	LLM, LLL, LMM

Source: Adapted from Lipp and Anklam (1998)

mostly used for making CBEs (Wang et al. 2006; Mohamed 2013). Among these techniques, addition of hydrogen in the unsaturated fatty acid helps to increase the saturated fats and make the melting point higher (Hassim et al. 2018). However, hydrogenation has not been well accepted because it adversely affects cholesterol level by producing higher amount of trans fats and medium chain fatty acid during the partial hydrogenation (Lonchampt and Hartel 2004). Interesterification process is beneficial in changing the glycerol backbone and rearranging the FAs into desirable positions. This provides an advantage in modifying the characteristics according to the necessity (Hassim et al. 2018). Oil combinations and processing techniques used to produce CBAs by different researchers are tabulated in Table 11.3.

11.5 Methods of Analysis

Different analytical methods have been used for analyzing the chemical and physical properties of vegetable fats (Table 11.4).

11.6 Properties and Compatibility of CBAs

11.6.1 Fatty Acid and Triacylglycerol Profile

CBE and CBR are non-lauric based fats whereas CBS is lauric based fat. The FA content of the vegetable oils may vary depending on the region and maturity level of the component. The chemical composition of CBE is similar to CB but that varies for CBR and CBS (Lipp and Anklam 1998). The TAG profile is different for CBR compared to CB. Sometimes it may contain trans-fat because of the use of hydrogenation process during fat modification (Reddy et al. 1990). The main two recommended oils are palm kernel oil and coconut oil for CBS production as these

Table 11.3 Production process to prepare CBE, CBR and CBS from different fats

Samples	Production process with substituted amount	References
CBE		
MSF + PMF	Blending (100/0, 90/10, 80/20, 70/30, 60/40, 50/50 and 0/100 (%wt))	Kaphueakngam et al. (2009)
HOSO and FA mixture	Interesterification by Lipozyme RM IM	Kadivar et al. (2013)
Refined PMF and palmitic–stearic FA	Lipase-catalyzed acidolysis	Mohamed (2013)
PMF and stearic acid	Enzymatic interesterification (Novo lipase Lipozyme™)	Undurraga et al. (2001)
Tea seed oil, methyl palmitate and methyl stearate	Interesterification (Lipase enzyme)	Wang et al. (2006)
Pentadesma butyracea butter with ethyl palmitate	Transesterification	Tchobo et al. (2009)
PO + methyl stearate	Interesterification (Carica papaya lipase)	Pinyaphong and Phutrakul (2009)
FPS + SS	Blending	Kang et al. (2013)
IB + PMF	Enzymatic interesterification	Bahari and Akoh (2018a)
Mango fat + PMF	Blending (100:0, 60:40, 50:50, 40:60, 0:100)	Momeny et al. (2013b)
CBR		
MSF + PS	Blending (90/10, 85/15, 80/20 and 75/25)	Jahurul et al. (2014a)
FPKO + PO	Blending	Zaidul et al. (2007)
CBS		
MKF	Substitution with CB (60% maximum)	Naeem et al. (2019)
CO	Substitution with CB (4.5% maximum)	Halim et al. (2019)
IGF/DPO	Enzymatic interesterification (90/10: IGF/DPO); Lipozyme IM TM	Yamoneka et al. (2018)
PMF/PKO/PS	Blending (14.9/59.6/25.5) (%w/w)	Biswas et al. (2017a)
CO + PO	Substitution with CB (60% for each by wt)	Limbardo et al. (2017)
HPKS	Commercially produced	Lillah et al. (2017)
PMF/PO/OO	Blending (66.7:16.7:16.7)	Ramli et al. (2013)
MKF	Enzymatic interesterification (40/60: MKF/CB); Lipozyme IM TM	Momeny et al. (2013a)
VCO	Substitution with CB (up to 5%)	Indarti et al. (2013)
PMF/RBO	Interesterification (75/25: PMF/RBO); Novozyme® 435	Saidin and Ramli (2010)
HPKO, HPKS	Commercially produced	Wang et al. (2010)
PKO	Two step fractionations (4 h, 22 °C and 19 °C)	Calliauw et al. (2005)

(continued)

Table 11.3 (continued)

Samples	Production process with substituted amount	References
HCO/OO	Enzymatic interesterification then fractionation; Mucor miehei	Chang et al. (1990)

CB Cocoa butter, *CBE* Cocoa butter equivalent, *CBR* Cocoa butter replacer, *CBS* Cocoa butter substitute, *MSF* mango seed fat, *PMF* palm mid-fraction, *FA* fatty acid, *HOSO* high oleic sunflower oil, *FPS* fractionated palm stearin, *SS* Shea stearin, *IB* Illipe butter, *FPKO* fractionated palm kernel oil, *CO* coconut oil, *PO* palm oil, *PKO* palm kernel oil, *OO* olive oil, *MKF* mango kernel fat, *PS* palm stearin, *RBD-PO* re-fined, bleached, and deodorized palm oil, *VCO* virgin coconut oil, *RBO* rice bran oil, *HPKO* hydrogenated palm kernel olein, *HPKS* hydrogenated palm kernel stearin, *IGF Irvingia gabonensis* seed fat, *DPO Dacryodes edulis* pulp oil, *HCO* hydrogenated cottonseed oil

Table 11.4 Analytical techniques used to determine chemical and physical properties of fats

Properties	Analysis	References
Chemical		
Fatty acid	Gas chromatography (GC)	Bahari and Akoh (2018a)
Triacylglycerols	High performance liquid chromatography (HPLC)	Dionisi et al. (2004)
Physical		
Polymorphism	X-Ray diffraction (XRD)	Danthine et al. (2015) and Biswas et al. (2018)
Melting profile	Differential scanning calorimetry (DSC)	Danthine et al. (2015) and Biswas et al. (2018)
Crystallization	DSC, polarized light microscopy (PLM)	Danthine et al. (2015) and Biswas et al. (2018)
Rheology	Rheometer	Bahari and Akoh (2018b)
Solid fat content	Pulsed nuclear magnetic resonance (p-NMR)	Danthine et al. (2015), Kadivar et al. (2016a) and Biswas et al. (2018)
Hardness	Texture analyzer	Bahari and Akoh (2018b)
Fat bloom	Scanning electron microscope (SEM), low vacuum scanning electron microscope (LV-SEM), 3D-laser scanning confocal microscopy, environmental scanning electron microscope (ESEM), Cryo-SEM	Rousseau and Smith (2008), Dahlenborg (2014) and Ashida et al. (2020)
Sensory properties	Hedonic scale	Biswas et al. (2017b)

contain higher level of lauric acids (Lonchampt and Hartel 2004). Previously different researchers made CBE, CBR and CBS from different vegetable fats and their FA and TAG profile are given in Tables 11.5 and 11.6.

Table 11.5 Fatty acid profile of CBE, CBR and CBS

Fatty acids (%)	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	References
CB (Malaysian)	NM	NM	NM	0.1	25.3	37.6	32.1	2.9	0.2	Sabariah et al. (1998)
CBE										
CBE (IB/PMF)	NM	NM	0.1	0.4	25.9	33.0	32.5	5.1	NM	Bahari and Akoh (2018a)
(FPS/SS): CB (30:70)	NM	NM	NM	0.2	24.9	37.4	33.2	2.7	1.2	Kang et al. (2013)
MKF/PMF (80/20)	NM	NM	0.08	0.26	16.3	37.2	39.6	3.9	0.36	Kaphueakngam et al. (2009) and Sonwai et al. (2012)
CBE 95 (SHS 95: PMF)	NM	NM	NM	NM	4.4	54.8	33.0	NM	NM	Bootello et al. (2012)
CBE 80 (SHS 80: PMF)	NM	NM	NM	NM	6.0	48.9	39.0	NM	NM	
CBE (HOSO): CB (5:95)	NM	NM	NM	NM	28.0	34.2	33.8	3.0	NM	Kadivar et al. (2016b)
CBE (HSHO): CB (5:95)	NM	NM	NM	NM	28.1	34.3	33.6	3.0	NM	
CBE (PO: Methyl stearate (1:4))	NM	NM	NM	NM	26.9	36.8	30.4	6.0	NM	Pinyaphong and Phutrakul (2009)
CBE (Tea tree oil + Methyl palmitate + Methyl stearate)	NM	NM	NM	NM	31.4	35.3	29.26	NM	NM	Wang et al. (2006)
CBE (Sal/Phulwara butter)	NM	NM	NM	NM	16	45.6	32.2	NM	NM	Reddy and Prabhakar (1989)
CBR										
MSF/PS (75/25)			0.06	0.46	23.45	30.59	37.89	5.27	0.43	Jahurul et al. (2014a)
CBS										
80% ternary blend (PMF/RBDPKO/RBDPS) + 15% stearic acid + 5% oleic acid	NM	0.2	8.52	1.13	24.81	32.33	29.10	3.91	NM	Biswas et al. (2016)
PMF/VCO/CO (90:5:5)	NM	NM	NM	1.5	50.6	5.2	33.0	4.4	NM	Mizan et al. (2016)
IGF	NM	2.5	40	50	3.5	0.9	1	1.1	0.2	Yamoneka et al. (2018)
DPO	NM	NM	NM	0.25	46.73	2.59	19.48	29.41	1.31	
(PMF/PO/OO) 16.7:66.7:16.7	NM	NM	NM	0.4	52.0	4.1	35.1	6.4	NM	Ramli et al. (2013)

CBS	5.79	40.06	13.60	13.00	26.68	0.44	0.14	NM	NM	Quast et al. (2013)
PKO	4.8	3.8	48	16.5	8.2	2.6	15.5	2.7	NM	Lonchampt and Hartel (2004)
CO	7.5	5.7	47	18.5	8.7	3	7.5	1.7	NM	Lonchampt and Hartel (2004)
HSO	NM	NM	NM	NM	16.1	83.9	NM	NM	NM	Abigor et al. (2003)
RBD-PO/HSO (1.6:1)	NM	NM	NM	0.9	32.2	38.5	23.8	2.3	NM	Abigor et al. (2003)
CBS (commercial)	2.3	2.9	54.6	20.7	9.2	8.7	NM	NM	NM	Sabariah et al. (1998)
AMF	1.2	2.6	3.3	11.5	31.2	11.4	19.9	1.1	1.0	Sabariah et al. (1998)

CB Cocoa butter, *CBE* Cocoa butter equivalent, *CBR* Cocoa butter replacer, *CBS* Cocoa butter substitute, *IB* Illipe butter, *PMF* palm mid-fraction, *FPS* fractionated palm stearin, *SS* Sal stearin, *MKF* mango kernel fat, *SHS* sunflower hard stearin, *HOSO* high oleic sunflower oil, *HSHO* high stearic high oleic sunflower oil, *PO* palm oil, *MSF* mango seed fat, *PS* palm stearin, *RBDPKO* re-fined, bleached, and deodorized palm kernel oil, *RBDPS* re-fined, bleached, and deodorized palm stearin, *VCO* virgin coconut oil, *IGF Irvingia gabonensis* seed fat, *DPO Dacryodes edulis* pulp oil, *PKO* palm kernel oil, *OO* Olive oil, *CO* coconut oil, *RBD-PO* re-fined, bleached, and deodorized palm oil, *HSO* hydrogenated soybean oil, *AMF* anhydrous milk fat, *NM* not mentioned

Table 11.6 TAG profile of different fats used as CBE, CBR and CBS in comparison to CB

TAG composition (%)	POP	POS	SOS	References
CB	17.55	43.61	29.62	Halim et al. (2019)
CBE				
IB/PMF	18.3	41.6	29.8	Bahari and Akoh (2018a)
CBE (HOSO): CB (5:95)	18.9	47.7	26.6	Kadivar et al. (2016a)
CBE (HSHO): CB (5:95)	19.6	49.4	26.9	
Hard PMF: CaO (3:1)	30.33	17.53	3.26	Mutia et al. (2015)
OO/Palmitic and stearic acid (1:3)	11.7	12.3	7.7	Mohamed (2014)
PMF and palmitic–stearic FA mixture	30.7	40.1	14.5	Mohamed (2013)
(FPS/SS; 70/30)	19.2	31.9	33.4	Kang et al. (2013)
CBE 95 (SHS 95: PMF)	0.3	8.1	68.5	Bootello et al. (2012)
CBE 80 (SHS 80: PMF)	0.4	8.8	55.5	
MKF/PMF (80/20)	12.7	6.7	26.9	Sonwai et al. (2012)
PMF/Stearic acid	23.4	38.5	20.2	Undurraga et al. (2001)
Purified PO/Tristearin	18	36.3	13.4	Liu et al. (1997)
CBR				
MSF/PS	8.6–17.7	12.6–19.6	37.2–31.4	Jahurul et al. (2014c)
CBS				
4.5% CNO + CB	13.54	32.38	21.65	Wang et al. (2006)
HPKO	5.4	2.4	1.6	Wang et al. (2010)
HPKS	0.8	NM	NM	
RBD-PO/HSO (1.6:1)	11	39	23	Abigor et al. (2003)
80% ternary blend (PMF/RBDPKO/RBDPS) + 15% stearic acid + 5% oleic acid	18	38.9	29.2	Biswas et al. (2018)

CB Cocoa butter, *CBE* Cocoa butter equivalent, *CBR* Cocoa butter replacer, *CBS* Cocoa butter substitute, *MKF* mango kernel fat, *IB* Illipe butter, *PMF* palm mid-fraction, *HOSO* high oleic sunflower oil, *HSHO* high stearic high oleic sunflower oil, *CNO* Coconut oil, *PO* palm oil, *CaO* Canola Oil, *OO* Olive oil, *FA* fatty acid, *MSF* mango seed fat, *PS* palm stearin, *FPS* fractionated palm stearin, *SS* Sal stearin, *SHS* sunflower hard stearin, *HPKO* hydrogenated palm kernel olein, *HPKS* hydrogenated palm kernel stearin, *RBD-PO* refined, bleached and deodorized palm oil, *HSO* hydrogenated soybean oil, *RBDPKO* refined, bleached and deodorized palm kernel oil, *RBDPS* refined, bleached and deodorized palm stearin, *NM* not mentioned

11.6.2 Physical Properties of CBAs

11.6.2.1 Melting Profile

Melting temperature is closely related to the FA composition of fats and oils. Saturated FA with higher molecular weight has higher melting point compared to saturated FAs with lower molecular weight. On the other hand, the melting point also varies between saturated and unsaturated FA molecules. Unsaturated FA has lower melting temperature than the saturated ones. For example, lauric acid's melting point is 44.8 °C whereas stearic acid and oleic acid have melting point of 70.1 °C and 16 °C, respectively. The temperature difference is significant for the saturated and unsaturated FAs although their carbon number is the same (Talbot 2015). CBE has similar FA profile as CB, so the melting profile is not so different compared to CB chocolate (29.49–36.6 °C). By physical blending or interesterification of different vegetable oils combination, researchers are able to prepare equivalents that have similar range of melting profile like CB. It is also worth to mention that with higher level of stearic acid, the melting profile range can be higher than the melting point of CB (Sonwai et al. 2012; Jin et al. 2018). Bahari and Akoh (2018b) produced CBE from illipe butter and palm stearin and analyzed the melting profile for dark chocolate and milk chocolate. The melting profile did not differ significantly from CB chocolate. Whereas white illipe butter chocolate showed the highest melting point (onset) because of higher level of stearic acid content (Bahari and Akoh 2018b). CBS are rich in lauric FA, so their melting temperature is lower than CB.

11.6.2.2 Polymorphism

Polymorphism is the property of any substance which allows it to crystallize in more than one crystalline form. This characteristic helps to describe the behavior of a fat in multiple melting points (De Clercq 2011). TAGs are binding in double-chain packing or triple-chain packing system. Most of the oils have this characteristic as they contain different TAGs. Usually DSC and powder XRD are used to identify the polymorphic forms (Marangoni and McGauley 2003).

Mainly three polymorphic forms have been mentioned on a gross classification by their level of stability: α, β′ and β (Himawan et al. 2006). Initially, it has been reported that CB belongs to six polymorphic forms but later van Malssen et al. (1999) reported that III and IV polymorphs do not exist. β′ has been reported as a phase range for the replacement of III and IV forms. But V and VI polymorphs were reported as separate identities (van Malssen et al. 1999). Timms (2003) made similar conclusion for V and VI polymorphs as the stable polymorphs by using X-Ray diffractions (Timms 2003). β′ polymorphs can be formed from liquid state whereas βV and βVI cannot be produced directly from liquid state. These two will be formed through the transformation of β′ state (van Malssen et al. 1999).

Most of the CBEs can form β polymorph similar to CB whereas CBS can form β′ polymorphs. They cannot go up to βV or βVI polymorphs. Polymorphism of CBAs is presented in Table 11.7.

11.6.2.3 Rheology

Chocolate rheology is complex as it is a combination of solid and liquid portion. Its solid compounds are sugar, cocoa with or without milk solids, where the liquid portion is cocoa butter. As a single component, cocoa butter flows like Newtonian fluid. At any flow rate, it will show single viscosity. But as chocolate with the solid and liquid portions, it behaves like non-Newtonian fluid. In case of non-Newtonian fluid, viscosity mostly depends on shear rate (Beckett et al. 2017). Chocolate viscosity is related to the composition, particle size distribution (PSD) and processing parameters (De Clercq et al. 2017). Afoakwa et al. (2008) mentioned about PSD, fat and lechitin content in chocolate which can influence on chocolate rheology (Afoakwa et al. 2008). Higher PSD value leads to higher viscosity or yield stress as the area for interaction becomes lower (Aidoo et al. 2014). Among other models, casson model has been widely used by the researchers to analyze the casson yield stress and casson plastic viscosity. Another study reported about higher crystalline mass as well as quicker transition from small to large crystals. They observed this characteristics especially at low crystallization temperatures, low cooling rates and under shear for chocolate (Bahari and Akoh 2018b; Ramel et al. 2018).

Three different crystallization types can be explained by oscillatory rheology. These are primary crystallization, microstructure crystallization and macroscopic crystallization (De Graef et al. 2008). In 2016, Kavidar et al. produced CBE from high oleic sunflower oil and high stearic high oleic sunflower oils. In both cases of 100% and 50% replacement for compound chocolates, crystallization was faster than CB. This may be described from their higher level of tri-saturate TAGs composition, which leads to the formation of more α network. Other replacements for CBEs (5% and 25%) did not take significantly longer time than CB for microstructure crystal network development (Kadivar et al. 2016a). Similar finding was reported by Sonwai et al. (2012). For 5% replacement of CB with CBE (MKF/PMF), crystallization was similar in which it involves two steps crystallization (Sonwai et al. 2012).

Biswas et al. (2017b) produced CBS with palm-based oil (PMF/RBDPKO/RBDPS). They fitted their data in casson model and concluded that 5–20% replacement of CBS with CB showed similar flow behavior for dark chocolate. The plastic viscosity was similar for 5% and 20% replacement of CB with CBS but the yield stress was slightly lower for 20% CBS replacement (Biswas et al. 2017b). Shamsudin et al. (2006) worked with three CBSs with different FA compositions to analyze their viscosity at 40–60 °C. They concluded that viscosity of CBS increment was directly related to the percentage of long chain saturated FA increment rather than the increment of the overall amount of saturated FA (Shamsudin et al. 2006).

Table 11.7 Different polymorphs associated with CBE, CBR and CBS

Oil combination	Modification process	Polymorph	References
CBE			
IB/PMF (10:3)	Interesterification	β polymorphism	Bahari and Akoh (2018a)
MKF/PMF (80:20)	Blending	Spherulites and densely packed	Sonwai et al. (2012)
Mango butter emulsion gel	Hot emulsification method	β polymorphism	Sagiri et al. (2014)
CBE 95 (SHS 95: PMF) and CBE 80 (SHS 80: PMF)	Solvent fractionation and blending	β polymorphism	Bootello et al. (2013)
HSHO sunflower oil stearin	NM	β′2 and/or β′1 polymorphism	Rincón-Cardona et al. (2013)
Malaysian MSO: PMF (50:50)	Blending	β polymorphism	Momeny et al. (2013b)
CBR			
MSF/PMF (85–70/15–30)	Blending	Crystals formed like CB	Jahurul et al. (2014b)
CBS			
(PMF/RBDPKO/RBDPS)	Interesterification	β′ polymorphism	Biswas et al. (2018)
IGF/DPO (9:1)	Blending and intersterification	β′ polymorphism	Yamoneka et al. (2018)
CB/CBS (80:20) CBS 100	Blending	β polymorphism β′ polymorphism	Da Silva et al. (2017)
CBS (PKO): CB (30–10:70–90) CBS 100	Chemical interesterification and blending	βV polymorphism β′ polymorphism	Quast et al. (2013)
HPKO/CB/MF (80:10:10)	Blending (Ternary)	β′ polymorphism	Wang et al. (2011)
HPKS/CB/MF (80:10:10)	Blending (Ternary)	β′ polymorphism (strong) β polymorphism (weak)	
HPKO and goat milk fat	Interesterification	β′ polymorphism	Ramli et al. (2005)
RT Chocolate SL chocolate	NM	β + β′ polymorphism β > β′ polymorphism	Osborn and Akoh (2002)
CBS/AMF/MCB (1:1:1) CBS/HMF/MCB (1:1:1) CBS/MCB (1:3)	Blending (Ternary) Blending (Binary)	β > β′ polymorphism β + β′ polymorphism β >>> β′ polymorphism	Sabariah et al. (1998)

CB Cocoa butter, *CBE* Cocoa butter equivalent, *CBR* Cocoa butter replacer, *CBS* Cocoa butter substitute, *IB* Illipe butter, *PMF* palm mid-fraction, *MKF* mango kernel fat, *SHS* sunflower hard stearin, *MSO* mango seed oil, *MSF* mango seed fat, *RBDPKO* refined, bleached and deodorized palm kernel oil, *RBDPS* refined, bleached and deodorized palm stearin, *HPKO* hydrogenated palm kernel olein, *HPKS* hydrogenated palm kernel stearin, *MF* milk fat, *GMF* goat milk fat, *IGF Irvingia gabonensis* fat, *DPO Dacryodes edulis* pulp oil, *PKO* palm kernel oil, *RT* randomized tallow, *SL* structured lipid, *MCB* Malaysian cocoa butter, *HMF* high melting fraction of milk fat, *AMF* anhydrous milk fat, *NM* not mentioned

Zzaman et al. (2014) studied rambutan fat to investigate the possibility of using it as a CBS with CB and they applied Newton, Bingham and Casson models for rheology analysis. They concluded that in all the three models, with the replacement of CB with rambutan fat as a CBS, the rheology has been changed significantly. It has also been mentioned that rambutan fat's viscosity was higher compared to CB. This is explainable by the chain length of FA and TAG composition of CB (Zzaman et al. 2014).

11.6.2.4 Hardness

The hardness of the chocolate is significantly affected by the increment of temperature. This happens because at higher temperature the solid fat content (SFC) decreases with temperature. It can also be influenced by the chocolate composition, its processing and tempering process. Particle size distribution, fat composition and lecithin content are correlated with the textural properties of the chocolate (Afoakwa et al. 2008; Torbica et al. 2016).

Addition of CBE lowered the hardness of chocolate compared to CB chocolate. The overall hardness increased with the increment of pre-crystallization temperature. CBI showed higher level of hardness in comparison with CBE (Torbica et al. 2016). CBE prepared by interesterification of illipe and PMF demonstrated similar hardness as CB (Bahari and Akoh 2018b). However, CBE prepared from 5% replacement of high oleic sunflower oil and high stearic sunflower oil showed similar CB like hardness up to 2 weeks. With the progression of time, the hardness lowered significantly for CBE chocolates. It may be because of the higher content of SOO in CBEs, which could impact in lowering the hardness level. On the other hand, CB chocolate continued with post-crystallization during long time storage (Kadivar et al. 2016b). In addition, coatings made with fractionated hydrogenated palm kernel oil demonstrated higher level of hardness compared to the fractionated palm kernel oil. It also showed similar hardness when replaced with CB by 2.5% (Williams et al. 1997). Filled chocolate made with PMF and desiccated coconut oil had been observed for 8 weeks and it was reported that at 18 °C storage temperature, hardness was not varying much from CB chocolate whereas for 30 °C storage the hardness was significantly lower than that of CB chocolate. With increasing temperature, oil migration of PMF increased and it softened the chocolate (Ali et al. 2001). Maheshwari and Reddy (2005) recommended kokum fat as CBI for warm climate. The hardness of chocolate increased with the replacement of kokum fat in chocolate at 30 °C. That effect was higher for milk chocolate compared to dark chocolate (Maheshwari and Reddy 2005). Tran et al. (2015) reported 10% replacement of Indian mango fat with CB showed similar hardness as CB chocolate (Tran et al. 2015).

Lillah et al. (2017) reported that CBS prepared by hydrogenated palm kernel stearin had an effect on the hardness of chocolate. Maximum hardness was observed with the addition of 80 mL/L of CBS emulsion at 36 °C and 40 °C. At 28 °C, all the

chocolates were very firm and brittle. With the increase of temperature to 32 °C, the hardness of dark chocolate decreased (Lillah et al. 2017).

In another study for CBS made from HPKO and HPKS, the authors concluded that with the addition of milk fat, hardness decreased because of the dilution effect. With the addition of 5%, 10% and 15% CB with CBS (HPKO), the hardness increased but the effect had not been pronounced with higher level of CB replacement. In contrast, with the addition of CB/CBS (HPKS), the hardness decreased due to the eutectic effect of the mixture. The combination of CB and milk fat with CBS had softening effect due to the united influence of dilution and eutectic effect (Wang et al. 2010).

Limbardo et al. (2017) worked with palm oil and coconut oil substitution separately with CB. They recommended up to 60% replacement of CB by these two oils as this replacement does not significantly vary the hardness and melting properties. They tested all the samples within 20–27 °C. For the first cycle which had been done at lower temperature, the hardness was higher as crystals could be formulated. On the second cycle (at 27 °C), the hardness was lowered because of the melting characteristics of the crystals at higher temperature. As palm oil and coconut oil both contain higher amount of unsaturated FA content, temperature increment resulted in the breakdown of the strong crystallization structures, leading to reduction of hardness. Coconut oil has higher amount of lauric fatty acids, which has the property of fast crystalizing at lower temperature and its change of state at ambient temperature is also fast. It melts quickly above room temperature. Therefore, the hardness decreased with the higher replacement of oils with CB (Limbardo et al. 2017).

Up to 5% replacement of CBS with CB can have significant difference in SFC content. SFC content is directly related to the hardness of chocolate compounds. The SFC content varies in a broader range (10.50 to 51.80%) at 25 °C. With more replacement of CB with CBS, the chemical incompatibilities increase, which also raises the eutectic effect. This makes the fat softer than before replacement (Quast et al. 2013; Tran et al. 2015). However, StOSt (St = stearic acid, O = oleic acid) rich fats show improved hardness characteristics compared to POP (P = palmitic acid, O = oleic acid) rich ones. Naeem et al. (2019) reported hardness characteristics for compound chocolate with mango fat substitution with CB and they found that hardness level started to rise after 60 days of storage (Naeem et al. 2019).

In another study, beef tallow was enzymatically modified to produce two CBS. Randomized tallow (RT) was prepared by mixing beef tallow with *Candida antarctica* (SP 435) lipase. Modification was also made by acidolysis reaction using *Rhizomucor miehei* (IM 60) lipase, beef tallow and stearic acid structured lipid (SL). RT had been substituted with CB and after analyzing its hardness, it was concluded that RT substitution with CB did not shift hardness level significantly. However, when the other combination was used to replace CB, it significantly softened the compound. This is because the SL contained higher level of unsaturated FA and this resulted in the eutectic softening effect (Osborn and Akoh 2002). Virgin coconut oil (VCO) was used as CBS in chocolate for up to 5% of replacement. 4% and 5% replacement of VCO were softer compared to 100% CB chocolate (Indarti et al. 2013).

11.6.2.5 Fat Bloom

This is the condition when chocolate surface loses its gloss with the appearance of small dots or large white spots. It can be uniform or marble like. Different aspects like storage temperature, SFC profile, TAG composition, degree of saturation and unsaturation, improper processing condition and composition of different oils can initiate or influence bloom formation in chocolates and compounds. Fat bloom happens in compound chocolate because of incompatible fat combination and/or storage bloom with/without phase transition of crystals. When two fats are not compatible, they try to separate themselves and that leads to lower SFC levels compared to their individual SFC levels. In the case of compatible fats with different levels of melting points, the dilution effect happens and the SFC lowering effect is proportional with the amount of added lower-melting point fat (Lohman and Hartel 1994; Lonchampt and Hartel 2004). In a recent study, Ashida et al. (2020) observed pre-bloom formation in CB non tempered chocolate by using 3D-laser scanning confocal microscopy. This technique helped to identify the pre-bloom stage of the chocolates when the bloom cannot be identified by any whitish haze spots (Ashida et al. 2020). Temperature can affect directly on bloom formation. It has been reported that 18 °C is better for chocolate storage compared to 30 °C. This is because at 30 °C, bloom formation started within 1 week of storage (Ali et al. 2001).

Torbica et al. (2014) added commercial CBE at three different concentrations (3, 5 and 7%) and analyzed the fat bloom properties. They reported that proper tempering at three different temperatures (25, 27 and 29 °C) for pre-crystallization influenced positively on fat bloom resistance (Torbica et al. 2014). Similar conclusion was observed for the MKF/PMF (80/20) CBE blend with CB chocolate and after 6 months of observation for fat bloom, the CBE blended with CB chocolate did not show significant difference with CB (Sonwai et al. 2012). CBE made with illipe butter and palm oil by interesterification process was also studied for bloom formation analysis and they showed similar properties as CB (Bahari and Akoh 2018b). Tran et al. (2015) recommended CBE from Indian mango fat/CB (10/90) blends as suitable for fat bloom retardation due to oil migration under non-tropical condition (Tran et al. 2015).

As mentioned previously, coconut oil and PKO has mostly been used as CBS and they have simple polymorphic behavior as β′ (Timms 1984). Rossell (1985) stated that fat bloom is related to the transformation of β′ to β polymorph. Lauric fat has simple type of polymorphism state as β′ where transformation plays a minor role (Rossell 1985). Tempering is not needed for the hydrogenated CBS fractions. Fast cooling to the temperature at 10–12 °C can settle β′ crystals (double chain packed) whereas CB crystalizes in βV (triple chain packed). Co-crystallization between CB and CBS will be in weak incorporation with trisaturated FAs and long chain mono unsaturated FAs (Lonchampt and Hartel 2004). As the compatibility of CB and CBS is very low, the addition of 4% of CB in coatings can cause bloom formation in a few months. When the addition is nearly 10% then the bloom formation will be faster, that is within a week (Laustsen 1991). Addition of hydrogenated PKO (up to

40–60 mL/L) as CBS emulsion in compound chocolate was reported to produce no chocolate bloom. But 80 mL/L addition of CBS can cause bloom formation (Lillah et al. 2017). Osborn and Akoh (2002) reported that randomized tallow and structured lipid chocolate compounds can have better bloom stability than dark chocolates. They mentioned the complex crystalline structure and high melting fats as possible reasons for this bloom stability (Osborn and Akoh 2002). Williams et al. (1997) analyzed compound coatings with fractionated and hydrogenated palm kernel oil (FHPKO), fractionated palm kernel oil (FPKO) and milk fat. They concluded that FHPKO bloomed faster compared to FPKO and milk fat addition facilitated the bloom compared to dark chocolate. Addition of more CB with milk fat enhanced the bloom rate (Williams et al. 1997). Biswas et al. (2017b) reported that in order to prevent bloom formation, compound chocolates made with CBS (PMF/RBDPKO/RBDPS ternary blends) can be stored at 24 °C. It is applicable for up to 20% fat replacement with CB. As a reason of bloom inhibition, they also mentioned about the complex formation of crystalline structure of the compounds as well as medium and high melting TAG profile of the fats (Biswas et al. 2017b). Similar statement was given by Halim et al. (2019) for coconut oil used as CBS in chocolate products. They mentioned replacement of coconut oil up to 4.5% can delay bloom formation compared to control chocolate. They mentioned that fast cooling of CBS and the formation of small and uniform β′ crystals may help to reduce the bloom formation rate (Halim et al. 2019). Wang et al. (2010) mentioned about the eutectic effect and dilution effect for incompatible fat phase separation in bloom formation. They reported that addition of CB/milk fat (MF) enhanced bloom formation compared to CBS compound chocolate whereas CB/MF/CBS (hydrogenated palm kernel olein and hydrogenated palm kernel stearin) mixture delayed bloom formation (Wang et al. 2010).

11.6.3 Sensory Properties

Snap, gloss, melting properties in mouth, fingerprint resistance and flavor were accounted as overall quality attributes for chocolate sensory evaluation by Maheshwari and Reddy (2005). They reported that sensory properties did not vary significantly when CB was replaced with 5% of kokum fat for milk and dark chocolates. But it showed higher fingerprint resistance compared to CB chocolate. That could be the reason of higher SFC content at 32.5 °C (Maheshwari and Reddy 2005). Similar findings were mentioned by Torbica et al. (2016) for commercial CBE chocolate up to 5% replacement. They analyzed chocolate structure, appearance, odor, taste and chewiness for overall quality assessment. It was mentioned, in order to achieve the similar quality for CBI replacement (by 5% and 7%), pre-crystallization is needed to be done at 27 °C (Torbica et al. 2016). Higher storage temperature (30 °C) is significantly less preferable for storage compared to the lower temperature (18 °C) for sensory evaluation of chocolate made with CBE (PMF and desiccated coconut oil). However, coconut can impart nice flavor along with overall

acceptability compared to CB based chocolate at 18 °C (Ali et al. 2001). Vítová et al. (2009) prepared three different chocolates namely plain chocolate with CB, chocolate for cooking with 5% addition of vegetable fat to replace CB and chocolate glaze which was full replacement of vegetable fats with CB. After comparing their sensory properties, they reported no significant difference between plain chocolate and chocolate for cooking with 5% addition of vegetable fat, which was similar to previous reports (Torbica et al. 2016; Biswas et al. 2017b). A 100% replacement of the vegetable fats, however, was not acceptable compared to the plain chocolate prepared with CB (Vítová et al. 2009).

Afoakwa et al. (2008) stated that a good quality chocolate should have a glossy appearance with light to deep brown color. Substitution of coconut oil in dark chocolate can affect color quality (Afoakwa et al. 2008). In another study, 4% and 5% substitution of VCO with dark chocolate had glossier appearance than the 100% dark chocolate (Indarti et al. 2013). However, with the substitution of mango fat in chocolate, significant color variation was not reported by Naeem et al. (2019).

Snap property is directly related to the tempering process during chocolate preparation. This helps to produce βV crystals that gives the chocolate a good snap property. For CBS, usually β′ polymorph forms. Due to the polymorphism, coconut oil substituted chocolate did not give as good snap property as dark chocolate. Coconut oil substituted chocolate had more acceptability in comparison to dark chocolate in most cases (Halim et al. 2019).

The overall acceptability of palm oil-based CBS was analyzed by Biswas et al. in 2017. They revealed that with 5% replacement the overall acceptability did not vary significantly whereas with 20% replacement the panel can detect the difference in the blend (Biswas et al. 2017b). Lillah et al. (2017) reported CBS emulsion used in chocolate compounds and found that overall acceptability increased up to 60% when CBS is replaced with CB in compound chocolate. At 80% replacement, the overall acceptability went downward but the value was still higher than the 100% CB chocolate (Lillah et al. 2017).

11.7 Application of CBAs

Table 11.8 shows the food applications of CBAs.

11.8 Regulation of CBA Usage

In EU Directive 2000/36/EC, it is allowed to add up to 5% of vegetable fats in replacement of CB in chocolate, which is applicable for all the member states of EU. The United States does not allow other vegetable fats in chocolate, but it is permitted for chocolate coating and compounds. In most countries, the addition level

Table 11.8 Application of CBAs in various products in replacement of CB

Products	CBA	Components	References
Chocolate	CBE	Palm mid-fraction	De Clercq et al. (2017)
	CBS	Mango seed fat	Naeem et al. (2019)
	CBS	Palm mid-fraction/palm kernel oil/palm stearin	Biswas et al. (2017b)
	CBR (Dark chocolate)	Milk fat fractions	Bystrom and Hartel (1994)
	CBS	Coconut oil, palm oil	Limbardo et al. (2017)
	CBS	Coconut oil	Halim et al. (2019)
	CBS	Mango seed fat	Momeny et al. (2013a)
	CBS	Rambutan fat	Zzaman et al. (2014)
	CBS	Virgin coconut oil	Indarti et al. (2013)
Chocolate for tropical countries	CBR	Palm stearin: Mango seed fat	Jahurul et al. (2014d)
	CBI	Kokum fat	Maheshwari and Reddy (2005)
	CBEx	Mahua: Kokum fat	Jeyarani and Reddy (1999)
	CBS	Hydrogenated palm kernel stearin	Lillah et al. (2017)
	CB like fat	Mango fat	Tran et al. (2015)
Coating	Compound coating	CB, palm kernel oil and anhydrous milk fat	Williams et al. (1997)
	CBE	CBE with sunflower oil, palm oil	Smith (2015)
	CBS	Palm kernel oil, coconut oil	Talbot (2009)
Confectionery filling	CBS	Palm mid-fraction/palm kernel oil/palm stearin	Biswas et al. (2018)
Confectionery products	CBE	Mango seed oil: Palm mid-fraction	Momeny et al. (2013b)
	CBS	Mango seed fat	Naeem et al. (2019)
	CBS	Palm mid-fraction/palm kernel oil/palm stearin	Biswas et al. (2018)
	CBEx	Shea (*Shorea robusta*) fat and Phulwara (*Madhuca butyracea*) butter	Reddy and Prabhakar (1989)
	CB modification	Fully hydrogenated palm oil (FHPO), fully hydrogenated cottonseed oil (FHCO), fully hydrogenated soybean oil (FHSO), fully hydrogenated crambe oil (FHCrO)	Ribeiro et al. (2013)
	CBR	Refined olive pomace oil (ROPO)	Ciftçi et al. (2009)

(continued)

Table 11.8 (continued)

Products	CBA	Components	References
	CBS	Rambutan fat	Issara et al. (2014) and Zzaman et al. (2014)
	CBS	Mango seed fat	Momeny et al. (2013a)
	CBS	Palm mid fraction: Palm oil: Olive oil	Ramli et al. (2013)
	CBS	Coconut oil, palm kernel oil	Talbot (2015)

CB Cocoa butter, *CBE* Cocoa butter equivalent, *CBR* Cocoa butter replacer, *CBS* Cocoa butter substitute, *CBEx* Cocoa butter extender, *CBI* Cocoa butter improver

of CBE in chocolate is higher than 5% and these are supposed to be declared as compounds (European Parliament and Council 2000). According to Malaysian Food Act 1983, chocolate must not contain more than 5% of vegetable fats or milk fat other than cocoa butter. Chocolate coating for biscuits or other similar confectionery products should contain minimum 12% of cocoa paste (Regulations 2015).

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Chapter 12
Oleogel: Production and Application

Sheah Yee Ghan, Lee Fong Siow, Chin Ping Tan, Kok Whye Cheong, and Yin Yin Thoo

Abstract In recent years, consumer awareness on their dietary intakes, especially in relation to solid fat, saturated fat and trans fatty acid contents has increased. Together with technological innovations, both scientists and industries have been looking for alternative delivery systems to improve both nutritional and quality characteristics of food products. Oleogel is defined as a semisolid delivery system consisting of a lipophilic liquid and a solid oleogelator mixture. Different edible oleogels can be tailored using different types of oleogelators and techniques to produce the required structural, mechanical and thermal characteristics. In the food industry, oleogels can be used (1) to minimize oil migration in multicomponent food products such as chocolates, (2) as fat replacer, to reduce the usage of hydrogenated fats and trans fatty acids in foods, (3) to enhance the oxidative stability of edible oils, and (4) delivery of bioactive compounds. Owing to its wide range of applications and versatility in the food industry, oleogel is foreseen as an important technological advancement to produce affordable healthy food products.

Keywords Oleogel · Oleogelators · Fat replacer · Oil migration inhibitor · Oil structuring · Delivery bioactive compounds

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12.1 Introduction

Oleogels are semisolid formulations which consist of apolar solvent as the liquid phase and oleogelator as the solid component. Oleogelator molecules undergo physical or chemical transformation to form fibrous structures which overlap and entangle with each other into a supramolecular three-dimensional (3D) architecture. Meanwhile, the apolar solvent is adapted within the spaces available in the 3D networked structure which is used to immobilise the flow of the solvent phase (Sahoo et al. 2011). The structuring networks form the structures *via* a combination of covalent, electrostatic, van der Waals or hydrogen bonding interactions, leading to formation of stable gel network (Fasolin et al. 2018).

Oleogels are thermodynamically stable below their gelation temperature, thermoreversible and possesses viscoelastic properties (Fasolin et al. 2018; Ogutcu et al. 2015). When oleogels are heated above their melting temperatures, the physical interactions among the oleogelator molecules are interrupted by the thermal energy. This causes the oleogels to lose their solid matrix-like structures and begin to flow like fluid. However, once the oleogels are subsequently cooled down, the physical interactions among the oleogelators prevail and the oleogels will reform to their semi-solid matrix (Sahoo et al. 2011). On the other hand, viscoelasticity is referred to oleogels having both viscous and elastic properties. At lower shear rate, oleogels behave like a solid and show elastic property. As the shear rate increases, the physical interactions among the oleogelator molecules are disrupted by the shear stress and the oleogels begin to flow.

Oleogels are initially developed as alternatives to partially hydrogenated fats in foods, with the aim to reduce the trans-fat content in food production. It is well known that excessive consumption of trans fatty acids poses a chronic threat to human health by increasing the risk of cardiovascular disease, systemic inflammation and type II diabetes (Da Pieve et al. 2011; Siraj et al. 2015). The detrimental effects of trans fatty acids has prompted the US Food and Drug Administration (FDA) to urge food companies to remove partially hydrogenated oils from the processed food, with a compliance period of 3 years, ending on June 18, 2018. In view of this, structuring edible oils has received considerable attention to formulate food products, as a replacement to hardstock fats without changing the physical and sensory of the food (Da Pieve et al. 2010; Co and Marangoni 2012; Rogers et al. 2017).

In recent years, oleogels are being studied extensively for use in medicine, pharmaceutical and food industries. This is owing to the advantages of easy preparation method, low production cost in which the formulation of oleogels require very small number of ingredients and have long-term stability (Jose and Gopalan 2018). Important parameters such as type of oleogelators and mechanism of the oleogelling must be considered prior to the oleogel development.

12.2 Types of Oleogelators

A wide variety of oleogelators have been discovered such as fatty acids, fatty alcohols, lecithins, phytosterols, monoacylglycerols, diacylglycerols, triacylglycerols, sorbitan monostearates, waxes and wax esters (Fasolin et al. 2018; Pernetti et al. 2007). These oleogelators are well known for their abilities to immobilize large volumes of liquid, following their self-assembly into a variety of aggregates such as rods, tubules and fibers (Garg et al. 2016). The oleogelation ability of oleogelator is reported to be dependent on their insoluble (hydrophilic head) and soluble (hydrophobic tails) portions. Hydrophilic head is responsible to induce crystal and network formation whereas the hydrophobic tail interacts with the oil moieties (Co and Marangoni 2012; Patel 2017).

Oleogelators can be categorized into two groups according to their molecular weight namely, polymers and low molecular weight (LMW) oleogelators (Lupi et al. 2018). Polymeric gels are based on the cross-linked network of their molecules established by covalent interactions; while for LMW gels, non-covalent such as hydrogen bondings and van der Waals interactions are responsible for the oleogelators assembly (Fasolin et al. 2018). For food applications, the oleogelators used must be of food grade, easily available and inexpensive. LMW oleogelators are currently considered to be the most popular option for application in food systems due to their versatility and compatibility (Lupi et al. 2018). Although wax-based oleogels provide the desired characteristics of food products but the added waxes are only approved as indirect additives (Patel and Dewettinck 2016). Thus, attempts to identify suitable food grade oleogelator is needed.

The use of food grade oleogelators is an emerging strategy and holds significant promise in the area of food and nutrition. To date, there is no oleogel products in the market yet. The major challenge on the use of oleogels in food application is to identify suitable food grade oleogelator in complex composite food products (i.e., multiphasic foods). Table 12.1 shows the list of oleogelators used in food and nutraceutical applications.

12.2.1 Mono Component

Oleogel systems can be classified into mono component or mixed components, as determined by the number of oleogelators used in the oleogel preparation (Bin Sintang et al. 2017). Among all the oleogelators tabulated, soy lecithin (SL), sorbitan (SMS) and glyceryl monostearate (GMS) have been widely used to immobilize vegetable oils. They are generally recognized as safe (possess “GRAS” status) by FDA (Cui et al. 2019). SL, SMS and GMS are able to work alone in structuring oil and produce oleogels which could be used as fat replacers and delivery systems. Due to the difference in the chemical structures of the head groups, glycerol-based oleogelators that have linear structure were reported to have stronger interactions

Table 12.1 List of oleogelators used in food and nutraceutical applications

Class	Applications	Reference
Polymeric oleogelators		
Ethyl cellulose	Fat replacers for fresh meat product	Gómez-Estaca et al. (2019)
Low molecular weight oleogelators		
Lecithin	Delivery systems for bioactive agents	Raut et al. (2012)
Sorbitan monostearate	Delivery systems for bioactive ingredients	Lupi et al. (2018)
Glyceryl monostearate	Delivery systems for bioactive ingredients	Cui et al. (2019)
Beeswax	Fat replacers for fresh meat product	Gómez-Estaca et al. (2019)
Rice bran wax	Fat replacers in ice cream	Zulim Botega et al. (2013)
Shellac wax	Fat replacers in spreads, chocolate and cakes	Patel et al. (2014)
Myverol	Delivery systems for bioactive ingredients	Ojeda-serna et al. (2019)
12-hydroxystearic acid	Delivery systems for bioactive ingredients	Iwanaga et al. (2010)
β-sitosterol / γ-oryzanol	Migration barrier in chocolate products	Wendt et al. (2017)

among each other, resulting in harder texture than sorbitan-based molecules with bulky sorbitan head groups (Fasolin et al. 2018; Trujillo-Ramírez et al. 2019). Despite the wide applications of lecithin oleogels, the major challenge on the preparation of lecithin oleogel remain unresolved. The purity of lecithin plays a key role in determining the success of oleogelling process. This in turn increases the cost of manufacturing.

12.2.2 Mixed Components

A mixture of two or more oleogelators was reported to exhibit synergism and resulting in a more stable structure than mono component gels (Garg et al. 2016). For mixed components gel, its properties can be tuned by altering the relative proportions of individual components, to provide superior structuring over the use of pure component. Some of such combinations currently being studied for their oil structuring properties are fatty acids and fatty alcohols, lecithin and sucrose ester, lecithin and sitosterol, and phytosterols and oryzanol (Wendt et al. 2017; Han et al. 2014; Dona et al. 2017; Truong et al. 2019). Although mixed component oleogels have been designed, more work needs to be done to understand the synergistic

interaction between the oleogelators and to exploit the full potential of structuring edible oils *via* oleogelation.

12.3 Mechanism of Oleogelation

Two common mechanisms used for the formation of oleogels are solid fiber and fluid-filled fiber mechanism. The main difference between these two mechanisms is the absence or presence of polar solvents (water) which is involved in oleogelation. In solid fiber mechanism, oleogel is formed by heating the mixture of oleogelators and apolar solvent at the temperature above their melting point (Garg et al. 2016). This is to ensure that the oleogelators are fully solubilized in the solvent prior to the mixing and cooling down to allow precipitation of oleogelators (Fig. 12.1). During the cooling process, there is a reduction in the gelator-solvent interactions, resulting in self-assembly of oleogelator molecules that attributes to the formation of a 3D network which immobilize the apolar solvent to produce oleogels (Jose and Gopalan 2018). Tube inversion method is used to confirm the formation of an oleogel, in which the oleogel refuses to flow when the tube is inverted.

In fluid-filled fiber mechanism, oleogelators formed reverse micelle and randomly dispersed in the apolar solvent. Upon addition of a polar solvent (e.g., water), the oleogelators continue to grow into tubular reverse micelle structures (Fig. 12.2). The presence of water acts as a bridge between two adjacent oleogelator

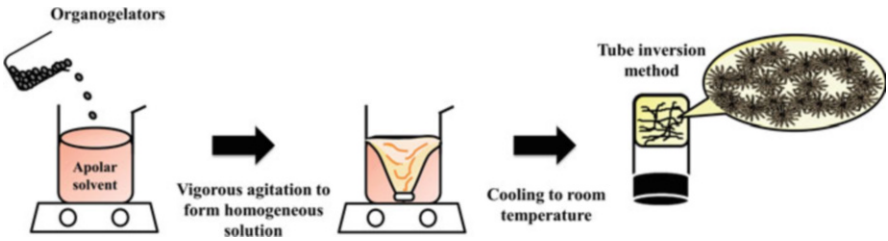

Fig. 12.1 Formation of oleogels by solid fiber mechanism

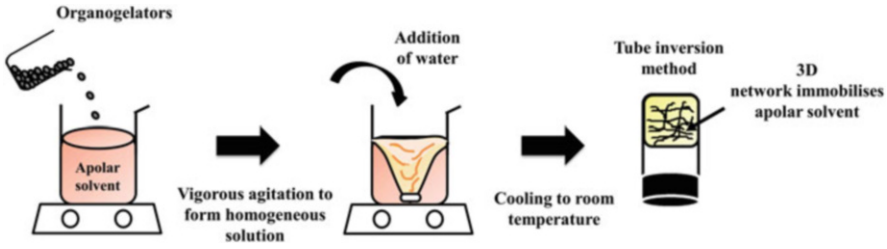

Fig. 12.2 Formation of oleogels by fluid-filled fiber mechanism

molecules by binding stoichiometrically to the hydrophilic head of the oleogelator molecules and form a linear network through hydrogen bonds (Garg et al. 2016). Further addition of water causes the oleogelator molecules to elongate, overlap and entangle with each other to a form a 3D gel network. The addition of water causes the oleogel to have well-balanced hydrophilic and lipophilic characters that allows it to be a potential bioactive compounds delivery vehicle (Sagiri et al. 2014). This was observed in the study by Yu et al. (2012), in which curcuminoids, that have poor water solubility, was successfully loaded into the oleogel and showed high bioaccessibility.

In short, for solid fiber mechanism, oleogels are produced by precipitation of the oleogelators in an apolar solvent with fluid-filled resulting from the entrapment of the water within the gelator structures. Solid fiber technology is simple, comparatively inexpensive and produces more stable oleogels as compared to the fluid-filled fiber mechanism. This is due to the highly ordered structures present in the solid fiber oleogels as compared to the simple chain entanglements in the fluid-filled fiber oleogels (Sahoo et al. 2011). However, the amphiphilic properties of fluid-filled fiber oleogels allow them to deliver a wide range of bioactive compounds.

12.4 Characterization of Oleogels

A variety of characterization studies on oleogels have been attempted by various researchers. These studies are crucial to provide comprehensive information on the properties of oleogels. Different properties, such as structural, mechanical and thermal properties, are required for various applications. These properties are influenced by the composition of oleogels. Therefore, it is important to understand the role of each of the component and how they influence the physicochemical properties of the oleogels (Fasolin et al. 2018). Factors affecting the oleogels properties include the concentration, molecular weight and structure of oleogelators used, type of organic solvents, etc. Table 12.2 describes the list of factors and their effects. These factors are key determinant factors to the physicochemical properties of oleogels.

12.4.1 Structural Properties

Various techniques are used to define oleogel structures, this includes microscopy analysis, Fourier transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD) study. The microscopy analysis is achieved using different types of microscopes such as polarized light microscopy (PLM), phase contrast microscopy (PCM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Zulim Botega et al. 2013; Yu et al. 2012; Liu et al. 2010). Among these methods, PLM is the most popular method used to visualize the internal structure of

Table 12.2 Various parameters affecting the property of oleogels

Factors	Effects on Oleogel's Property
1. Oleogelator	Gel characteristics can be varied by adjusting the proportion and concentration of the ingredients
2. Organic solvent	Different polarity of the solvent determines the strength and possibility of oleogelation
3. Temperature	The effect of temperature depends on the melting properties of the oleogelator and its interaction with the organic solvent
4. Molecular weight	Low molecular weight oleogelator requires a high concentration to build up viscosity and set to gel possibly
5. Molecular structure	The structure of the oleogelator backbone determine its self-assembly capability
6. Gelation time	Varies depending on the molecular self-assembly of oleogelators in a system and their interaction with the medium

oleogels. Fat crystals are birefringent, in which they are able to appear brightly against the dark background of liquid oils (Liu et al. 2010). Such difference allows the micro-structure of the fats to be observed clearly under PLM. As for PCM, it is frequently used for gaining contrast in a translucent specimen without staining process (Singh et al. 2015). SEM and TEM have a higher resolution of visualization than other imaging techniques. However, samples have to be dehydrated prior to the examination. The tedious preparation steps which involve utilization of solvents to remove water from the sample might cause some modification to the structure of the oleogels (Garg et al. 2016). Hence, PLM and PCM are more widely used due to low cost and easy sample preparation.

FTIR is used to understand the intermolecular interactions between oil-gelator in oleogel. Both hydrogen bonding and van der Waals interactions are required for the formation of oleogels. However, the existence of intermolecular interactions is varied when different oleogelators are used. Lupi et al. (2016) showed that hydrogen bonding was dominant in oleogels formed by monoglycerides of fatty acids (MAGs) but it was not the main driving force for policosanol oleogel formation. Although hydrogen bonding in MAGs was stronger than van der Waals interaction but the gel strength of policosanol oleogel was higher. This was attributed to the longer alkyl chain length and lower solubility of policosanol in the virgin olive oil, which promote the formation of gel through van der Waals force (Suzuki et al. 2003). This suggests the importance of determining the intermolecular interactions between oleogelators whenever a new oleogel is developed.

During the formation of oleogel, the long hydrocarbon chains of triacylglycerol in oleogels tend to pack into various crystal lattices, resulting in different polymorphisms. Each of the crystal polymorph represents different melting points and play key roles in predicting structural and mechanical properties of the oleogel. By using XRD, the fat crystal conformation of the oleogels can be determined. The three main polymorphs are α, β′ and β, which have subcell structure of hexagonal, orthorhombic-perpendicular and triclinic-parallel, respectively (Szydłowska-Czerniak et al. 2005). Among these three polymorphs, α-form (Tm 17–24 °C) has the lowest

Table 12.3 Short spacing crystal polymorphs based on XRD

Polymorphic form	Short (d) spacing (Å)	Subcell
α	4.15	Hexagonal
β′	4.20, 3.80 4.27, 3.97, 3.71	Orthorhombic perpendicular
β	4.6, 3.8	Triclinic parallel

Adapted from deMan (1992)

melting point, followed by β′ (Tm 24–30 °C) and β-form (Tm 32–36 °C) (Biswas et al. 2016). Due to the lowest thermal stability of α-form, it can be easily transformed to either β′ of β-form. Table 12.3 shows the short spacing characteristics of various crystal polymorphs as determined by XRD.

For application in food industry, β′-form is commonly observed in margarine, shortening and other spread product, owing to its optimal fat crystal lattice which gives rise to the optimal rheological and textural properties. On the other hand, the most stable β-form is required in chocolate and confectionary products made of cocoa butter (Sato and Ueno 2011). Since the properties of polymorphs are different, it is important to understand the polymorphic behavior of triacylglycerols in oleogels so that they can be used effectively in food applications.

12.4.2 Mechanical Properties

In order to apply oleogel in margarine or spread production, the softness and good spreadability of the final products are essential properties. Measurement of firmness, spreadability and rheological characterization are useful tools to understand the mechanical strength of an oleogel. The type of oleogelators and solvent used were reported to have significant effect on the mechanical properties of oleogels. This is explained by Gravelle et al. (2014), where the solubility balance between oleogelator-solvent and the interactions among oleogelators play a major role in determining the network structure and gel properties (Gravelle et al. 2014). Hence, to improve the applicability of oleogel in food industry, it is necessary to optimize the composition of an oleogel to achieve desired mechanical properties that meet customers' expectations.

12.4.3 Thermal Properties

Differential scanning calorimetry (DSC) is a reliable technique for determining the melting and crystallization point of an oleogel. It can also be used to measure the strength of the intermolecular interactions in gels. Gel network strength is directly proportional to the magnitude of enthalpy change (ΔH). A higher ΔH indicates that

the network is made of stronger bond while a lower ΔH values indicates a weaker bonded network is formed in the oleogel. Besides this, the temperature at which oleogel transforms to liquid is also important to indicate the thermal properties of an oleogel. Oleogels which are able to maintain their structure at room temperature is an advantage as they do not require refrigeration during storage and transportation.

12.5 Potential Application of Oleogels

The exceptional physical, functional, and nutritional properties of oleogels have attracted the attention of food and pharmaceutical industries. In food industry, oleogels could be used to minimize the oil migration in composite food products, maintain the texture of the foods without addition of trans fatty acids, enhance the oxidative stability of oil and as a delivery system for bioactive molecules. Owing to its wide range of applications in food production oleogel has been claimed to be "the fat of the future" (Rogers 2009).

12.5.1 Oil Migration Inhibitor

One of the interesting applications of oleogels is their use as oil migration inhibitor in multi-component food products such as chocolates, spreads, and margarines. These foods contain both solid and liquid lipid phases where a small amount of the liquid fats can move to the surface of the product during storage (Da Pieve et al. 2010). Surface fat bloom in chocolates is one of the primary causes of consumer dissatisfaction and rejection of confectionary products. In order to maintain the quality of the food product during storage, it is crucial to restrict the migration of oil between its components. Wendt et al. (2017) have developed oleogels from sunflower oil using β-sitosterol and γ-oryzanol as structuring agents. The produced oleogels successfully reduced the oil migration rate by half. This study demonstrates that oleogelation is an alternative strategy to immobilize the liquid oils within a gel network. It helps to reduce the movement and migration of triacylglycerols out of or through the materials and slow down the creation of fat bloom.

12.5.2 Fat Replacer

Oleogelation is one of the promising strategies in reducing the usage of hydrogenated fats and trans fatty acids in foods. In a study performed by Patel et al. (2014), the shortening used in bakery products has been replaced by oleogels from rapeseed oil and shellac (a food grade resin). Results showed that the batter produced by oleogel-based emulsions led to a runnier consistency as compared to the standard

cake margarine. When added to the cake, the oleogel product showed lower uniformity in cell size due to the lack of solid fat crystals, resulting in uneven distribution of air in the batter. However, it was still able to achieve texture, sponginess and moistness standards of a cake margarine. In another study, oleogel containing 5% of MAGs in sunflower oil were added in bread making resulting in higher firmness (Calligaris et al. 2013). Also, for bread application, oleogels of 9% sodium stearoyl lactylate in sunflower oil maintained the typical shape of bread with a well leavened structure. There was a 30% reduction in the content of saturated fatty acids (Meng et al. 2019).

12.5.3 Oil Structuring

A few studies have been conducted on the effect of oleogels on the storage stability of edible oils. Lipids are susceptible to oxidation. The formation of oxidation products not only poses health risk but also produce undesirable flavor and color changes, results in loss of sensory and nutritional qualities, making the food unacceptable for consumers. The role of carnauba wax and beeswax in structuring canola oil and grapeseed oil has been studied by Yi et al. (2017) with the intent to evaluate the oxidative stability of oleogels. Results obtained from this study confirmed the ability of wax to structure liquid oils and the oxidative stability of oleogels were significantly higher than liquid oil. In a similar study, MAG oleogel was reported to be ineffective in affecting the production of primary oxidation products but poses a hurdle to second step of oxidation (Da Pieve et al. 2011). In view of this, it is believed that oleogels are effective in hindering the development of oxidative reactions. The application of oleogel in food can be a promising strategy to extend the shelf-life of the product, thereby opening interesting perspectives to produce novel healthy food products.

12.5.4 Incorporation of Bioactive Compounds

Recent efforts have been made to improve the nutritional value of oleogel, by incorporating bioactive compounds into the oleogel. Bioactive compounds, such as polyphenols, flavonoids and vitamins, are nutritional constituents that naturally occur in plant and food products in small quantities (Aditya et al. 2017). They exhibit beneficial effects such as anti-inflammatory, antitumor and antioxidant activities. As most of the bioactive compounds are susceptible to physical or chemical degradations during processing, bioactive compounds cannot directly be added into food products. It is vital to incorporate them into a suitable delivery system before they can be successfully introduced into a food matrix. Incorporation of bioactive compounds into oleogels, such as β-carotene, curcuminoids, lycopene, phytosterols and omega-3 fatty acids, have been studied previously. Their benefits include antioxidant

properties, lower the incidences of cardiovascular disease and certain cancers (Zetzl et al. 2012). Clearly, the nutritional values of an oleogel can be enhanced significantly by addition of bioactive compounds.

Due to the amphiphilic properties of oleogels, both hydrophilic and hydrophobic food substances can be successfully incorporated into the oleogels system. However, most of the bioactive compounds will degrade easily when exposed to high temperature. This is one of the challenges in incorporating the bioactive compounds into oleogels since high temperature is required for oleogel formation. In order to minimize the degradation of bioactive compounds, bioactive compounds have been added during the cooling process, before gelation occurs. This helps to maximize the quality and effectiveness of any added bioactive compounds into the oleogels.

12.5.5 Delivery of Bioactive Compounds

The application of oleogels as fat replacers has been well documented, however, limited studies have been focused on the use of oleogels as a delivery system. Most of the delivery system studies are confined to the pharmaceutical field. This is attributed to their lipid medium which increases the bioavailability of lipid soluble drugs and gel matrix that offers sustained or controlled release of the drug molecules (Thet et al. 2018). Although there have been some applications of oleogels in dermal and transdermal delivery, the oral delivery of bioactive compounds using oleogel is relatively new in food science. Based on the literature, a variety of delivery systems with different compositions and structure have been developed to be used in the food industry, such as oil-in-water emulsions, nanoemulsions and microemulsions (Tavano et al. 2014; Yi et al. 2014; Huang and Zhou 2019).

Bioactive compounds are often reported to have low bioavailability due to their poor water solubility, low digestion stability and low absorption in gastrointestinal tract (Yu et al. 2012). The main reason to incorporate bioactive compounds into delivery systems is to deliver them to small intestine to maximize their absorption and bioavailability. During the digestion process, secretion of hydrochloric acid causes the pH in stomach to drop to below 3.0. This acidic environment acts as a bactericidal barrier and tends to kill pathogenic bacteria that enter the stomach (Lund et al. 2014). In the meantime, it will also cause degradation of bioactive compounds. In order to improve the performance of these compounds, oleogels have been used to delay the release of bioactive compounds. Bioactive compounds are being entrapped within the gel matrix and protected from the acidic environment in stomach before release in small intestine, where the absorption of compounds take place.

Yu et al. (2012) successfully developed novel oleogel-based nanoemulsions for oral delivery of curcuminoids with high loading and bioavailability. In this study, curcuminoid was initially added into medium chain triacylglycerol emulsion with Tween 20 as the emulsifiers to improve the loading and bioaccessibility of curcuminoids. However, the precipitation of curcuminoids was observed after

1 month of storage. This problem has been overcome by using the oleogel as the oil phase in the nanoemulsion formulation with monostearin functioning as an oleogelator. The bioaccessibility of the curcuminoids remained at 80%. This novel formulation approach may also be used for oral delivery of other poorly soluble nutraceutical with high loading capacity, which has significant impact on functional foods, dietary supplements and pharmaceutical industries. Furthermore, the capability of oleogel networks to delay the release of bioactive compounds must be studied in detail in order to evaluate their full potential in this area.

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Chapter 13
Characterization of Nanoemulsions: The Way Forward

Tai Boon Tan, Phui Yee Tan, and Yee Lin Gan

Abstract Studies on nanoemulsion have gained traction in recent years due to nanoemulsion's huge application potential in various industries. However, studies to date have tended to focus on the effects of processing and formulation parameters on the fabrication of stable nanoemulsions, with a lack of emphasis on the characterization of the fabricated nanoemulsions. As such, most of these studies reported only the fundamental characteristics of the nanoemulsions, such as particle size, particle size distribution, viscosity, colour and stability. Although these aforementioned characteristics are important and should inherently be included in all studies related to nanoemulsions, we believe that it is time for researchers to move forward and explore other characterization analyses that complement conventional characterization analyses and would provide a better understanding and a more complete picture of the developed nanoemulsion. Thus, in this chapter, analyses based on microscopy techniques, in vitro simulated gastrointestinal models and in vitro cellular models are presented. Under microscopy techniques, the difference between two primary techniques, scanning electron microscopy and transmission electron microscopy, is discussed. In addition, the steps involved in both techniques, as well as their pros and cons in gaining deeper insight into the morphology of nanoemulsions are also described. Meanwhile, for in vitro simulated gastrointestinal analysis, the protocol, advantages and disadvantages, and the significance of this analysis are reviewed. Finally, for analyses based on in vitro cellular models which can be used to assess intestinal absorption and permeability of compounds encapsulated and delivered via nanoemulsion system, the characteristics, advantages and

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limitations of various cellular models are discussed in detail. Recent published studies on nanoemulsions which incorporated the aforementioned analyses are also summarized in tables for ease of reading. We hope that the information presented in this chapter could provide a positive point of view and inspire fellow researchers to initiate the incorporation of additional complementary characterization analyses in their studies on nanoemulsion systems, and consequently elevate our understanding of this subject matter.

Keywords Nanoemulsion · In-vitro simulated gastrointestinatl model · In-vitro cellular model · INFOGEST · CaCo-2 cell line · Particle size distribution

13.1 Introduction

Nanoemulsions are essentially emulsions containing dispersed particles in the nanoscale range. With nanoemulsions possessing numerous advantages (such as being highly bioavailable, kinetically stable, and optically transparent) over emulsions in their bulk form, it is of little wonder that there has been an ever-growing interest in the use of nanoemulsions as encapsulation and delivery systems for various compounds of interest. Nanoemulsions, be it simple oil-in-water (O/W) or water-in-oil (W/O) systems, to more complex variants such as the oil-in-water-in-oil (O/W/O) or water-in-oil-in-water (W/O/W) systems, are constantly being developed for food, pharmaceutical and cosmeceutical applications. A quick search on Scopus using the term 'nanoemulsion' returned a hit of 6721 articles, with one article being published in year 1995 to over 1000 articles in year 2019. A vast portion of the articles described the preparation or fabrication of nanoemulsions using various materials and processes, followed by the characterization and stability evaluation of these nanoemulsions. Compulsory characteristics of the developed nanoemulsions, specifically the particle size, is necessary as the defining criterion for a system to be labelled as nanoemulsion is its particle size. From a technical point of view, a nanoemulsion is defined as emulsions with particle size of 100 nm and below. However, from literature, it is very common for authors to report emulsions with particle sizes up to 1000 nm as nanoemulsions, partly due to the difficulty of achieving $<$100 nm sizes using conventional processes and/ or materials. The particle size (along with particle size distribution) are usually measured using standard characterization procedures based invariably on dynamic light scattering technique. This technique is convenient and non-invasive, and provides relatively accurate results within minutes. Although useful and is considered the "gold standard" especially when it comes to the reporting of data, it is worth taking note that the outcomes of a dynamic light scattering-based analysis is estimated based on the Stokes-Einstein equation with assumptions that the particles measured are non-interacting and have a spherical geometry (Tan et al. 2016). As such, it is recommended that the particle size results derived from this technique is validated using visualization techniques, i.e. microscopy-based analyses. This is also in line

with the latest development needs in the context of research in which more sophisticated and informative analyses are highly valued.

As mentioned earlier, nanoemulsions are touted to be effective delivery systems because theoretically, they confer high bioavailability to the compounds that they encapsulate. In early period research in the area of nanoemulsions, this theory is often neglected and not tested as researchers focused on the effects of various preparation methods and materials, followed by basic characterization and stability evaluation of the resulting nanoemulsions. However, as research progresses, researchers begin to investigate the bioavailability of compounds delivered via nanoemulsion systems, often through in vitro simulated gastrointestinal models. And in recent times, cellular uptake of compounds when delivered via these systems is also gaining traction.

Hence, in line with the theme of the book, this current chapter will attempt to provide insights into the latest development in the characterization of nanoemulsion systems. Specifically, complementary characterization analyses involving microscopic techniques, in vitro simulated gastrointestinal models and in vitro cellular models will be reviewed. These analyses will be briefly defined and protocols/methodologies that are commonly applied will be outlined. The advantages of each analysis as well as their limitations will also be presented. In addition, the significance of the findings obtained using the aforementioned analyses will be discussed. In other words, we strive to rationalize the relevance of these analyses in an attempt to provide researchers with more options for their future studies as these analyses may help in enhancing the impact of their research. In general, there are no experimental protocols for the said analyses that could be considered as truly accurate as they very much depend on the types of compounds being studied. Most of the time, researchers adapt and modify established protocols based on their sample and to suit their experimental needs. As such, examples of protocols based on the studies that have been carried out by various research groups in recent years will be provided.

13.2 Microscopy Techniques

As mentioned earlier, it is advisable to validate particle size results derived from dynamic light scattering technique using visualization techniques, such as microscopic-based analyses, as the former technique calculates the size of particles based on assumption that the particles are non-interacting spheres. However, this assumption is not always met as nanoparticles hardly exist as perfect spheres. Furthermore, recent advances in the use of different nanoemulsion preparation techniques (such as layer-by-layer deposition) and various types of emulsifiers (such as branched polysaccharide emulsifiers and protein nanofibrils) mean that the resulting nanoparticles could be of varying shapes, i.e., not perfectly spherical.

As such, it is essential to complement particle size measurement using microscopy-based analyses in order to have a certain degree of certainty to the

obtained results. In this context, traditional optical light microscopy can and has been widely used for the particle size measurement of conventional emulsion systems. However, in nanoemulsion systems, especially those that contain particles with sizes below 500 nm, the effectiveness of light microscopy is severely diminished. The limitation of light microscopy is even more apparent when researchers are also interested in the morphology of the nanoparticles as this information is often used to gauge the effectiveness of the emulsifiers in encapsulating the core compound of interest (Klang et al. 2012).

Thus, this is where electron microscopy comes into the picture. In general, the two techniques of electron microscopy that are commonly used to visualize nanoemulsions are scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

13.2.1 Scanning Electron Microscopy (SEM)

SEM works by scanning samples with a beam of electrons. These electrons interact with the surface of the samples and produce secondary electrons which are then detected and interpreted. Due to its greater depth of field, SEM can be used to provide highly-defined three-dimensional images of a sample. This enables users to scrutinize the sample's surface structure and obtain useful topographical information. The ability to visualize surface morphology is one of SEM's primary advantages over TEM that can only be used to obtain two-dimension projections of a sample. On the other hand, a lack of internal details and a relatively lower resolution as compared to TEM are some of SEM's limitations.

In terms of experimental protocol, fresh nanoemulsion samples are usually dried and subjected to a fixation procedure. Then, the samples are sputter-coated with gold particles at appropriate thickness and then examined using scanning electron microscope. This is the general experimental protocol undertaken by researchers although the reagents used for the fixation procedure may differ (Hatziantoniou et al. 2007; Sharma et al. 2017). Besides conventional SEM, its variants which include freeze-fracture-SEM and cryo-SEM can also be employed to visualize nanoemulsion samples. In fact, these variants are often considered to be more suitable for nanoemulsion samples, although they are relatively more expensive and require additional steps in terms of sample preparation. For example, Sedaghat Doost et al. (2018) utilized cryo-SEM to characterize their nanoemulsion samples. In their study, nanoemulsion samples were placed on a copper grid and frozen in a nitrogen slush prior to fracturing. Then, the samples are sputter-coated with platinum before being subjected to image recording.

13.2.2 Transmission Electron Microscopy (TEM)

Unlike SEM which scans a sample, TEM operates by transmitting a beam of electrons through the sample. As such, a vacuum condition is required as the presence of gas molecules will deflect the electrons. In a typical TEM setup, the microscope column is equipped with an electron gun and a series of electromagnetic lenses to focus the electron beam on the sample. An image is then formed and subsequently magnified and focused by an objective lens, and finally captured using an imaging device. In a way, TEM is similar to optical microscopy but offers a much higher resolution because of the small de Broglie wavelengths of the electrons. The resolution of an image obtained via TEM can be adjusted by manipulating the acceleration voltage of the electrons; higher resolution can be achieved by increasing the voltage acceleration, and vice versa. However, it is worth taking note that when voltage acceleration is increased, the scattering of electrons would decrease and this will consequently affect the contrast of the image obtained. All things considered, acceleration voltage of 70–200 kV are commonly applied in TEM analysis of nanoemulsions (Sharma et al. 2017).

Similar to SEM, TEM is expensive and requires extensive sample preparation. However, simplified, rapid and less expensive sample preparation methods involving the use of Formvar-coated copper grids and negative staining have been developed (Klang et al. 2012). This negative staining method is often performed using common salts or acids of heavy metals such as tungsten, molybdenum or uranium. According to Massover (2008), the reagents used for staining purpose must have the ability to form a thin glassy layer upon drying and not chemically alter the sample. They must also be sufficiently stable against the electron beam radiation in order to protect the sample from damage, besides providing structural support to prevent the air-dried sample from collapsing.

In general, the sample preparation method for TEM analysis involves placing a drop of the nanoemulsion sample onto a carbon-coated grid that is left to stand for a few minutes to allow the sample to be absorbed. Then, any excess sample will be removed (using filter paper) and the grid is left to air-dry. This is followed by the negative staining step in which phosphotungstic acid is commonly used (Tan et al. 2016; Wang et al., 2020a, b). Then, the sample is again left to dry (up to a few hours) and finally viewed using a transmission electron microscope. Resulting TEM images often depict bright nanodroplets against a dark background as a result of the weak scattering capacity of droplets against strong scattering capacity of the negative stains (Fig. 13.1). Through these images, the shape and internal structure of the droplets can be observed, and their size measured using appropriate tools that usually come together with the transmission electron microscope software. Subsequently, these results can be used to compare with those obtained via dynamic light scattering technique to validate the particle size of a nanoemulsion.

In terms of result interpretation, users have to be cautious and bear in mind that since the sample preparation method involves drying steps, sample deformation (such as shrinkage) as water evaporates during the drying process may affect the

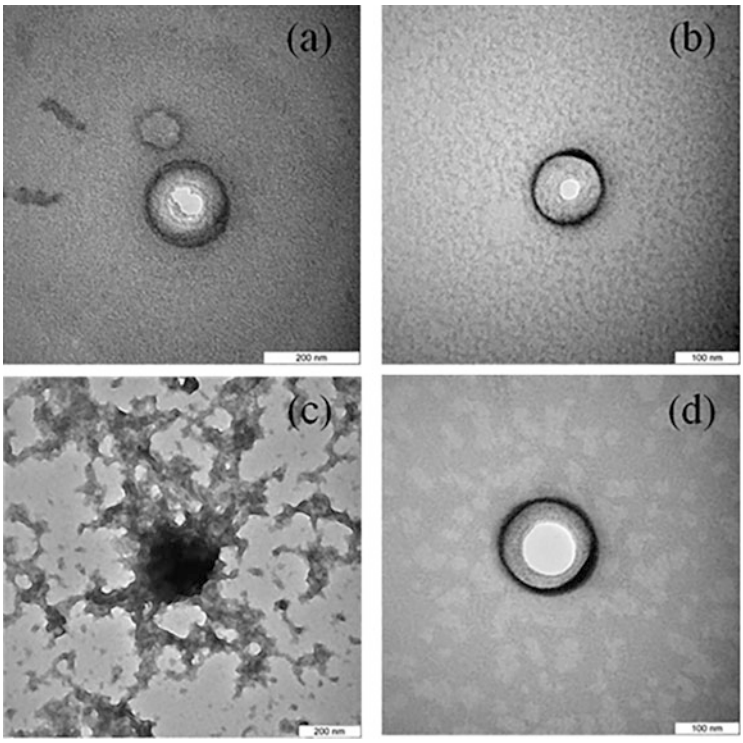

Fig. 13.1 Transmission electron micrograph showing particle shape of lutein nanodispersions prepared by using (**a**) Tween 80, (**b**) sodium dodecyl sulfate (SDS), (**c**) sodium caseinate and (**d**) SDS-Tween 80 (Tan et al. 2016). Reproduced by permission of The Royal Society of Chemistry

final captured image of the sample. Moreover, uneven or incomplete staining during the staining step can also negatively affect the result. As such, sample preparation methods (such as cryofixation) or the use of cryo-TEM have been proposed to improve the accuracy of analysis results. However, having said that, the use of cryo-based techniques or equipment are relatively more costly as compared to conventional ones and thus, may not be feasible for the purpose of simple characterization of a nanoemulsion's morphological properties.

The following table (Table 13.1) summarizes some of the studies related to the use of SEM and TEM in characterizing nanoemulsions. Generally, the latter is more often used for the characterization of nanoemulsions while the former is found to be more widely used to observe the changes in bacterial cells after being treated with nanoemulsions containing antimicrobial agent.

Table 13.1 Studies related to nanoemulsions which carried out analysis using scanning electron microscopy (SEM) and/or transmission electron microscopy (TEM)

Sample	Analysis	Findings	Reference
Sage oil nanoemulsions	SEM Changes in bacterial morphology after application of sage oil nanoemulsions	The rod-shaped cells of Escherichia coli showed signs shrinkages and destruction	Moghimi et al. (2016)
PIT-fabricated cinnamon oil nanoemulsions	Field emission scanning electron microscopy (FE-SEM) Visualization of bacterial morphology treated with cinnamon oil nanoemulsions	Impaired membrane structure and leakage of inner cell materials	Chuesiang et al. (2019)
Antibacterial hydroxypropyl methyl cellulose edible films containing nanoemulsions of Thymus daenensis essential oil	FE-SEM Morphological characteristics of edible films made using nanoemulsions of Thymus daenensis essential oil	Proper incorporation of nanoemulsions into the edible film was confirmed via observation of small pores on vertical cross section of the films	Moghimi et al. (2017)
Nanoemulsions and solid lipid nanoparticles containing high amounts of ceramides	SEM Morphological observation of nanoemulsions and solid lipid nanoparticles	Particles with smooth surfaces and almost spherical shapes	Hatziantoniou et al. (2007)
Sodium caseinate stabilized clove oil nanoemulsion	SEM and TEM Surface and internal morphologies of nanoparticles	Spherical nanoparticles with slight aggregation or adherence Discrete spherical slightly adhered nanoemulsion droplets	Sharma et al. (2017)
Lutein nanodispersion	TEM Morphology of nanoparticles	Nanodispersions stabilized using Tween 80, sodium dodecyl sulfate (SDS) and SDS-Tween 80 contained well-defined spherical particles Nanodispersion stabilized using sodium caseinate contained less-defined particles (coral-like thread image)	Tan et al. (2016)
Cinnamon oil nanoemulsion	TEM Morphology and size of nanoparticles	Emulsion particles were spherical in shape and in nanometric range Size of particles were similar to results of DLS	Yildirim et al. (2017)

(continued)

Table 13.1 (continued)

Sample	Analysis	Findings	Reference
		measurement (approximately 100 nm)	
Sunflower oil nanoemulsion stabilized using casein hydrolysate-carboxymethyl chitosan (CHs-OCC) conjugates	TEM Morphological characteristics of nanoemulsions	Oil nanodroplets were spherical in shape, evenly dispersed and appeared as bright round white spots Diameter of about 200–300 nm Rough structure observed on the surface of nanodroplets which was attributed to the adhesion of CHs-OCC conjugates	Wang et al. (2020b)
Lime essential oil nanoparticles	TEM Morphology and size of nanoparticles	Nanoparticles were spherical in shape with sizes within the nanometer range (20–200 nm)	Liew et al. (2020)

13.3 In Vitro Simulated Gastrointestinal Analysis

As its name implies, the in vitro simulated gastrointestinal (GI) analysis involves a series of digestion procedures which are carried out to simulate the conditions of the human gastrointestinal system (McClements and Li 2010). Be it the pharmaceutical or food industry, it has become a norm to screen developed nanoemulsions using this analysis.

13.3.1 Protocol

Throughout the years, researchers have been developing different in vitro simulated GI models, ranging from single static to dynamic models for the screening of numerous products (Li et al. 2020). Among these models, the static in vitro digestion models are often applied in the studies of nanoemulsions. The existence of various methods has caused difficulties in terms of comparing the results obtained from different laboratories, thereby leading to continuous attempts to standardise the in vitro digestion protocol. The United States Pharmacopeia has developed a range of static methods with different complexities for the digestion assessment of pharmaceutical products. These methods, however, are not suitable for the studies of food products which tend to be more complicated than pharmaceutical products. As such, the international INFOGEST network has established a standard static in vitro simulated GI method especially for the studies of food products (Minekus et al.

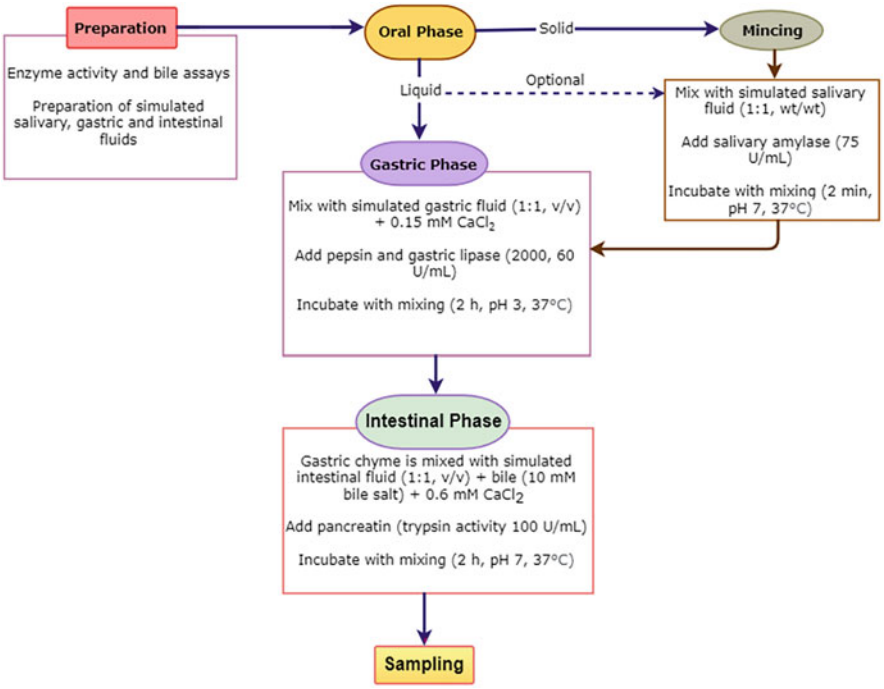

Fig. 13.2 Flow chart of the INFOGEST protocol

2014). Based on the adult physiological conditions, the standardised protocol, which is now regarded as the INFOGEST method, specifies the digestion conditions in the three major digestive stages, i.e. mouth, stomach and intestine. This method was then further improved in 2019 to eliminate a few issues in its original protocol (Brodkorb et al. 2019).

The INFOGEST method has listed step-by-step guidelines, from the preparation to the end of digestion phase (Fig. 13.2). In the initial preparation stage, a set of enzyme activity (i.e. amylase, pepsin, lipase and trypsin activity assays) and bile assays with specific units of activity are recommended to standardise activities of the enzymes utilized in the protocol. The standardisation of enzyme activity is emphasized as one of the critical steps to achieve comparable results among the different laboratories. During the simulated digestion process, the experimental conditions, i.e. dilution ratio of food, concentrations of electrolyte solutions and enzymes, pH and duration of digestion, are fixed for each phase. The oral phase can be performed without salivary amylase, provided that the tested food product does not contain any starch. If starch is present, the food is mixed with amylase for 2 min at pH 7. It is suggested that solid food has to be minced to simulate the mastication step in the mouth. As for nanoemulsion that is in liquid form, the mincing step can be omitted. The oral bolus or mixture obtained from the oral phase is then diluted with simulated gastric fluid (SGF) and gastric enzymes (pepsin and gastric lipase) in the subsequent gastric phase. The pH of the mixture is fixed at pH 3 and the mixture is subsequently incubated for 2 h under agitation. Physiologically, the digestion of emulsified lipids

normally starts in the stomach, wherein the gastric lipase binds to the surface of lipid droplets, thus decomposing the triacylglyercols into di- and mono-acylglycerols and free fatty acids (McClements and Li 2010). Theoretically, lipid droplets of smaller size would experience higher degree of digestion as compared to those of bigger size due to their larger surface area for lipase adsorption (Mat et al. 2020). The lipids continue to be digested in the subsequent small intestine and the digestion products are absorbed via formation of micelles with the help of bile salts and phospholipids secreted by the liver. Taking into account the actual intestinal conditions, the in vitro intestinal phase utilises simulated intestinal fluid (SIF), bile salts and pancreatic enzymes to dilute the gastric chyme at 1:1 volume ratio, which is then incubated for 2 h at pH 7. After the completion of simulated digestion process, the enzyme activities are inhibited through instant freezing or addition of enzyme inhibitors, such as Pefabloc SC (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride) for the inhibition of trypsin and chymotrypsin (Brodkorb et al. 2019). The last stage of the protocol involves sampling, sample post-treatment, storage and sample analyses which should be carefully planned in advance, as it might vary according to sample types and would critically affect the final results of the study.

13.3.2 Advantages

It is known that the in vivo approach is the ideal way to perform digestion studies on nanoemulsion products. However, certain prototype nanoemulsions may not yet be safe or suitable for human clinical trials, therefore causing ethical restrictions (Mat et al. 2016). Animal studies of prototype nanoemulsions are also not suitable as the samples may harm and/or even sacrifice the animals. In addition to the safety and ethical setbacks, in vivo study is usually time-consuming as it takes a considerable amount of time to collect sufficient data. Furthermore, a group of trained and experienced personnel is required for the well handling of animals. Thus, the in vivo approach is often expensive to be conducted (Minekus et al. 2014). In contrast to the in vivo approach, the in vitro method is rather straightforward, making it a rapid method to be applied for digestion studies. By avoiding the need to involve humans or animals, the in vitro approach provides lower inter-subject data variations and contributes to high reproducibility (Sandoval-Cuellar et al. 2020). With precise control over the experimental conditions, the findings of in vitro tests could be used to predict the outcomes and mechanisms of in vivo digestions (Bohn et al. 2018). Therefore, the in vitro simulated GI analysis serves as an economic alternative, especially for the industries to test prototype emulsions prior to animal or clinical studies.

13.3.3 Disadvantages

Technically, designing an in vitro simulated GI system has always been a challenging task. Considering that the human GI tract is a complex system, the in vitro

simulated GI system is far too simple. In fact, the static in vitro protocol only screens the delivery systems in beakers that are stirred continuously. This protocol is thus claimed to have failed to completely mimic the GI anatomy, mechanical forces, the dynamic flow of food or fluids and absorption of the targeted compound within the GI tract (Li et al. 2020). For example, given that the pH of the in vitro gastric phase is constant, the gradual addition and emptying of gastric fluid that actually occurs within the stomach are lacking in the in vitro protocol. In terms of structure, despite being a sequential system comprising of duodenum, jejunum and ileum, the intestinal phase is assumed as a single phase having constant dilution ratio, mineral content, pH, etc., which are totally different from the in vivo conditions.

As described previously, the standardised INFOGEST method was developed based on the adult digestive system. Hence, further adaptations may be necessary to apply the method for different groups of people such as the elderly or patients with specific health conditions, such as cystic fibrosis (Brodkorb et al. 2019). The human diet, on the other hand, comprises multiple food components of different compositions, causing it to be more complicated than the in vitro model systems that are tested under controlled in vitro environments. As a result, the understanding of the interactions between the delivery system and the other food components under the actual digestive conditions is limited.

13.3.4 Significance of Analysis

As in the case of the nutraceutical industry, the food industry is consistently developing emulsion-based delivery systems for the encapsulation and controlled release of lipophilic bioactive compounds. By designing a suitable delivery system, the bioavailability of the compounds of interest could be enhanced, leading to the fabrication of food products that could serve a specific functional purpose for the well-being of mankind. In order to assess the bioaccessibility of the encapsulated compounds, it is necessary to study the stability and digestive behaviour of the nanoemulsion-based delivery system when it passes through every stage of the human gastrointestinal tract. In this context, the application of in vitro simulated gastrointestinal analysis allows the researchers to accomplish the digestion study in a reproducible manner.

Thus far, the INFOGEST method has proven to be suitable for bioaccessibility study of bioactive compounds and oils entrapped in various forms of emulsion systems. Some of the related studies are listed in the following Table 13.2.

13.4 In Vitro Cellular Models: Prediction of Human Oral Bioavailability

As a promising delivery system to enhance the oral bioavailability of encapsulated compounds, one significant challenge that nanoemulsions need to overcome is to enable the encapsulated compounds to transport across the formidable intestine

Table 13.2 Bioaccessibility studies of various compounds via adaptation of INFOGEST method

Compounds	Sample matrix	Aims	Findings	References
Resveratrol	– Biopolymer nanoparticles consisting of caseinate or caseinate-dextran shell with zein-resveratrol core – Biopolymer complexes of resveratrol and caseinate or caseinate-dextran conjugates	Evaluating effect of encapsulation process on bioaccessibility of resveratrol	Biopolymer nanoparticles and complexes improved bioaccessibility of resveratrol	Davidov-Pardo et al. (2015)
Beta-carotene	– Nanoemulsions of medium chain triglycerides (MCT) – MCT-glyceryl stearate or MCT-partially hydrogenated palm oil solid lipid nanoparticles (SLNs)	Investigating the effect of different concentrations of solid lipid on *in vitro* lipid digestibility kinetics and beta-carotene bioaccessibility	– SLNs with increasing hydrogenated palm oil concentration shows slower lipolysis kinetics – Hydrogenated palm oil-containing solid lipid nanoparticles exhibited higher beta-carotene bioaccessibility	Helena de Abreu-Martins et al. 2020
Zeaxanthin dipalmitate	– O/W emulsions with or without mixture of *L. barbarum* fruit, containing oils of different unsaturation degree	Comparing the bioaccessibility of zeaxanthin dipalmitate among emulsions of different oils	– Effect of unsaturation degree of oil on the bioaccessibility of zeaxanthin dipalmitate was confirmed	Kan et al. (2020)
Fatty acids in high-oleic palm oil	Nano- and macroemulsions of high-oleic palm oil stabilized by whey protein and soy lecithin	– Comparing the digestion behavior of high-oleic palm oil in the nano- and macroemulsions – Evaluating the bioaccessibility of fatty acids released from digested emulsions	– Different digestion behavior of high-oleic palm oil in both emulsions was observed – Bioaccessibility of unsaturated and saturated fatty acids in the palm oil of both emulsions were different from that of the non-emulsified palm oil	Sandoval-Cuellar et al. (2020)
Short-chain fatty acids	Pickering emulsions stabilised by modified cellulose nanocrystals	Comparing the release of short-chain fatty acids between the emulsions of sequential digestion and emulsions of direct intestinal digestion	Higher release of short-chain fatty acids was observed in direct intestinal-digested emulsions than that of sequentially-digested emulsions	Le et al. (2020)

barrier in order to reach the systemic circulation. It is always estimated that encapsulated compounds would have enhanced bioavailability due to the tiny particle sizes of nanoemulsions providing a higher surface area which eases the permeation through the intestinal epithelial layer. However, using this assumption may lead to false prediction as physiological conditions have not been incorporated in this physicochemical parameter-based estimation methods and how much encapsulated compounds will be bioavailable following administration remains unknown. Physiologically, following gastrointestinal digestion, the transport of digesta through human intestinal is a complex and dynamic process that involves various mechanisms in parallel including passive diffusion (paracellular and transcellular), carrier-mediated influx process (facilitated diffusion and active transport), endocytosis and efflux mechanisms (Sarmento et al. 2012). Numerous studies have demonstrated that physicochemical properties and composition of nanoemulsions play a key role in modulating the transepithelial transport behaviours of encapsulated compounds (Fan et al. 2017b; Liu et al. 2019; Silva et al. 2019). Therefore, characterizations of intestinal absorption and permeability of the formulated nanoemulsions under physiologically relevant conditions are crucial for the evaluation of its delivery efficiency. The data is useful for the researchers in fabricating more effective nanoemulsions for oral application by tuning the composition and physicochemical properties of nanoemulsions.

A variety of physiologically relevant models are currently available for the assessment of intestinal absorption and permeability of test compounds, including in vivo human intestinal perfusion, in vivo and in situ animal intestinal perfusion, ex vivo models using excised intestinal tissue from animals or human (Ussing chamber) and in vitro cellular models (Lefebvre et al. 2015). Nevertheless, evaluation in human and animal models is less favorable due to ethical, cost, and time concerns. It is also problematic to obtain viable animal and human excised intestinal tissue frequently as well as maintaining its viability over a long period for regular assessments. On the other hand, in vitro cellular models which show good correlation result with human intestinal permeability serve as an attractive alternative to the in vivo and ex vivo animals and human models (Sarmento et al. 2012; Youhanna and Lauschke 2020). It is a relatively high-throughput, cost-effective, reproducible, and easier-to-perform assay. The parameters and conditions of in vitro cellular models can be changed and controlled easily, allowing a better manipulation to examine a vast number of factors that affect transepithelial transport of test compounds. Therefore, in vitro models have been extensively used to evaluate the delivery efficiency of the test compounds and to predict their in vivo bioavailability.

The human intestinal epithelium consists of a heterogeneous population of cells including enterocytes (absorptive cells), goblet cells (mucus-secreting cells), microfold (M) cells (immunosurveillance cells), enteroendocrine cells (hormone-producing cells), Paneth cells (antimicrobial peptides-producing cells), tuft cells and cup cells. Ideally, isolated primary epithelial cells from gastrointestinal should be the best cellular model for the evaluation of intestinal absorption and permeability of the test compounds. However, they are not commonly used due to their inability to form an organized monolayer cell to display apical and basolateral sides and have a restricted lifespan in culture (Gordon et al. 2015; Lefebvre et al. 2015). Therefore,

immortalized cell lines, capable of reconstructing the human differentiated epithelial cell monolayer, are often used instead. Different in vitro cellular models have been established which include Caco-2 monoculture, Caco-2/HT29-MTX co-culture, Caco-2/Raji B co-culture, and Caco-2/HT29-MTX/Raji B co-culture (Pereira et al. 2016). Although monoculture is relatively easier-to-perform and cost-effective, culturing of various cell lines in a model is often preferred in order to reproduce the heterogeneous cell population of the intestinal epithelium to generate more predictable experimental results.

An earlier study found no significant difference in the transport rate of curcumin loaded nanoemulsions across Caco-2 monoculture and Caco-2/Raji B co-culture (Memvanga et al. 2013). However, in a study conducted by Xia et al. (2017), which compared the cellular uptake and permeability of various sizes of medium chain triglyceride-based nanoemulsions in Caco-2 monoculture, Caco-2/HT29-MTX co-culture, Caco-2/Raji B co-culture, and Caco-2/HT29-MTX/Raji B co-culture, the results suggested that the cellular uptake and intestinal permeability of MCT-based nanoemulsions followed the sequence of: Caco-2/Raji B > Caco-2/HT29-MTX/Raji B > Caco-2 > Caco-2/HT29-MTX. In addition, a more recent study has shown that cellular uptake of β-carotene-loaded nanoemulsions in Caco-2 monoculture was significantly higher than in Caco-2/HT29-MTX co-culture (Gasa-Falcon et al. 2021). The above-mentioned results imply that the selection of right cellular models that correspond to the experiment purposes is important for the prediction of bioavailability of test compounds. Hence, the characteristics, advantages and limitations of each cellular model are reviewed below and summarized in Table 13.3.

13.4.1 Caco-2 Monoculture

The intestinal epithelium is dominated by enterocytes (make up more than 80% of the epithelium), the highly polarized epithelial cells which are responsible for the primary absorption of bioaccessible substances in the small intestine (Pereira et al. 2016). Although Caco-2 cell line is derived from human colon adenocarcinoma, a colonic origin, it undergoes spontaneous enterocytic differentiation in culture after reaching confluent to resemble enterocytes. Upon 18 to 21 days of culture, a polarized monolayer cell is well-formed with the presence of brush border on their apical surface, expressing typical membrane transporters and intestinal enzymes, and forming tight junctions between the adjacent cells (Araújo and Sarmento 2013; Lozoya-Agullo et al. 2017; Schimpel et al. 2014; Volpe 2020). Hence, Caco-2 cell monolayer behaves closely like mature enterocytes, both structurally and functionally. It has been accepted as a gold-standard model for the prediction of intestinal absorption in human by both regulatory authorities and industrial companies (Costa and Ahluwalia 2019).

Nevertheless, Caco-2 monoculture does not fully mimic the human intestinal epithelium, making further improvement of this model necessary. Caco-2 monolayer

Table 13.3 Characteristic, limitation and application of in vitro cellular models

Cellular model	Origin	Main Characteristic	Limitation	Culture for transport studies	Application	References
Caco-2	Human colon adenocarcinoma	Polarized cells with the presence of tight junctions and brush borders; express several relevant influx and efflux membrane transporters; express metabolizing enzymes (e.g. esterase, sulfatase, aminopeptidase)	The phenotype of cells varies according to cell culture conditions; absent of mucus layer; expression level of some active and efflux transporters differs from human's intestine; permeability through tight junctions is lower than in human; low expression of metabolizing enzymes from cytochrome P450	Caco-2 cells are seeded onto the apical side of transwell plates (pore size: 0.4 μm) and grown for 21 days. Transepithelial electrical resistance (TEER) value: >250–350 Ω cm^2	Undifferentiated Caco-2 model is useful to study cytotoxicity of test compounds Differentiated Caco-2 model is used to study the delivery efficacy, safety and transport mechanisms of test compounds	Pereira et al. (2016), Sarmento et al. (2012)
Caco-2/HT29-MTX Co-culture	Human colon adenocarcinoma	Incorporates a mucus layer on the Caco-2 monolayer; enhances paracellular permeability; express a lower level of efflux transporters	Absent of M cells; availability of various cell sub-clones contributes to interlaboratory variability	Caco-2 and HT29-MTX cells are grown separately, and the pre-determined ratio for Caco-2 to HT29-MTX cells (9:1) is mixed and seeded onto the apical side of transwell plates (pore size: 0.4–3 μm). The cells are grown for 21 days TEER value: > 150–250 Ω cm^2	On top of the application of Caco-2 monoculture, it is used to evaluate the role of mucus layer on transepithelial transports	Lozoya-Agullo et al. (2017), Pan et al. (2015), Pereira et al. (2016)

(continued)

Table 13.3 (continued)

Cellular model	Origin	Main Characteristic	Limitation	Culture for transport studies	Application	References
Caco-2/ Raji B co-culture	Human colon adenocarcinoma/ Human Burkitt's lymphoma	Develops human follicle-associated epithelium morphology (Peyer's patch)	Lack of a mucus layer and stromal cells; co-culture characteristics may vary with discrepancies preparation of primary lymphocyte, results in interlaboratory variability; no established quantitative correlation to human bioavailability	Caco-2 and Raji B cells are grown separately. Caco-2 cells are seeded onto the apical side of transwell plates (pore size: 0.4–3 μm). After day 14, the Raji B cells at the pre-determined ratio for Caco-2 to Raji B cells (1:2) are added to the basolateral compartment and maintained for 4–7 days TEER value: >150–250 Ω cm^2	It is used to study the delivery efficacy, safety and transport mechanisms of test compounds. Specific assessment of internalization of test compounds through M cells	Kleiveland (2015), Pereira et al. (2016), Sarmento et al. (2012)
Caco-2/ HT29-MTX/ Raji B co-culture	Human colon adenocarcinoma/ Human Burkitt's lymphoma	Incorporates a mucus layer on the Caco-2 monolayer; develops human follicle-associated epithelium morphology (Peyer's patch)	Co-culture characteristics may vary with discrepancies preparation of primary lymphocyte, results in interlaboratory variability; no established quantitative correlation to human bioavailability	Caco-2, HT29-MTX and Raji B cells are grown separately. The pre-determined ratio for Caco-2 to HT29-MTX cells (9:1) is mixed and seeded onto the apical side of transwell plates (pore size: 0.4–3 μm). After day 14, the Raji B cells at the pre-determined ratio	It is used to study the delivery efficacy, safety and transport mechanisms of test compounds when different absorption pathways present in the same model	Araújo and Sarmento (2013), Lozoya-Agullo et al. (2017), Schimpel et al. (2014)

			for Caco-2 to Raji B cells (1:2) are added to the basolateral compartment and maintained for 4–7 days. TEER value: >150–250 Ω cm^2		

consists solely of absorptive cells, lacking a mucus layer that interferes with intestinal absorption. The tightness of Caco-2 monolayer resembles colon than the looser junctions in the small intestine, causing reduced paracellular permeability. Also, Caco-2 cells over-express P-glycoprotein, an efflux transporter, and this may lead to lower absorption due to higher efflux rate (Alqahtani et al. 2013). Thus, co-culture of Caco-2 cell line with another cell line that further mimics the human intestinal has been developed to improve the Caco-2 cell model.

13.4.2 Caco-2/HT29-MTX Co-culture

In the small intestine, goblet cells represent 10% to 25% of the epithelium and secrete mucus continuously to form one layer of mucus that covers over the tips of microvilli (Hilgendorf et al. 2000). It is the first absorption barrier at the outermost region of the mucus layer. The mucus is loosely adhered and easily shed to clear the pathogens, thus, affecting the residence time of delivered compounds. The test compounds may be absorbed by enterocytes only if they can permeate through the mucus layer.

HT29 cell line, similar to the Caco-2 cell line, is derived from human colon adenocarcinoma. When adapting HT29 cells with a high dose of methotrexate (MTX), these cells transform to exhibit almost exclusively mucus-secreting phenotype (Martínez-Maqueda et al. 2015). HT29-MTX cells behave like goblet cells, differentiate and grow into a polarized monolayer. It does not form tight junction as tight as Caco-2 cells nor express P-glycoprotein but it can produce mucus to cover the whole monolayer of Caco-2 cells, shaping the Caco-2/HT29-MTX co-culture model to closely resemble the human intestine (Araújo and Sarmento 2013). Different studies have demonstrated the best-fit ratio to mimic physiological conditions for Caco-2 to HT29-MTX cells to be 9:1. However, other ratios like 8:2 and 7:3, are also used in various studies (Pan et al. 2015; Schimpel et al. 2014).

13.4.3 Caco-2/Raji B Co-culture

As multivariate processes and heterogeneous cell population are involved in the intestinal absorption, another in vitro cellular model has been established by co-culturing Raji B and Caco-2 cell lines, representing human intestinal follicle associated epithelium (FAE) morphology (des Rieux et al. 2007). Raji B cells originated from a human Burkitt's lymphoma. When cultured with Caco-2 cells, it can induce some Caco-2 cells to acquire M cell phenotype. M cells are specialized epithelial cells that internalize the antigen from the lumen of human intestines and transport it to the immune cells of mucosal lymphoid tissues for the initiation of immune responses (Dillon and Lo 2019). They are characterized with higher transcytotic capacity, lack of glycocalyx layer, sparse and irregular microvilli, and

can transport a broad range of compounds without digesting them (Pereira et al. 2016; Sarmento et al. 2012). Therefore, Caco-2/Raji B co-culture model is useful for the assessment of uptake of encapsulated compounds through M cell-associated pathway.

13.4.4 Caco-2/HT29-MTX/Raji B Triple Co-culture

To further mimic the human intestinal epithelium, a triple co-culture consisting of Caco-2 cells, HT29-MTX cells and Raji B cells has been developed (Araújo and Sarmento 2013; Schimpel et al. 2014). All the main components involved in the intestinal absorption and transepithelial transport are found in this model, i.e. Caco-2 cells to resemble the enterocytes, HT29-MTX cells to resemble the goblets cells, and Raji B cells to promote M cell phenotype on some Caco-2 cells. On the grounds that these three cell lines are capable of maintaining their own functions during the co-culturing, it is thus far the in vitro cellular model that best resembles the human intestinal epithelium and produces more reliable results of bioavailability.

13.4.5 Cellular Uptake Assay

Cellular uptake of nanoemulsions in the gastrointestinal tracts relies on the diffusion through the mucus layer (first barrier) and interaction with the intestinal epithelium (second barrier) (Cui et al. 2017). Evaluation of the uptake efficiency of nanoemulsions by intestinal cells can be done by selecting the right cellular model that corresponds to the experiment purposes and is a useful screening assay during the early stage of formulation.

All the cell types listed in Table 13.3 are generally maintained using the Dulbecco's modified eagle medium (DMEM) (high glucose) supplemented with 10% to 20% of fetal bovine serum (FBS), 1% nonessential amino acid, and 1% penicillin and streptomycin and incubated in a humidified cell incubator supplied with 5% CO_2 at 37 °C (Kleiveland 2015; Lea 2015; Martínez-Maqueda et al. 2015). Prior to the cellular uptake experiment, the cells are harvested and seeded into the plates and incubated to reach approximately 90% to 95% confluency (Fan et al. 2017b; Yi et al. 2015). Thereafter, the cells are co-incubated with nanoemulsions for a specific duration (typically 1–6 h). In order to determine the suitable range of concentrations used for nanoemulsions, cytotoxicity assay is usually conducted beforehand. After incubation for a predetermined period, the nanoemulsions are removed and the cell monolayer is washed for three times to stop the cellular uptake. The dissociated cell monolayer is then collected and lysed prior to the quantification of amount uptake. The uptake concentration of the encapsulated compounds in the samples can be determined using analytical instruments such as ultraviolet spectrophotometer, high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), gas chromatography (GC), and gas

chromatography-mass spectrometry (GC-MS). The cellular uptake efficiency is then calculated based on the following equation:

$$\text{Cellular uptake efficiency } (\%) = \text{A\_cells}/\text{A\_total} \times 100\% \quad (13.1)$$

where A_{cells} is the amount of encapsulated compounds associated with the cells, A_{total} is the initial amount of encapsulated compounds present in the feed (Wei et al. 2015).

Alternatively, flow cytometry analysis can be performed to evaluate the cellular uptake efficiency if the nanoemulsions are coupled with a fluorophore. Nanoemulsions can also be labelled with Nile red for a qualitative measurement. After the co-incubation, the cell membrane and nucleus of the fixed cells are labelled with Alexa flour 488 and 4′,6-diamidino-2-phenylindole (DAPI), respectively (Walia and Chen 2020). The cellular uptake can be detected by viewing under a confocal laser scanning microscope (CLSM) and the images are taken for qualitative comparison.

Additionally, the possible cellular uptake pathways of nanoemulsions can be characterized in the cellular uptake study by pre-incubating cells with specific inhibitors prior to the addition of nanoemulsions. For instance, sodium azide inhibits energy-dependent transport (Fan et al. 2017b), 5-(*N*-ethyl-*N*-isopropyl)-amiloride inhibit macropinocytosis, cytochalasin D inhibits phagocytosis and micropinocytosis (Dutta and Donaldson 2012), chlorpromazine inhibits clathrin-mediated endocytosis (Dutta and Donaldson 2012), and nystatin inhibits lipid raft/caveolae-mediated endocytosis (Fan et al. 2017b), and filipin inhibits caveolae-mediated endocytosis (Vocelle et al. 2016). The reduction of cellular uptake efficiency following incubation with the inhibitors indicates the involvement of a specific pathway during the cellular uptake of nanoemulsions.

13.4.6 Transport or Permeability Assay

In this assay, a Transwell system is utilized to create apical and basolateral compartments which mimics in vivo intestinal lumen and the epithelial layer. This setup is useful for the investigation of intestinal lumen-to-blood permeability of test compounds. Hence, the rates of permeability of the test compounds generated from this assay can be used to predict the in vivo human absorption.

Prior to the experiment, the cells are seeded onto the apical side of transwell inserts and cultured to form monolayers (around 21 days). The integrity of the cell monolayer is determined by measuring the transepithelial electrical resistance (TEER) using an epithelial volt-ohm-meter. The TEER value ($\Omega\ cm^2$) is determined based on the following equation:

$$\text{TEER value} = \text{TEER\_monolayer}/\text{TEER\_blank} \times \text{A\_well} \tag{13.2}$$

where $TEER_{monolayer}$ (Ω) is the resistance of cell monolayer in culture medium together with the filter membrane, $TEER_{blank}$ (Ω) is the resistance of culture medium with filter membrane alone, and A_{well} (cm^2) is the surface area of the membrane (Silva et al. 2019).

Transport studies are performed with the cell monolayers that achieved the TEER values stated in Table 13.3, depending on the type of in vitro cellular models. Nanoemulsions are then added to the donor compartment, which is apical side for apical to basolateral (A-to-B) transport study or basolateral side for basolateral to apical (B-to-A) transport study. At predetermined time intervals, the samples are withdrawn from the receiver compartment, the basolateral compartment for A-to-B transport study or apical compartment for B-to-A transport study. At the end of the experiment, the TEER values are measured again to ensure the integrity of cell monolayers. The concentration of the encapsulated compounds in the collected samples is analysed for the calculation of apparent permeability coefficient (P_{app}) (cm s^{-1}).

Normally, the P_{app} of the encapsulated compounds is calculated from the linear slope of the plot based on the following equation:

$$\text{P\_app} = \text{dQ}/\text{dt} \times 1/(\text{A} + \text{C\_0}) \tag{13.3}$$

where *dQ/dt* (μmol s^{-1}) is the steady-state flux (the slope of the concentration of encapsulated compounds against time), A (cm^2) is the surface area of the membrane, and C_o (μM) is the initial concentration of encapsulated compounds in the donor compartment (Heinlein et al. 2014).

Calculation of P_{app} using this equation is only applicable in the instance of sink conditions when the concentration in the receiver compartment does not exceed 10% of the concentration in the donor compartment. Thus, to maintain sink conditions throughout the transport study, sampling intervals should be chosen carefully so that the receiver concentration does not exceed 10% of the donor concentration per time interval (Hubatsch et al. 2007). If the sink conditions are not achievable, especially with higher permeable compounds, P_{app} is determined from a non-linear curve-fitting equation (non-sink conditions) as stated below:

$$\begin{aligned}&\text{C\_(R(t))} = \text{M}/(\text{V\_D} + \text{V\_R}) + (\text{C\_(R(0))} + \text{M}/(\text{V\_D} + \text{V\_R}))\\ &\text{e\^{}}(-\text{P\_app A}(1/\text{V\_D} + 1/\text{V\_R})\text{t})\end{aligned} \tag{13.4}$$

where $C_{R(t)}$ (μM) is the concentration of the encapsulated compounds in the receiver compartment at sampling time t measured from the start of the time interval, M (μmol) is the total amount of encapsulated compounds present in the system,

V_D (μL) and V_R (μL) are the volumes of donor and receiver compartments, respectively, $C_{R(0)}$ (μM) is the concentration of the encapsulated compounds in the receiver compartment at the start of the time interval, and A (cm^2) is the surface area of the membrane (Heinlein et al. 2014).

Generally, if the P_{app} of A-to-B transport is more than 1.0×10^{-5} cm s^{-1}, it indicates that the encapsulated compounds are well transported. In contrast, if the P_{app} of A-to-B transport is less than 1.0×10^{-7} cm s^{-1}, the encapsulated compounds are poorly transported (Yang et al. 2017).

Subsequently, the efflux ratio is determined according to the following equation:

$$\text{Efflux ratio} = \text{P\_(app B} - \text{A)/P\_(app A} - \text{B)} \tag{13.5}$$

where $P_{app\ A\text{-}B}$ is the permeability coefficient of apical-to-basolateral transport, and $P_{app\ B\text{-}A}$ is the permeability coefficient of basolateral-to-apical transport.

Efflux ratio is used to determine the extent of efflux transporters influence on the intestinal absorption, where efflux ratio of more than two indicates that the encapsulated compounds are possibly the substrates of efflux transporters (Crowe and Wright 2012). Efflux transporters such as P-glycoprotein, breast cancer resistance protein (BCRP) and multidrug resistance-associated protein 2 (MRP2), which act as the first line of defence in the intestine epithelium, may forward the absorbed compounds back to the intestinal lumen and limit the intestinal absorption (Murakami and Takano 2008). An encapsulated compound can be a substrate for one or more than one efflux transporters. Co-incubation of nanoemulsions with a specific inhibitor for each efflux transporter or a combination can help to discover the particular efflux transporter that takes part in attenuation of intestinal absorption. Commonly, verapamil, cyclosporine A, or ketoconazole are added as an inhibitor for P-glycoprotein while probenecid or indomethacin are the inhibitors for MRP2, and fumitremorgin C is the inhibitor for BCRP (Murakami and Takano 2008). After adding the inhibitors into the permeability study, a reduction of efflux ratio when compared to the efflux ratio determined without inhibitor is an indication of the involvement of the specific efflux transporter in attenuating intestinal absorption.

At the end of the experiment, the sample should be collected from the donor compartment in order to determine the concentration change at the end of the experiment and calculate the mass balance of the transport study. It is calculated based on the following equation:

$$\begin{aligned}\text{Mass balance } (\%) = \Big(&\text{C\_(D(End))V\_D} + \sum \text{(C\_(S(t))\ V\_(S(t)))} \\ &+ \text{C\_(R(End))V\_(R(End)))/((C\_(D(0))\ V\_(D(0))))} \\ &\times 100\end{aligned} \tag{13.6}$$

where $C_{D(End)}$ and $C_{D(0)}$ (μM) are the respective concentration of the encapsulated compounds in the donor compartment at the end and start of the experiment, $C_{R(End)}$ (μM) are the respective concentration of the encapsulated compounds in the receiver compartment at the end of the experiment, $C_{S(t)}$ (μM) is the concentration of the encapsulated compounds in the samples withdrawn at different time intervals, V_D, $V_{S(t)}$ and $V_{R(End)}$ (μL) are the respective volumes (Heinlein et al. 2014).

For an acceptable approximation of the P_{app}, high mass balances of more than 80% are desirable to ensure an unambiguous comparison of P_{app} of different nanoemulsion formulations (Hubatsch et al. 2007). Low mass balances have been reported frequently in the transport study, leading to underestimation of the P_{app} and thus, impair the prediction of bioavailability of test compounds. Usually, low mass balances are due to non-specific adsorption to the transwell apparatus, retention in monolayer cells, or metabolization of test compounds. To improve the mass balance of the transport study, bovine serum albumin can be added to prevent non-specific binding where the concentration of test compounds retained in the cells can be determined and included in the calculation and metabolites can be measured to compensate the loss of test compounds (Heinlein et al. 2014; Hubatsch et al. 2007). Improvement of mass balance is particularly essential to ensure an accurate comparison of P_{app} of different formulations of nanoemulsions.

Often, intact or undigested nanoemulsions are used in the experiments. Hence, to mimic the route of the gastrointestinal tract following oral administration, digested nanoemulsion obtained from in vitro digestion should be used. Many studies have confirmed that the physicochemical properties and composition of formulated nanoemulsions influence the cellular uptake efficiency and intestinal permeability of nanoemulsions (Fan et al. 2017b; Liu et al. 2019; Silva et al. 2019). At the same time, several researchers have revealed that the physicochemical properties of digested nanoemulsions were significantly different from that of pristine nanoemulsions (Cheong et al. 2016; Teixé-Roig et al. 2020). Also, substantial pieces of evidence have indicated that the cellular uptake efficiency and P_{app} of the digested nanoemulsions were also considerably different from that of undigested nanoemulsions (Akbari et al. 2017; Yu and Huang 2012). Hence, a tiered approach should be adopted to obtain more conclusive data; in vitro digestion followed by cellular uptake or permeability studies. Table 13.4 summarizes the studies that emphasize the significant effects of physicochemical properties and composition of formulated nanoemulsions on intestinal permeability as well as cellular uptake efficiency.

Table 13.4 Recent nanoemulsions studies incorporating cellular uptake and transport assays using in vitro cellular models

Nanoemulsion	Characteristic of nanoemulsion	In vitro digestion	In vitro cellular model (experimental design)	Main findings	References
Oil phase: Labrafac lipophile WL1349 Emulsifier: Solutol HS15	Particle size: 80 nm, 200 nm, 550 nm and 1000 nm	No	Caco-2, Caco-2/HT29-MTX, Caco-2/Raji and Caco-2/HT29-MTX/Raji (cellular uptake and permeability assay)	Cellular uptake and permeability of nanoemulsions across different cellular models followed the sequence of Caco-2/Raji B > Caco-2/HT29-MTX/Raji B > Caco-2 > Caco-2/HT29-MTX, and it was size-dependent	Xia et al. (2017)
Encapsulated compound: β-carotene Oil phase: Corn oil Emulsifier: lecithin or sodium caseinate	Lecithin-based nanoemulsion: Particle size: 350 nm Zeta potential: -58.81 ± 2.56 mV PDI: 0.603 ± 0.006 Sodium caseinate-based nanoemulsion: Particle size: 290 nm Zeta potential: −53.41 ± 1.83 mV PDI: 0.773 ± 0.001	Yes	Caco-2 and Caco-2/HT29-MTX (Permeability assay)	Higher concentration of β-carotene was found in Caco-2 monoculture than Caco-2/HT29-MTX co-culture. In both cellular models, the concentration of β-carotene was significantly higher in lecithin-based nanoemulsion as compared to sodium caseinate-based nanoemulsion	Gasa-Falcon et al. (2021)
Encapsulated compound: β-carotene Oil phase: Corn oil Emulsifier: Tween 20	Organogel-tween 20-based nanoemulsion: Particle size: 218 nm PDI: 0.280	Yes	Caco-2 monoculture	Cellular uptake of β-carotene was higher in micelles form as compared to the corresponding undigested nanoemulsion and suspension. Caveolae/lipid raft-dependent endocytosis was the main endocytosis pathway	Fan et al. (2017a)
Encapsulated compound: curcumin Oil phase: medium chain	Organogel-Tween 20-based nanoemulsion: Particle	Yes	Caco-2 monoculture (permeability assay)	Permeation rate across Caco-2 monolayer of digested nanoemulsion was significantly	Yu and Huang (2012)

triglyceride (MCT) and monostearin Emulsifier: Tween 20	size: 218 nm PDI: 0.280			higher than intact (undigested) nanoemulsion.	
Encapsulated compound: Pterostilbene Oil phase: MCT, sunflower oil and olive oil Emulsifier: Sodium caseinate	MCT-based nanoemulsion: Particle size: 175 nm Digested: 159 nm Sunflower oil-based nanoemulsion: Particle size: 202 nm Digested: 183 nm Olive oil-based nanoemulsion Particle size: 205 nm Digested: 170 nm	Yes	Caco-2 monoculture (transport or permeability assays)	Higher bioaccessibility of pterostilbene was observed in MCT-based nanoemulsion due to higher polarity of oil compared to sunflower oil and olive oil. Permeation rate across Caco-2 monolayer showed similar observation with the highest rate found in MCT-based nanoemulsion.	Liu et al. (2019)
Encapsulated compound: β-carotene Oil phase: olive oil, canola oil, corn oil, palm oil, coconut oil, and MCT Emulsifier: Sodium caseinate	Six types of nanoemulsions: Particle size: 167.4–178.8 nm	Yes	Caco-2 monoculture (cellular uptake assay)	Higher bioaccessibility of β-carotene was observed in olive oil-, canola oil- and corn oil-based nanoemulsion as compared to palm oil-, coconut oil-, and MCT-based nanoemulsion. Still, no significant difference was observed among their corresponding micelles fraction	Yi et al. (2015)
Encapsulated compound: 5-demethylnobiletin Oil phase: MCT, canola oil (long chain triglycerides (LCT)) Emulsifier: Sodium caseinate	MCT-based nanoemulsion: Particle size: 173 ± 3 nm Zeta potential: 40.9 ± 1.7 mV LCT-based nanoemulsion:	Yes	Caco-2 monoculture (transport or permeability assays)	Higher bioaccessibility of 5-demethylnobiletin was found in MCT-based nanoemulsion as compared to LCT-based nanoemulsion. However, transport of 5-demethylnobiletin in mixed micelles was higher in LCT-based nanoemulsion than MCT-based	Yao et al. (2020)

(continued)

Table 13.4 (continued)

Nanoemulsion	Characteristic of nanoemulsion	In vitro digestion	In vitro cellular model (experimental design)	Main findings	References
	Particle size: 178 ± 2 nm Zeta potential: 40.9 ± 1.7 mV			nanoemulsion due to higher metabolic activity in the latter.	
Encapsulated compound: curcumin Oil phase: MCT Emulsifier: whey protein isolate and chitosan for multilayer nanoemulsion	Nanoemulsion: Particle size: 186.0 ± 3.9 nm PDI: 0.124 ± 0.014 Zeta potential: −51.9 ± 2.4 mV Multilayer nanoemulsion: Particle size:189.0 ± 3.4 nm PDI: 0.167 ± 0.004 Zeta Potential: 40.1 ± 1.2 mV	Yes	Caco-2 monoculture (transport or permeability assays)	Significant changes were observed in particle size and zeta potential of nanoemulsion and multilayer nanoemulsion following in vitro digestion. Chitosan layer-by-layer technique increased the apparent permeability coefficient of curcumin through Caco-2 monolayer cells	Silva et al. (2019)

13.5 Conclusion

With the continuous progression in research related to nanoemulsion system, it is inevitable for researchers to incorporate more advanced analyses in their studies in order to provide a more insightful and comprehensive understanding of this subject area. In this chapter, a few relevant analyses that complement fundamental analyses commonly used in the characterization of nanoemulsions are described. It is hoped that the complementary techniques described in this chapter would be useful and act as a platform for researchers to continuously explore newer analytical techniques so that greater advancement in this field can be achieved.

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Chapter 14
Processing Contaminants in Edible Oil

Kok Ming Goh, Kar Lin Nyam, and Chin Ping Tan

Abstract Mono-chloropropane-diol (MCPD) esters and glycidyl esters (GE) are processing contaminants that occur during vegetable oil refining processing. Their presence in various source of refined vegetables oil was reviewed and reported in EFSA journal 2016. From toxicological assessments, MCPD esters and GE are harmful when a free MCPD and/or glycidol are released from the parent esters, along a human gastrointestinal tract. To date, 3-MCPD is classified as a potential carcinogen which can deplete kidney function and male fertility in animal studies, while glycidol shows mutagenic properties. A refined palm oil is reported to contain highest amount of MCPD esters and GE among other refined vegetable oils such as sunflower and soybean oils. This chapter critically reviewed the formation pathways, and the formation of MCPD esters and GE as process-contaminants during the refining process. Although, it is known that a deodorization step which involved high temperature will cause MCPD esters and GE formation, the presence of other precursors such as chlorine and partial acylglycerol are contributing to the elevated MCPD esters and/or GE content. On the other hand, the detection method of MCPD esters and GE is also an important element to evaluate a palm oil contamination level. This chapter discussed the different available methods such as indirect and direct detection methods. A recently published method which uses a modified AOCS official method by gas-chromatography tandem mass spectrometry method (GC-MS/MS) was also discussed. The analysis of MCPD esters and GE by GC-MS/MS is highly recommended as it increases sensitivity, repeatability and recovery of the compounds. Most importantly, the chromatogram and peak shape are improved as compared to conventional GC-MS detection by selected ion mode (SIM). In

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addition, sample preparation method prior chromatography also being reviewed in this chapter to provide references to difference needs. Finally, the occurrence and mitigation of MCPD esters and GE in edible oil should be an all-rounded approach.

Keywords 3-Mono-chloropropane-diol esters · Glycidyl esters · Deodorization · Carcinogen · Genotoxicity · Direct method · In-direct method

14.1 Introduction

The ester forms of mono-chloropropane-diol (MCPD) and glycidyl esters (GE) are processing contaminants in the fat and oil industries. MCPD esters were first discussed as 3-mono-chloropropane-1,2-diol (3-MCPD) esters in fat and oil samples, and more related research has been conducted thereafter (Cheng et al. 2017). However, the isomer of 3-MCPD ester, 2-mono-chloropropane-1,3 diol (2-MCPD) ester, should receive the same attention from researchers because its toxicological effects on the human body are considered equal to that of 3-MCPD ester (Wallace et al. 2016). MCPD esters and GE occurrences in the food system are a complex chain. Contamination is normally caused by contaminated fats and oil as a part of food ingredients, and these compounds can be formed during different processing methods, such as frying.

According to toxicological assessments, MCPD esters and GE are harmful to the human body when 3-MCPD and/or glycidol are released from the parent esters (MCPD esters and GE) via lipase-catalyzed hydrolysis along the human gastrointestinal tract (Bakhiya et al. 2011). The European Scientific Committee on Food classified 3-MCPD as a potential carcinogen to the human body, and this toxicity depletes kidney function and male fertility (study using rats). On the other hand, glycidol shows mutagenic properties in a range of in vitro and in vivo genotoxicity tests. Although toxicological studies involving 2-MCPD are still rare, the toxicological effects may be different from those of 3-MCPD. However, the suggestion from EFSA 2016 still recommends treating 2- and 3-MCPD equally in terms of toxicological effects because the release of MCPD from their parent esters is assumed to be a 100% rate along the human digestion system (Wallace et al. 2016).

Since edible oil is a very important component in our daily diet that is almost impossible to avoid, the intake limit of these contaminants present in fats and oils should be addressed. The European Scientific Committee on Food has suggested that the tolerable daily intake (TDI) of 3-MCPD is 2 μg/kg body weight per day (kg/w per day). Next, glycidol was classified by the International Agency for Research on Cancer as a Group 2A genotoxic carcinogen that is probably carcinogenic to humans. Therefore, the recommended intake of glycidol is based on the ALARA (as low as reasonably achievable) principle (Wallace et al. 2016).

According to EFSA 2016, palm oil contains the highest amount of MCPD esters and GE among the investigated edible fats. It is well understood that MCPD esters and GE are process contaminants, mostly formed during the physical refining

process (Wallace et al. 2016). Palm oil is a low-cost, versatile and easily available ingredient that is widely used in many consumer products. Currently, most commercial infant formulas are fortified with refined palm oil due to its abundance of palmitic acid. Human milk is rich in C 16:0 fatty acids, which provide approximately 10% of the nourishment for breastfed infants (Innis 2016). Therefore, palm oil is used as the main ingredient in infant formula to match the human milk composition.

Recently, the EU regulation set the maximum level for GE at 1 ppm in vegetable oils and fats placed on the market for the final consumer or for use as an ingredient in food. In addition, the GE maximum level for vegetable oils and fats destined for the production of baby food and processed cereal-based food is set at 0.5 ppm (Bonwick and Birch 2019). However, the maximum level of 3-MCPD ester is suggested by the European Commission to be 1.25 ppm for oils and fats from coconut, maize, rapeseed, sunflower, soybean and palm kernel oil and mixtures of oils and fats from this category. The European Commission also defined the 2.5 ppm 3-MCPD limit applied to other vegetable oils and fish oil and mixtures of oils and fats from this category, as well as for mixtures of oils and fats from the two aforementioned categories.

The studies of 3-MCPD esters and GE are mainly focused on the formation pathway (with the presence of different potential precursors) and mitigation strategies (removal of precursors), especially during the vegetable refining process and/or food processing processes. As the global demand for refined vegetable oil, especially refined palm oil, increases the safety issues related to these contaminants should always be highlighted. Likewise, in accordance with the enforcement of maximum limits for GE and MCPD esters in food products, analysis advancement to detect the presence of these contaminants must be revised and improved periodically.

14.2 Formation Pathway of MCPD Esters and Glycidyl Esters

To date, there are primarily four mechanisms for the formation of 3-MCPD esters, which involve Sn2 nucleophilic attack by chlorine ions. MCPD esters can be formed via the following possible pathways: first, direct substitution of a chlorine anion for a glycerol carbon atom, replacing the OH group; second, direct substitution of a chlorine anion for a glycerol carbon atom, replacing the fatty acid ester chain; third, via an intermediate epoxide ring before nucleophilic attack by a chlorine anion at the Sn2 position; and fourth, via an intermediate alkyloxonium group before nucleophilic attack by a chlorine anion at the Sn2 position (Bakhiya et al. 2011). In addition, a more recent study suggested that chlorine radicals or chlorine compounds attack a cyclic acyloxonium free radical (CAFR) intermediate through a free radical mechanism. (Zhang et al. 2013, 2015). Zhang et al. described a proposed mechanism for the formation of 3-MCPD diesters in the presence of DAG under high temperature and low moisture conditions. The mechanism is described as follows: a

hydroxyl radical on the C-3 position of a DAG is eliminated at high temperature to form radicals; then, this carbon-centered radical can attack the ester carbonyl group at the C-2 position to form a cyclic acyloxonium free radical (CAFR). CAFRs react with Cl radicals to form 3-MCPD diesters, or a CAFR extracts a Cl radical from any chlorine-containing agent (e.g., HCl or NaCl) to form a 3-MCPD diester and release new radical species (from the chlorine-containing agent, e.g., •H or •Na) (Zhang et al. 2013).

In addition, the presence of water and high temperature causes TAG hydrolysis, which cleaves the components into smaller constituents. The products of TAG hydrolysis are diacylglycerol (DAG) isomers, monoacylglycerol (MAG) isomers and free fatty acid chains. These acylglycerols undergo nucleophilic ring-opening substitution by chlorine ions through an intermediate acyloxonium cation to form MCPD fatty acid esters. These reactions can be triggered without the intermediate acyloxonium, but the products are formed via a direct nucleophilic substitution of ester or hydroxyl groups by a chlorine ion instead. In other options, cyclic acyloxonium free radicals can be formed by water elimination at high temperature, reacting with the chlorine radicals. For these reactions to occur, a high temperature is always favored for an MCPD to be formed (Crews et al. 2013; Rahn and Yaylayan 2011).

In an edible oil (palm-oil) refinery, the majority of MCPD esters are formed during the deodorization process (Craft et al. 2012). Chlorine is a critical compound that is responsible for the formation of MCPD esters during palm oil refining when the organochlorine decomposition rate in a partially refined palm oil has a strong relationship with the MCPD esters formed (Tiong et al. 2018). To date, the proposed mechanism of 3-MCPD esters involves the presence of chlorine, clearly showing that chlorine is an important precursor for MCPD to be formed in a system. In addition, organic and inorganic chlorine-containing substances are detected in edible oil during oil production (Craft et al. 2012). Therefore, controlling the chlorine-containing agent during the palm oil refining process serves as a good strategy to reduce the formation of 3-MCPD esters.

However, the formation of GE is more often related to DAG and MAG as the predominant precursors. GE can be formed by intramolecular rearrangement, followed by removal of the fatty acid chain from a DAG under high temperature. Theoretically, at high temperature, both 1,2- and 1,3-DAG readily release the fatty acid chain through hydroxyl group (proton) abstraction by the vinyl carboxyl group. This results in oxirane ring formation from the nucleophilic reaction of the alkoxide group. MAG, as another important precursor, although mostly present at lower levels in nondeodorized palm oil, has the tendency to form GE through the thermal dehydration of vicinal diol. However, it is believed that only 1(3)-MAG could undergo the reaction to form GE (Destaillats et al. 2012).

14.2.1 Overview of Refining of Crude Palm Oil

Since the 1960s, the palm oil refining industry has become one of the most important industries in Malaysia, contributing a significant portion of the nation's GDP. The number of refining factories in Malaysia has been growing since 1976. There were 15 refineries in 1976, and the number of refineries then increased; 74 and 75 refineries were recorded in 1987 and 1988, respectively (Sze Yi et al. 2018). Then, the number of refineries changed with the market demand over the years, and 52 refineries were recorded up to 2016 by the Malaysian Palm Oil Board, from data collected from the Ministry of Primary Industry (MPI) in Malaysia (Ramli 2017).

In 2018, crude palm oil (CPO) production declined compared to that in 2017, and the recorded production declined from 19.92 million tons to 19.52 million tons, an approximately 2% decrease. It is believed that the low CPO production in 2018 was mainly due to the lower fresh fruit bunch (FFB) yield performance. The Malaysian oil palm industry performance is summarized in Table 14.1 (Kushairi 2019).

The refining process of CPO involves pretreatment (degumming and bleaching), followed by deodorization. A refined CPO is called refined, bleached and deodorized (RBD) palm oil. The refining process is aimed at removing undesired components from CPO. As CPO is extracted from the fresh fruit bunches of palms, the amount of nonglyceride components, such as free fatty acids (FFAs), oxidation products, carotenoids and odoriferous substances, can be substantial. The refining process must be efficient to turn a CPO into quality edible oil for consumers that has the lowest amount of unwanted impurities and preserves the highest possible amount of triglyceride content.

To date, physical refining is a better option for refining CPO than chemical refining, which uses alkali to neutralize FFAs. The physical refining process is commonly used by refiners because of its maximum efficiency with minimum oil loss and lower chemical usage and waste effluent. However, the conditions applied during the physical refining process inevitably induce the formation of MCPD esters

Table 14.1 Malaysian oil palm industry performance during year 2017 and 2018

	2018	2017	Difference Vol./value	%
Opening stocks (mil tonnes)	2.73	1.67	1.07	63.9
CPO production (mil tonnes)	19.52	19.92	(0.40)	(2.0)
Fresh food bunch (FFB) yield (tonnes/hectare)	17.16	17.89	(0.73)	(4.1)
Oil extraction rate (%)	19.95	19.72	0.23	1.2
Palm oil exports (mil tonnes)	16.49	16.56	(0.07)	(0.4)
Palm oil imports (mil tonnes)	0.84	0.56	0.29	51.3
Closing stocks (mil tonnes)	3.22	2.73	0.48	17.7
CPO price (RM/tonne)	2232.50	2783.00	(550.50)	(19.80)
Export revenue (RM billion)	65.12	74.75	(9.63)	(12.9)

Adapted from MPOB and Kushairi (2019)

and/or GE, especially when the CPO is a lower quality grade. Generally, a CPO with high FFA often leads to higher MCPD and GE contents in refined palm oil.

14.2.2 Physical Refining

The physical refining of a CPO includes three major processes, namely, degumming, bleaching and deodorization. The degumming and bleaching steps are considered the pretreatment process for reducing impurities, preparing the CPO for the intense refining conditions during deodorization. A palm oil after the refining process should be clear, low in FFA (<1.0%) and free of any off-flavors (Tan et al. 2009). RBD palm oil can be further processed by fractionation for more specific applications (Habi Mat Dian 2018).

14.2.3 Degumming and Bleaching

Degumming is a crucial step that aims to remove impurities, including phosphatides, carbohydrates, proteins and trace metals. Few approaches can be used to achieve a refined oil with low phosphorus content (<5 ppm), namely, water degumming, acid degumming, dry degumming, enzymatic degumming, modified acid degumming and membrane filter degumming. Dry degumming is the most commonly practiced degumming in the palm oil refining process because palm oil has relatively low phosphatide contents. In the dry degumming process, metal ion and phosphatide complexes are decomposed by acid (food-grade phosphoric acid or citric acid) treatment. Bleaching earth is used after the dry degumming process to remove the degumming acid, phosphatides, pigment and other impurities by filtration (O'Brien 2008; Sim et al. 2020).

Dry degumming is normally started by adding 85% phosphoric acid at a proportion of 0.05–1.2% of the oil at 80–100 °C. At the same temperature, the reaction is held for a short time, between 15–30 min, and then treated with bleaching earth under vacuum (Sim et al. 2020). The basic bleaching earths used in edible bleaching processes are natural bleaching earth, activated bleaching earth, activated carbon, and synthetic amorphous silica (O'Brien 2008).

The bleaching earth functions as an absorbent to remove excessive phosphoric acid, soaps, trace metals, moisture, carotenoids, pigments, and oxidation products, and eventually, all of these impurities are removed by filtration.

14.2.4 Deodorization

Deodorization is the last step in a refining process, resulting in an edible oil that is stable, neutral in taste, colorless, and low in FFA content (Chong 2012). After degumming and bleaching, the oil is subjected to a vacuum steam distillation process that operates at elevated temperature. The oils are treated using stripping steam under vacuum conditions and normally at high temperatures (> 230 °C). The FFA and odoriferous substances can be removed with the aid of low pressure and high temperature because their boiling point is decreased under such conditions. In addition, vacuum conditions are necessary for the deodorization step to minimize the oxidation of the treated oil by reducing the air exposure (Gibon et al. 2007).

Although deodorization is a necessary process, this process inevitably induces the formation of MCPD esters and GE in the refined oil, mostly due to the high temperature used during deodorization. At 240 °C or below, the formation of 3-MCPD and GE is relatively low. However, when the temperature increases to higher than 240 °C, the content of contaminant esters can drastically increase with increasing temperature. With deodorization at 290 °C for 120 min, the GE content can reach 37 mg/kg. The high temperature conditions during deodorization indeed encourage the formation of MAGs and DAGs as a result of TAG hydrolysis, which serves as the precursor of GE formation. On the other hand, the 3-MCPD ester content can decrease when the deodorization time is extended at 290 °C. However, the use of such high temperatures is not significant from a practical refining point of view (Matthäus and Pudel 2014).

It is reported that a higher dosage of bleaching earth does not necessarily reduce the 3-MCPD ester and GE contents in the refined oil due to the actual optimal dosage to reduce esters in oil, which may vary for different types of bleaching earth. In terms of pH, a low pH bleaching earth (approximately pH 3) is not a good choice because it might facilitate 3-MCPD ester and GE formation. In addition, bleaching earth with a pH value of approximately 5 is a better option for the bleaching step because it can reduce the formation of 3-MCPD ester and GE while not compromising the refined oil qualities, such as color and FFA content (Hew et al. 2020). In short, the findings from most studies agreed that an acid-activated bleaching earth with acidic pH value should be avoided to control the formation of 3-MCPD ester and GE.

14.3 Detection Techniques for MCPD Esters and Glycidyl Esters

The advancement of detection and quantitation of MCPD esters and GE is a greater concern now as the limit of these contaminants is being enforced in regulations. To date, mainstream analysis still relies on chromatography techniques with or without sample preparation (derivatization). In most cases, an indirect method is favored.

The European Commission has not specified a standard analytical method for analysis and detection, but a method without a derivatization step can be presumed to face problems of lower sensitivity and selectivity, especially when a low concentration of MCPD esters and GE are present. To date, the three indirect methods validated by the American Oil Chemists' Society (AOCS) in 2013 are widely used in research and relevant industries.

14.3.1 Indirect Analysis Methods

Generally, MCPD ester and GE analysis detection often requires sample preparation to convert the compounds into derivatives detectable by the instruments. The molecular weights of free MCPD and glycidol are 110.5 g/mol and 74.08 g/mol, respectively. Additionally, the boiling points of MCPD and glycidol are considered to be very high at 213 °C and 167 °C, respectively (Wallace et al. 2016). Therefore, by considering their low molecular weights and high boiling points, detection by a chromatography technique could be relatively difficult, especially using gas chromatography. Furthermore, if a low molecular weight compound is directly injected into gas chromatography with mass spectrometry, it can be difficult to distinguish the targeted compound from the background noise, thus lowering the sensitivity. However, the application of liquid chromatography coupled with ultraviolet or fluorescent detection is also seemingly not viable for MCPD and glycidol detection because they are lacking in chromophores (Baer et al. 2010).

Therefore, a derivatization process can solve the problems mentioned in detecting these contaminants. Indirect analysis of MCPD ester and GE often requires tedious sample preparation. In most cases, the MCPD and glycidol must be cleaved from their parent esters and derivatized by a volatile agent. The derivatization allows higher repeatability in the sense that all of the MCPD and glycidol liberated from their parent esters are converted into a uniform structure (Hamlet et al. 2014; Reece et al. 2005). On the other hand, the direct technique requires minimal to no sample preparation. Although the direct technique is straightforward and able to provide highly detailed information on the chemical structure of esters, there is a need to obtain all possible standard compounds as references during quantification (Dubois et al. 2012).

Indirect methods are common among users because they are more convenient and affordable. To date, there are three indirect methods validated by the American Oil Chemists' Society (AOCS). Among the methods, the Official Method Cd 29a-13: 2- and 3- MCPD Fatty Acid Esters and Glycidol Fatty Acid Esters in Edible Oils and Fats by Acid Transesterification is widely used, especially for research purposes. This method quantitates 2-MCPD ester, 3-MCPD ester and GE as the three core compounds to be reflected in the chromatography. The transesterification process using mild acid (1.8% v/v sulfuric acid in methanol) and requires approximately 16 h to completely release MCPD and glycidol equivalent (GE is converted into monobromopropanediol during the sample preparation) from the parent esters.

Table 14.2 Comparison of analytical and statistical parameters between SIM and MRM modes using standards

Parameter		SIM			MRM	
	3-MCPD	2-MCPD	GE	3-MCPD	2-MCPD	GE
n	5	5	5	5	5	5
b	1.335	0.612	1.027	1.343	0.397	0.197
s_b	0.079	0.150	0.024	0.049	0.065	0.037
s_a	0.008	0.006	0.013	0.010	0.003	0.002
$S_{y/x}$	0.016	0.023	0.036	0.029	0.012	0.003
R^2	0.998	0.998	0.997	0.999	0.998	0.998
LOD, (mg/kg)	0.100	0.300	0.240	0.010	0.010	0.024
S/N	3.67	6.58	4.64	3.15	6.99	3.64
LOQ, (mg/kg)	0.300	0.500	0.600	0.050	0.050	0.060
S/N	10.27	11.80	13.96	11.13	13.81	10.12

n number of replications, *b* slope of calibration curve, S_b standard deviation of slope, S_a interception of y-axis standard deviation, $S_{y/x}$ regression line standard deviation, R^2 coefficient of determinations, *S/N* signal to noise ratio, *LOD* limit of detection, *LOQ* limit of quantification

Then, the derivatized MCPD and MBPD by phenylboronic acid is detected using GC-MS application with selected ion mode (SIM) detection.

However, the advanced detection method using the AOCS official method Cd 29a-13 coupled with GC-MS/MS application is also being adapted and studied by several research groups. A GC with a dual MS detector was able to enhance the sensitivity, selectivity and repeatability of the MCPD esters and GE detection and quantification. By using GC-MS/MS, targeted compounds are ionized as precursor ions, followed by fragmentation created by inert gas (normally an argon gas) bombardment. The detection of targeted compounds is based on the precursor/fragmentation ion pairs instead of the selected ion, which is then called multiple reaction monitoring (MRM) detection mode. In the case of MCPD esters and GE detection, MRM is particularly useful when the sample matrix is complex. The detection of MCPD esters and GE using tandem mass spectrometry has been demonstrated in fish oil samples (Garballo-Rubio et al. 2017), vegetable fats and oil samples (Zwagerman and Overman 2016) and baby food products (Nguyen and Fromberg 2020). These reports suggested that MRM is a robust, sensitive and repeatable method for MCPD esters and GE detection, especially at low concentrations. In addition, another study extended the GC tandem mass spectrometry application in chocolate products, margarine products and naturally low contaminated samples such as CPO and olive oil. Furthermore, the research critically evaluated the improvement made by MRM detection compared to SIM detection, including the sensitivity, recovery, repeatability, and chromatography peak shape (Goh et al. 2019b). Generally, MRM can detect the presence of these contaminants at much lower concentrations with a signal-to-noise (S/N) ratio above 3. The comparison between SIM and MRM detection by Goh et al. (2019a) is listed in Table 14.2.

14.3.2 Direct Analysis Methods

The direct analysis technique of MCPD esters and GE is advantageous to the user whenever the detailed lipid profile is of concern. To date, the official method of direct detection approved by AOCS, Method Cd 28–10: Glycidyl Fatty Acid Esters in Edible Oils, is the only method. This method describes the uses of liquid chromatography-mass spectrometry (LC-MS) to detect the different species of glycidyl fatty acid esters using five standards (C16:0-GE, C18:0-GE, C18:1-GE, C18:2-GE and C18:3-GE). Generally, the direct detection of GE is more established and feasible than that of 3-MCPD esters. GE naturally has less variation in its chemical structure, with a single bound fatty acid ester. However, MCPD esters have more variation due to being a combination of any two fatty acids among the long list of available fatty acid esters (Sim et al. 2020). Although the quantitation of MCPD esters and/or GE is still workable using a direct technique, the cost of analysis is increased due to the need for a standard for each ester species.

Solid phase extraction (SPE) clean up made up of C18 and a silica cartridge is typically involved in the direct analysis of MCPD esters (MacMahon et al. 2013). A reported study that compared the direct methods (SPE extraction and LC-ToF-MS) and indirect methods (acid methanolysis, HFBI derivatization and GC-MS) concluded that the proposed direct analysis is suitable for palm oil and palm olein samples. Also, other edible oils, such as sunflower and soybean oil, are also applicable when analytical standards are available. The results obtained from both methods are comparable (Dubois et al. 2012). However, a direct method is less applicable due to the extensive need for analytical standards.

14.3.3 Main Steps in the Analysis of MCPD Esters and GE via an Indirect Quantitation Approach

During indirect analysis, all the species of the MCPD esters are converted into a single compound. In this case, the mean of the indirect analysis is a total 3-MCPD ester equivalent, which is usually presented in units of ppm or mg/kg. To convert all the MCPD ester species into a single and recognizable compound, transesterification (removal of fatty acid chains or release of free MCPD) and derivatization procedures are required.

Generally, the removal of fatty acid chains can be achieved using acid (Divinova et al. 2004; Kusters et al. 2011; Zelinkova et al. 2017) or alkaline-catalyzed (Kusters et al. 2010; Weißhaar 2008) transesterification. In addition, the lipase enzyme (lipase from Aspergillus oryzae) is an applicable method for MCPD transesterification but is the least reported scenario (Hamlet and Sadd 2004), most likely due to its high cost and instability. Similarly, GE is cleaved into glycidol and one ester chain during the analysis by one of the transesterification methods mentioned above.

The AOCS Official Method Cd 29a-13 is a good example of the indirect analysis of MCPD esters and GE. This method is a process of converting GE into monobromopropandiol esters (MBPD) followed by MPCD and MBPD transesterification using an acid methanolic solution. Then, the free forms of MCPD and MBPD are derivatized using phenylboronic acid.

First, an acidic bromide solution is introduced to the sample to convert GE into MBPD. Because bromide ions are less reactive than chloride ions, the MCPD ester structure remains unreacted, provided the reaction temperature is controlled at 50 °C.

Then, transesterification of the esterified MCPD and MBPD is carried out using an acidic methanol solution. During transesterification, MCPD and MBPD in free form are released from their triacylglycerol or partial acylglycerol structures. Fatty acid methyl esters (FAMEs) and glycerol are also formed as unwanted components, which are then discarded using n-heptane. A 16 h transesterification duration is proposed by AOCS Official Method Cd 29a-13. This is because the acid concentration used in the method is relatively mild (1.8% sulfuric acid) and therefore allows a stable and steady reaction condition. Interestingly, a 4 h reaction time is sufficient to complete 3-MCPD ester cleavage, as concluded by Ermacora and Hrncirik (Ermacora and Hrncirik 2011). Another study by Abd. Razak et al. suggested that an 18 h reaction time is preferred due to the higher recovery observed among 16 h, 18 h, and 20 h reaction times (Abd. Razak et al. 2012). In addition, neither of the studies extended the test to a reaction time beyond 20 h. Therefore, it is suggested that the transesterification reaction time should remain anywhere between 4 h and 20 h during actual practice. Additionally, the derivatization process (samples and standards) should be controlled using deuterated 3-MCPD-d5 as an internal standard and preferably performed within a single preparation.

Although an alkaline-catalyzed transesterification method requires a relatively shorter reaction time during the transesterification reactions, 3-MCPD in the free form is reported to be unstable under alkaline solution (Hrncirik et al. 2011) (Kuhlmann 2011). AOCS Official Method Cd 29c-13 demonstrated the uses of strong alkaline (saturated sodium methoxide or sodium hydroxide) transesterification. The reaction time is recommended to be between 3.5 and 5 min, and any short delay can cause significant 3-MCPD loss due to poor stability in a strong alkaline. Even so, degradation of 3-MCPD can be maintained at a negligible level when the reaction conditions are carried out at extremely cold temperatures (−25 °C). Cold incubation during transesterification is proposed in AOCS Official Method Cd 29b-13 (Kuhlmann 2011). However, cold incubation requires a long reaction (18 h), a suitable cooling system (extra cold temperature incubation) and well-trained personnel to minimize errors.

After a 16 h transesterification in AOCS Official Method Cd 29a-13, the FAMEs and remaining TAGs should be separated from the mixture using liquid-liquid extraction. During the liquid-liquid extraction, a saturated sodium sulfate solution is used to trap MCPD and MBPD in their free form at the bottom layer, which is then derivatized using phenylboronic acid (PBA). Sodium sulfate is considered a better option for salting out agents because it does not contain any chloride components. It is known that a chloride salt can react with glycidol, partial acylglycerols and TAGs

to form 3-MCPD (Weißhaar 2008). This results in positive biased data in the analysis.

Finally, a derivatization process is the most crucial procedure to make MCPD and MBPD detectable. MCPD and GE (or glycidol) do not have good properties for separation by GC-MS application because of their low volatility. PBA and heptafluorobutyryl imidazole (HFBI) are the two commonly used derivatizing agents. PBA is considered a slightly better option than HFBI because HFBI derivatization requires strict anhydrous conditions. PBA usage was eventually adapted in the AOCS official methods (Crews et al. 2013). PBA is generally used in most reported studies due to its higher repeatability and being easy to handle. However, during general practices, excessive PBA accumulation over a long period of injection into a GC system can be detrimental to the system.

14.3.4 Other Methods

The detection and quantitation prediction of total MCPD esters using Fourier transform infrared spectroscopy (FTIR) coupled with chemometrics have been demonstrated recently. This is considered a rapid method for detecting the presence of MCPD esters and predicting the total MCPD ester content in the sample through FTIR spectroscopy. A study performed by Wong et al. (2019) evaluated the relationship between the 3-MCPD ester content in palm olein samples with the 800–700 cm^{-1} regions in the FTIR spectrum. It is known that a wavenumber between 800 and 700 cm^{-1} represents C-Cl bonds. The findings from the partial least square (PLS) model showed a positive correlation between the FTIR spectrum and the data obtained from indirect GC-MS analysis (Wong et al. 2019).

The aforementioned study provided fresh insight into how chemometric analysis (machine learning) can be used to predict the content of the total MCPD ester in palm oil samples. Goh et al. (2019a) extended the study into a high-level fusion model that operates at an optimal level to reduce the redundancy and provide complementarity among the established models. A total of six models, namely, the cubist, random forest, neural network, average neural network, PLS and consensus models, were developed using coding software (R software). The consensus model is a fusion model that consists of the other models as member models (Goh et al. 2019a). The findings suggested that a consensus model is optimal for total MCPD ester prediction compared to the member models (performing as individual models) in terms of coefficient of determination (R^2), root mean square error (RMSE), slope value and y-axis interception (Fig. 14.1).

Alternatively, sample preparation by automation is a growing trend penetrating the market of MCPD ester and GE analysis. Indeed, the invention of automated working stations to perform sample preparation has provided convenience to users. An automated working station can reduce uncertainty and error opportunities during manual preparation. A publication by Zwagerman and Overman transformed AOCS Official Method Cd 29c-13 into an automated indirect method. The adapted method

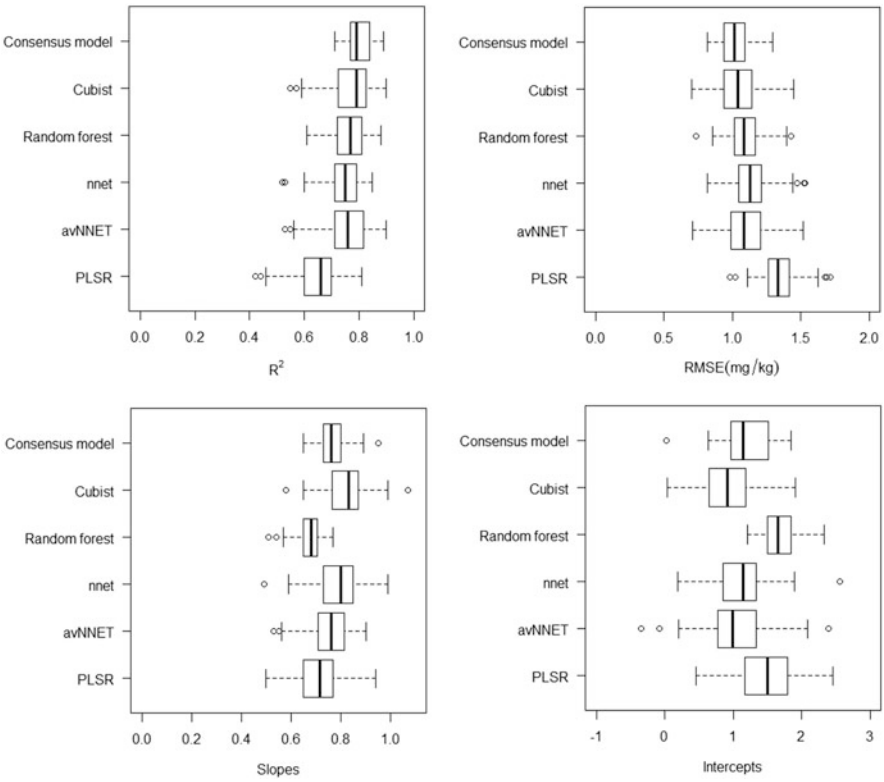

Fig. 14.1 Box plots of R^2, RMSE, slope values and Y-axis interception values of the models in total MCPD esters prediction

is able to ensure the separate detection of glycidol and minimize overestimation due to the conversion of 3-MCPD into glycidol during alkaline transesterification using a carbon-13 labeled internal standard (Zwagerman and Overman 2016). It is said that the automated method is an appropriate method for use in operational quality control, especially for routine checks of MCPD ester and GE analysis.

14.4 Conclusion

GE and 3-MCPD esters are processing contaminants formed during vegetable oil and palm oil processing. It is clearly shown that physical refining of palm oil produces most MCPD ester and GE formation due to the high temperature used and the presence of precursors. Chlorine is identified as one of the main precursors to form MCPD, but the forming mechanisms, root sources of chlorine atoms and the method of chlorine introduction into the refining process are still minimally

understood. The footprint of chlorine transfer from an oil palm plantation to refined palm oil should be investigated and be a focus of future studies to efficiently reduce the introduction of chlorine atoms into palm oil processing. Alternatively, MCPD esters and GE can be further formed during secondary food processing, especially in frying applications. In fact, the mitigation of MCPD esters and GE in the food system should be an all-around approach. Additionally, analysis advancement must be reviewed from time to time, especially regarding novel and rapid detection methods. Rapid detection of MCPD ester and GE using chemometric analysis or other approaches should be evaluated. The database of MCPD ester and GE contamination patterns in fat and oil samples (e.g., FTIR spectra) should be collected and processed by centralized executives for research purposes. In short, MCPD ester and GE contamination is an issue that involves the whole palm oil production chain, and every segment along the chain should play a role in delivering palm oil products with extremely low MCPD ester and GE contents.

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Chapter 15
Enzymatic Biodiesel Production from Low-Quality Waste Oils and Non-edible Oils: Current Status and Future Prospects

Mun Yuen Chang, Jun Mann Loh, Cher Pin Song, and Eng-Seng Chan

Abstract Depletion of fossil fuel, increasing energy demand and environmental degradation have motivated the development and application of biofuels. Biodiesel is a promising replacement for fossil diesel because it can be derived from renewable sources and it lowers the emission of greenhouse gases. This chapter comprehensively reviews the process for biodiesel production, especially from low-quality and non-edible feedstocks that contain a high amount of impurities. The enzymatic process is highlighted as it provides more environmental benefits as compared to the conventional chemical-catalysed transesterification process. An overview of the reaction scheme and mechanism of an enzymatic process is presented along with a critical discussion on the different types of enzymes that have been studied for biodiesel production. The industrial adoption of enzymatic processes for biodiesel production is also presented. Finally, the limitations and challenges of enzymatic processes for biodiesel production are discussed to provide a direction for process improvement in the future.

Keywords Biodiesel · Second generation feedstock · Immobilized enzyme lipase · Acid-base process · Glycerolysis · Free enzyme lipase

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15.1 Current Outlook

Biodiesel, also known as fatty acid methyl ester (FAME), is one of the best substitutes for fossil fuels. Besides contributing to the rapid reduction in greenhouse gases (GHGs), biodiesel is low in sulphur content and free of aromatic compounds, thus offering unrivalled environmental benefits. Additionally, biodiesel can be used directly in all diesel engines without any forms of modifications.

The global production of biodiesel has reached approximately 38 billion litres in 2019 (OECD, Food, and Nations 2019). The main producing and consuming countries include EU, USA, Argentina, Brazil, Indonesia, Malaysia and Thailand. In the past, biodiesel was conventionally produced from edible oils. However, due to the 'food versus fuel' conundrum, there is a shift towards the use of secondary lipid feedstock such as non-edible oils and low-quality feedstocks for biodiesel production.

The EU's Renewable Energy Directive (RED) has calculated the default GHG savings for various lipid feedstocks based on their production, transportation and distribution (Flach et al. 2018). It was found that the biodiesel production from waste vegetable oils or animal oils could result in substantial GHG savings up to 83%, which is 27–64% higher than the biodiesel produced from edible oils such as soybean, rapeseed, sunflower and palm. There-fore, many EU state members have implemented a double-counting system to encourage the use of waste-based feedstocks for biodiesel production.

This chapter presents an overview of the current status and new developments of biodiesel production technologies, especially enzymatic biodiesel production since it offers numerous advantages compared to the conventional chemical-catalysed process when low-quality lipid feedstocks are used.

15.2 Second-Generation Feedstocks

Non-edible oils can be obtained from plant species, such as Jatropha (*Jatropha curcas*), rubber seed tree (*Hevca brasiliensis*), neem (*Azadirachta indica*), mahua (*Madhuca indica* and *Madhuca longifolia*) and many more. These oils have been viewed as potential feedstocks for biodiesel production as they are readily available from many parts of the world and are much cheaper than edible oils. For instance, mahua is mainly found in India with an annual oil production of 60 million tonnes (Aransiola et al. 2019). Rubber seed trees, on the other hand, are mostly found in Southeast Asia, such as Indonesia, Malaysia and Thailand. In 2011, approximately 9.7 million ha of rubber plantation was available worldwide. The rubber seed yield was estimated to be 80–120 kg of rubber seed oil per ha (Zhu et al. 2014; Aransiola et al. 2019). Furthermore, neem trees are typically found in Nigeria, Sri Lanka, Malaysia and Burma. Aransiola et al. (2019) reported that the neem seed contains

20–30 wt% of oil, which translates to a total of approximately 2670 kg of neem oil per ha. Also, Jatropha oil which is obtained from *Jatropha curcas L.* can be found in subtropical countries, including Africa and Asia (Bart et al. 2010). The seed contains 20–60 wt% oil while the kernel contains about 40–60 wt% oil. Aransiola et al. (2019) stated that around 1900–2500 kg of Jatropha oil can be produced per ha.

Aside from non-edible plant oils, the use of waste cooking oils (WCOs) as the raw materials for biodiesel has been the focus for years. These waste oils have been used for cooking and frying at elevated temperatures in the food processing industry, fast-food restaurants, as well as in households. It is reported that most of the WCOs originate from vegetable oils, while a minority of them are produced from animal fats. The global availability of WCO is estimated to be 16.76 million tonnes per annum, whereby the supply is dominated by the United States that holds 55% of the global production, followed by China that accounts for a quarter of the world production (Gui et al. 2008; Nur and Wan 2016). Loh et al. (2006) stated that Malaysia only covers 3% of the global production, contributing about 0.5 million tonnes of WCO annually. Owing to the large consumption of vegetable oils, an enormous amount of WCO is generated and is expected to increase by 2% annually (Nur and Wan 2016). As such, a considerable amount of WCO could be utilised as a feedstock in the biodiesel industry.

Inedible animal fats are gaining substantial attention in the biodiesel sector in recent years. The main sources of animal fats are beef tallow, lard, poultry fat and fish oils (Feddern 2010). Animal fats can easily be sourced from slaughterhouses because they are required to follow a strict food regulation in managing their meat products and by-products. One of the animal fats producers stated that nine million tonnes of by-product is generated out of 258 million tonnes of meat produced globally (Vuure 2016). However, only approximately 33% of the animal fats is supplied to the biofuel industry in the EU (2.97 million tonnes) (AOCS 2020). Fuelled by higher customer demand, meat production has seen a dramatic rise in the past years and will continue to increase the animal fats production.

Recently, palm oil wastes such as sludge palm oil (SPO) and palm fatty acid distillate (PFAD) are found to be the potential feedstocks for biodiesel production. They are abundantly available in the leading palm oil producer countries. The world production of palm oil has reached 75.7 million tonnes in 2019, with Indonesia dominating the market (57%), followed by Malaysia (28%) (Statista 2020). According to industry source, fresh fruit bunches (FFB) typically contains 25% of palm oil. However, the oil extraction rate (OER) of palm oil is only about 20% per FFB; whereby approximately 1–2% will be lost into the waste stream, while the remaining oil are not extracted. Usually, the oil is lost during the milling process, such as sterilisation, decantation, and washing. These oil losses are known to be SPO which can be converted into biodiesel. It is estimated that up to 7.57 million tonnes of SPO is generated each year. On the other hand, PFAD is generated at the deodorisation stage in palm oil refineries. It has been reported by Panapanaan (2009) that approximately 8.2 kg of PFAD is produced from 1 tonne of FFB. Assuming all the CPO is sent to the refinery plant, 2.98 million tonnes of PFAD can be generated annually.

These waste feedstocks are generally high in free fatty acids (FFA), ranging from 5–90 wt%. Also, it contains water varying between 0.3–11 wt%. These impurities are found to interfere with the traditional alkaline-catalysed transesterification process for biodiesel synthesis. The presence of FFA and water would react with the alkaline catalyst to form soap that causes emulsification, thus reducing the biodiesel yield significantly. Several different process routes have been investigated in the past to convert high FFA feedstock into biodiesel and they will be discussed in the next section.

15.3 Process Routes for High FFA Feedstocks

15.3.1 Acid-Base Process

One of the common methods to convert high FFA feedstock into biodiesel is by using a two-step acid-base process. In this process, an acid catalyst is first used to esterify the FFA to biodiesel, hence reducing the FFA level in the feedstock. Subsequently, an alkali catalyst is used to convert the triglycerides (TAG) into biodiesel via the transesterification process. Conventionally, the two-step process would require 2–17 h to achieve a high biodiesel yield. The reaction time is dependent on several parameters such as initial FFA, catalyst loading, reaction temperature and alcohol-to-oil molar ratio. In the esterification step, sulphuric acid is commonly used as the acid catalyst, with a catalyst loading varying from 2 to 20 wt%. During this process, the initial FFA of the feedstock could reduce to less than 2 wt%. As for the subsequent transesterification step, the use of potassium hydroxide or sodium hydroxide as the alkaline catalyst is commonly adopted. The amount of alkaline catalyst used in this step is ranging from 1 to 2 wt%. Overall, the reaction temperatures for both stages are considered mild (i.e., ranging from 40 to 100 °C). Nevertheless, a high alcohol-to-oil molar ratio is typically required during esterification in order to push the reaction forward. As a result, the total alcohol-to-oil molar ratio required for both process is between 12:1 and 48:1. Furthermore, the corrosive nature of acid catalyst used in the esterification process could damage the process equipment. Moreover, the acid catalyst needs to be neutralised before the transesterification process. This additional step would increase the amount of wastewater generated.

15.3.2 Glycerolysis

Another well-known process for the pre-treatment of high FFA feedstock prior to biodiesel synthesis is glycerolysis, which is also known as re-esterification. In this process, FFA is converted into acrylglycerides. Glycerolysis involves the addition of glycerol to high FFA oils at a high temperature, ranging from 120 to 200 °C, in the presence of a zinc-based catalyst or without catalyst (Kombe et al. 2013). The FFA

in the feedstock reacts with crude glycerol to form monoglyceride (MAG), diglyceride (DAG) and triglyceride (Mićić et al. 2019). This technique produces low FFA oil (less than 3% FFA level) that makes it suitable for the conventional alkali transesterification process. In this approach, the high FFA oil is pre-treated without the need of alcohol, unlike the acid esterification. In addition, the potential of using crude glycerol for glycerolysis can save the glycerin purification cost. This could reduce the overall biodiesel production cost (Tu et al. 2017).

Nevertheless, glycerolysis is an energy intensive process because it requires high operating temperature. In fact, operating at high temperature tends to cause low MAG yield, undesirable decomposition and oxidation reaction. The oxidation reaction blackens the product and it produces a strong odour (Felizardo et al. 2011). Moreover, the yield of acylglycerides is limited by the reaction equilibrium. Hence, an excess amount of glycerol (4–70% of oil) is required to achieve a desirable yield. For instance, 40% excess glycerol was required to convert 99.6% of FFA in WCO into acylglycerides at 210 °C in 4 h (Cai et al. 2015).

15.3.3 Enzymatic Process

Recently, lipases have gained considerable attention as biocatalysts for low-quality feedstocks. Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are enzymes that catalyse the hydrolysis of esters and TAG to produce DAG, MAG, FFA and glycerol (Aguieiras et al. 2015). Lipases are also capable of catalysing transesterification, interesterification and esterification in non-aqueous media. In contrast to chemical catalysis, the enzymatic process offers a greener processing route to synthesise biodiesel. Enzyme provides numerous advantages over chemical catalysts such as no soap formation with low-quality lipid feedstocks that naturally contains a high amount of FFA and water. The absence of soap formation gives a higher biodiesel yield and will ease the subsequent product recovery processes. Furthermore, the by-product glycerol produced is of higher purity, which has a higher market value. In addition, enzyme catalyzed reaction requires lower methanol dosage compared to catalysis route, and the process can be carried out at ambient pressure and a milder temperature (30–50°). (Andrade et al. 2017a, b; Avhad and Marchetti 2019; Christopher et al. 2014).

Lipases are usually derived from animals, plants and microorganisms. Microbial lipases from fungal, bacterial or yeast are one of the most popular sources for biodiesel synthesis because they are generally more stable than animal and plant lipases (Hasan et al. 2006). Besides, microbial lipases can be mass-produced at ease and they showed great versatility in industry application. The most commonly used microbial lipases for biodiesel production include *Candida antartica* lipase B (CALB), *Candida rugosa*, *Pseudomonas cepacia*, *Thermomyces lanuginosus*, *Rhizomucor miehei*, *Aspergillus niger* and *Rhizopus orzyae*.

15.4 Reaction Scheme and Mechanism

Figure 15.1 shows the reaction schemes of the enzymatic route for biodiesel production. Lipases can catalyse transesterification, esterification and hydrolysis simultaneously during the biodiesel synthesis using low-quality lipid feedstocks.

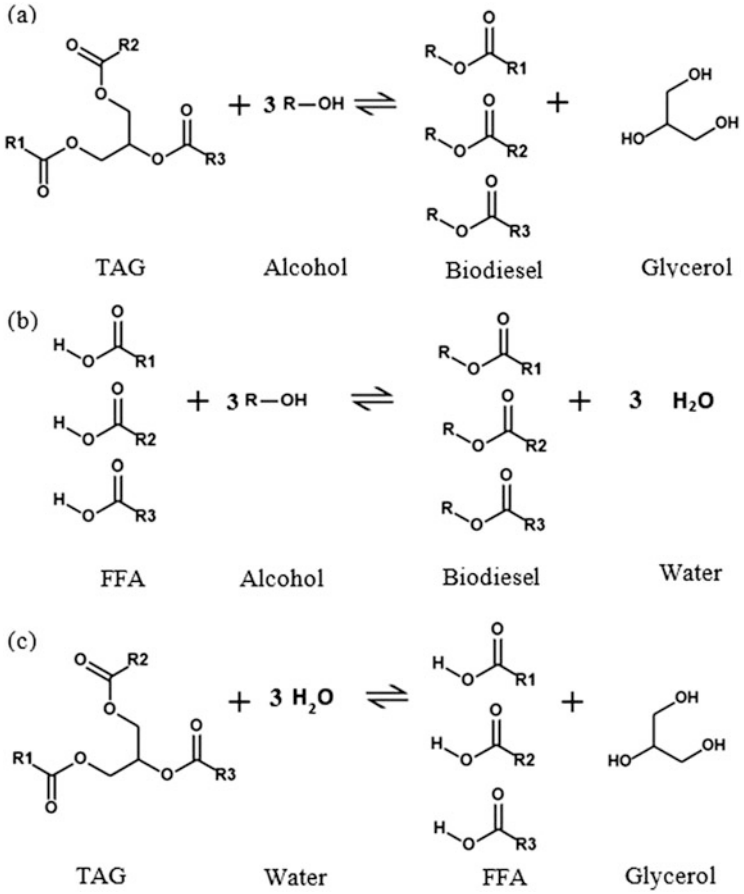

Fig. 15.1 Reaction schemes of enzyme-catalysed biodiesel production. (**a**) Transesterification; (**b**) Esterification; (**c**) Hydrolysis

For example, TAG and FFA are converted into biodiesel via transesterification and esterification, respectively. On the other hand, the presence of water can promote the hydrolysis of TAG to FFA. Subsequently, the FFA produced can be esterified to biodiesel. All these reactions are equilibrium-limited, hence the presence of excess substrates are required to promote the forward reaction towards the formation of products. For instance, a methanol concentration higher than the stoichiometric ratio necessary would favour the forward reaction of transesterification and esterification to generate more biodiesel.

The kinetics of the enzymatic reaction is commonly described by the Ping-Pong Bi-Bi mechanism (Clark and Blanch 1997). This mechanism is a double displacement reaction which involves the combination of an enzyme and a substrate to form two different products that happen consecutively (Fig. 15.2). The active site of the enzyme consists of two important functional groups, namely the hydroxyl group (nucleophile) and the amine group (eletrophile) (Panalotov and Verger 2000). When the enzyme is catalysing a reaction, protons are transferring across these two functional groups within the active sites of the lipase, which is similar to an acid-base reactions.

For transesterification reaction, an enzyme molecule (E) first attacks TAG to form an active E-TAG complex. The E-TAG complex quickly rearranges to an acylated E-TAG intermediate and liberates a DAG as the first product. Then, the acylated E-TAG complex will combine with an alcohol molecule (A), forming a secondary acylated E-TAG-A complex. FAME will be released as the second product, and the E will return to its original state. DAG and MAG will follow the same mechanism as illustrated in Fig. 15.2 to produce FAME through transesterification.

The mechanism of esterification and hydrolysis was then deduced based on the Ping-Pong Bi-Bi mechanism illustrated by Al-Zuhair et al. (2006). For instance, the esterification reaction is initiated when an E molecule attacks an FFA molecule to form an E-FFA complex (Fig. 15.3). Water (W) is the first product that is liberated from this mechanism; then alcohol will combine with the acylated E-FFA complex to generate FAME as the second product. On the other hand, the E molecule first attacks a TAG molecule in the mechanism of enzymatic hydrolysis, which is similar to the mechanism of enzymatic transesterification (Fig. 15.4). However, water is the second substrate that will attach to the acylated E-FFA complex in hydrolysis reaction, and FFA is produced as the second product in this mechanism.

15.5 Type of Enzyme

15.5.1 Immobilised Lipase

Enzymes are produced from microbes and it is typically more expensive than chemical catalysts. Therefore, there has been a substantial interest to recycle the enzyme in order to lower the production cost. One of the most popular methods to effectively recycle the enzyme is by enzyme immobilisation. The immobilised

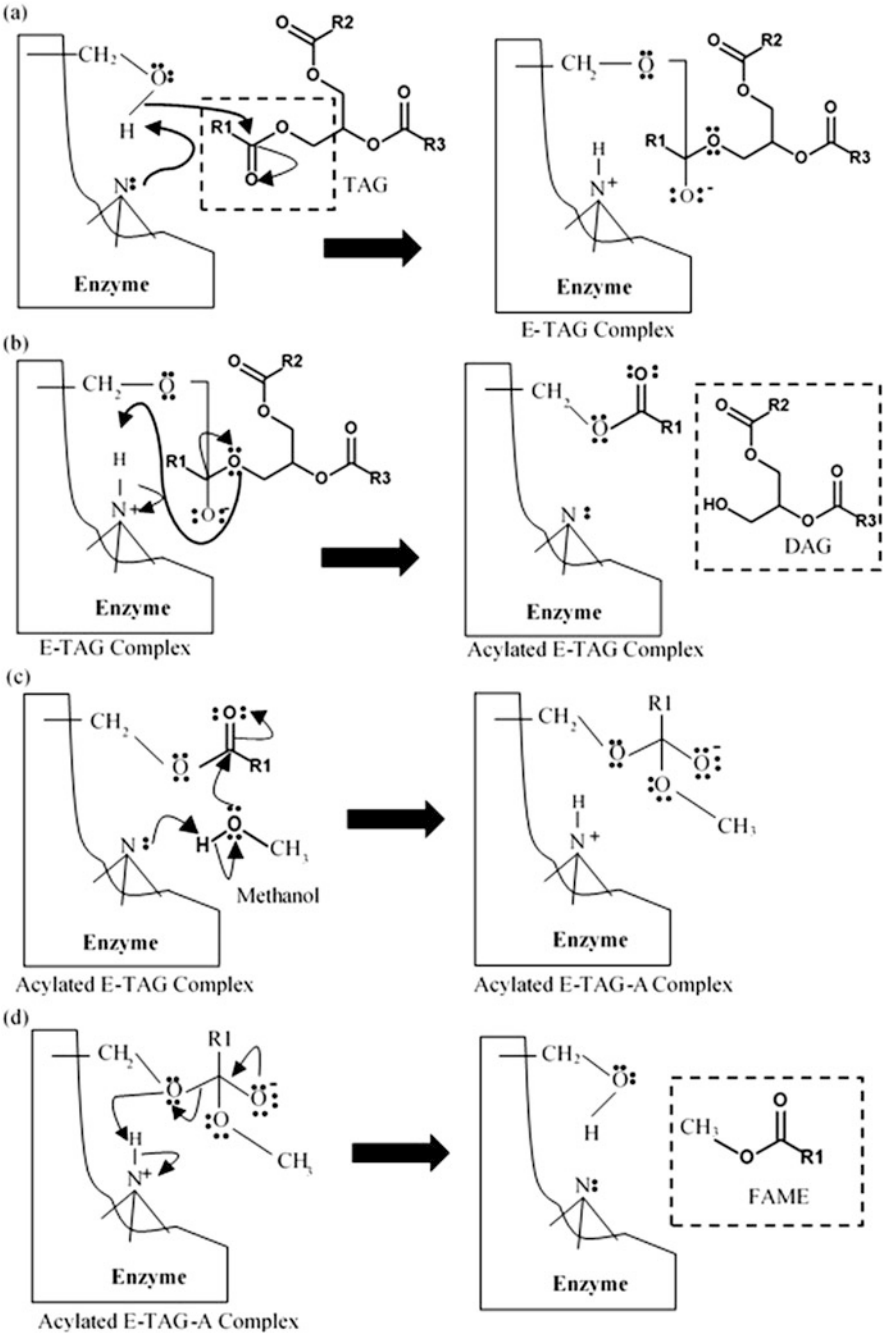

Fig. 15.2 Graphical illustrations of the mechanism of enzymatic biodiesel production through transesterification (Al-Zuhair et al. 2006)

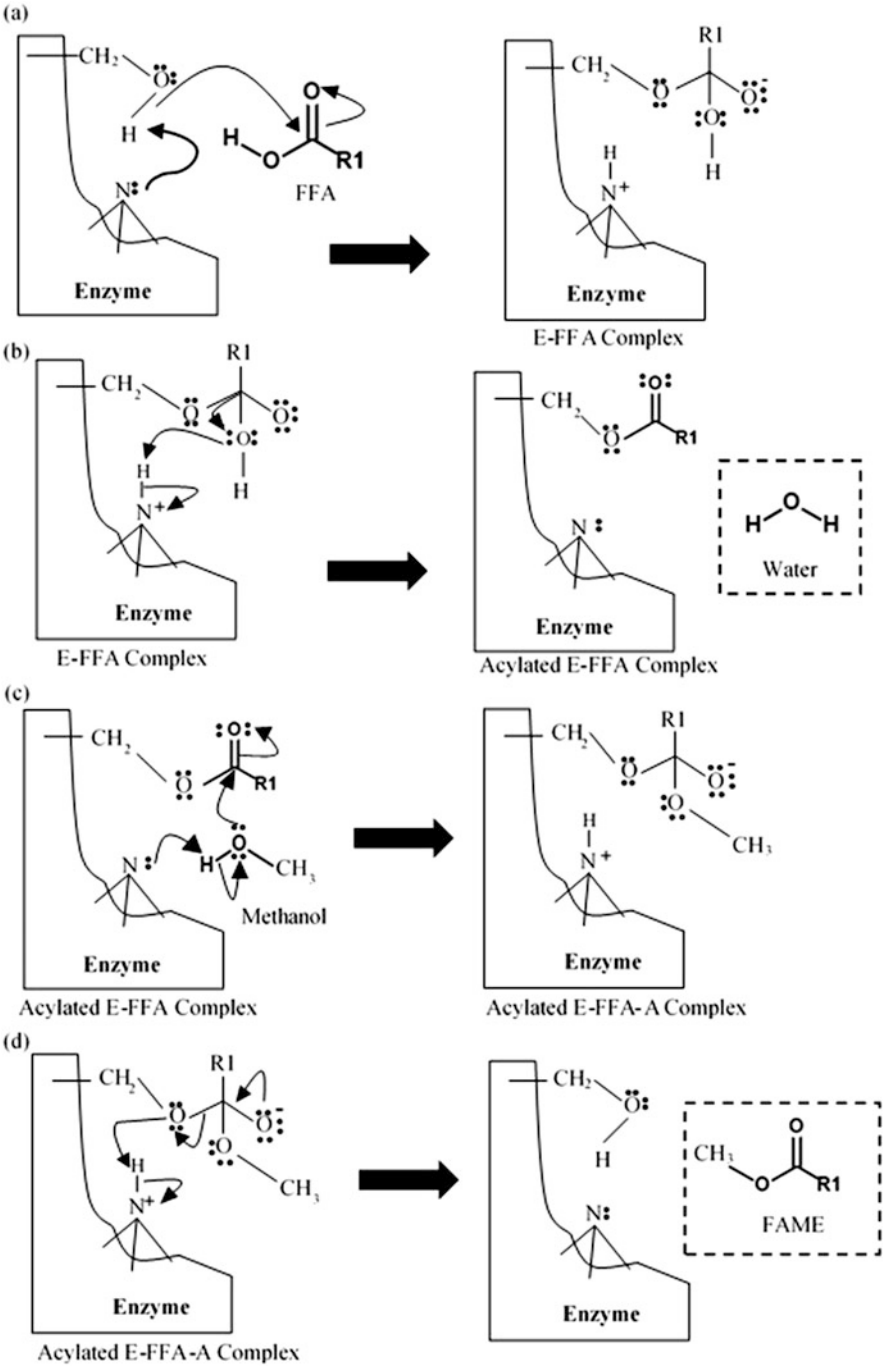

Fig. 15.3 Graphical illustrations of mechanism of enzymatic biodiesel production through esterification

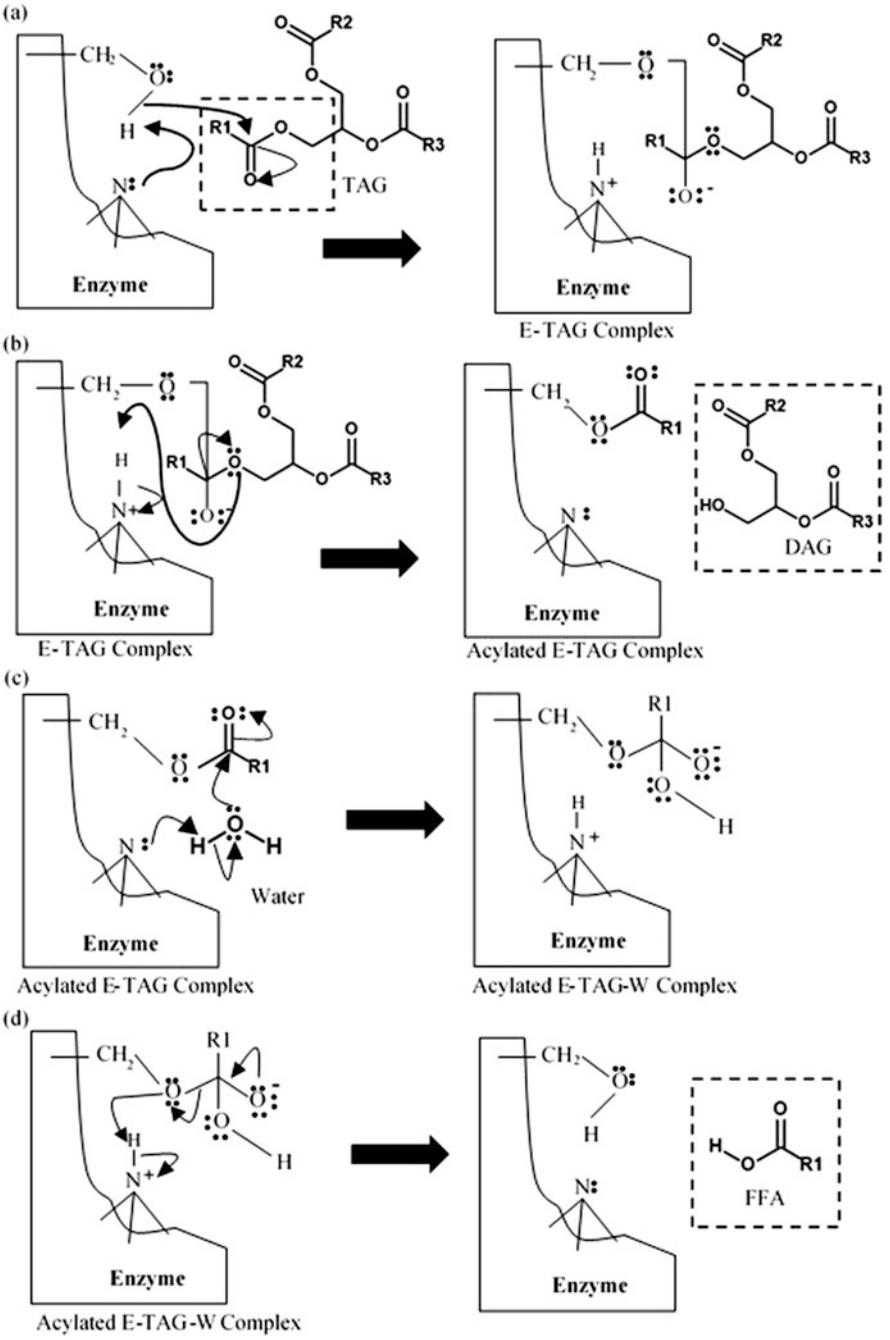

Fig. 15.4 Graphical illustrations of mechanism of enzymatic hydrolysis of TAG

enzymes can be easily recovered using simple physical separation methods (i.e., settling or filtration). Besides, the immobilised enzymes allow the conversion of a batch process to a continuous process since the enzymes are retained in a reactor whilst substrate and product can flow in and out of the reactor continuously. Furthermore, immobilisation helps to increase the tolerance limit of the enzyme towards alcohol, heat and shear force (Gog et al. 2012; Amini et al. 2016; Wang et al. 2017).

The selection of support material for immobilisation is vital as it would affect the resultant enzyme activity. Porous materials such as silica, polymer resins, ceramics, magnetic particles and carbon nanotubes are commonly used as the support for enzyme immobilisation due to their extensive surface areas responsible for enzyme loading (Zhao et al. 2015). The pore size of the support must be large enough to allow free-flowing of substrates and products into and out from the enzymes. Other important factors in selecting a suitable support to ensure the enzyme activity is preserved during the immobilisation stage include hydrophobicity, particle size and surface area.

The typical enzyme immobilisation methods are carrier binding, encapsulation, entrapment and cross-linking (Sankaran et al. 2016). Carrier binding of enzyme and support can be reversible or irreversible based on the immobilisation methods. Physical adsorption is the only reversible process categorised under carrier binding as compared to ionic binding, covalent binding and affinity binding. Physical adsorption is the most widely studied method because the process is straightforward while keeping a high enzymatic activity after immobilisation. The lipase is generally adsorbed to a support material through the van der Waals forces, hydrophobic interactions, electrostatic interaction or affinity adsorption. However, the enzymes immobilised by the adsorption method could leach out from their support after multiple times of reuse due to the weak interaction forces (Minteer 2016).

Until now, Novozym 435 (*Candida antartica* lipase B) is the most widely studied immobilised enzyme in the literature because of its high efficiency in converting edible oils and low-quality feedstocks to biodiesel. Novozym 435 is immobilised on macroporous acrylic resin support through physical adsorption that has a certain level of hydrophobicity. For instance, Watanabe et al. (2002) reported that 93.8% of biodiesel was successfully produced from degummed soybean oil in 48 h using Novozym 435 as the biocatalyst. Meanwhile, Modi et al. (2007) reported that Novozym 435 could effectively catalyse the transesterification of non-edible karanj oil and ethyl acetate, where 90% of biodiesel content was achieved in a solvent-free system.

Besides the adsorption method, enzymes can be immobilised via the encapsulation or entrapment methods. The working principle of these methods is that they confine the movement of enzyme molecules using a semi-permeable polymer material but allowing the diffusion of substrates and products through the material. However, these methods have a major drawback. The diffusion of the substrates and products through the permeable layer could be severely hindered. As a result, the immobilised enzyme through encapsulation was reported to have a low biodiesel

conversion and low enzyme stability. For example, Jegannathan et al. (2010) encapsulated the lipase from *Burkholderia cepacia* in κ-carrageenan biopolymer and used the immobilised enzyme for biodiesel production. It was found that the biodiesel conversion decreased significantly in each cycle of reuse and only 40% of biodiesel conversion was achieved at the tenth cycle of reaction.

On the other hand, enzymes can be immobilised via a cross-linking method. In this approach, enzymes are immobilised by adding a cross-linking agent to form aggregates of enzymes through non-covalent bonding. The immobilised enzymes produced using this method is known as cross-linked enzyme aggregates (CLEAs). So far, glutaraldehyde is the most commonly used cross-linking agent reported in the literature (Gupta et al. 2009). By using this method, the use of expensive support materials for immobilisation can be eliminated, thus reducing the enzyme cost. However, the formation of CLEAs clusters by filtration or centrifugation during the enzyme recovery process was often reported (Wang et al. 2011; Montoro-García et al. 2010). The clumping of CLEAs increased the mass transfer limitations during the reaction, and the catalytic efficiency of the enzyme is greatly reduced after multiple times of reused. In recent years, there has been growing interest in producing CLEAs through magnetic cross-linking (mCLEAs) so that the enzyme can be recovered easily from the reaction mixture by using a magnet. For example, mCLEAs produced from CALB were investigated by Pico et al. (2018) for biodiesel production. Lipids that extracted from *Chlorella vulgaris* var. L3 was used as the feedstock and more than 90% of biodiesel conversion was achieved in 3 h of reaction. Besides, the mCLEAs of CALB retained 90% of its initial enzyme activity even after 10 cycles of reuse. Badoei-dalfard et al. (2019) synthesised CLEAs of Km12 lipases, which were then covalently coupled to the amino coated magnetite nanoparticles to improve the reusability of mCLEAs. A FAME conversion of approximately 70% was obtained in 42 h using WCO as a feedstock and no significant loss of enzyme activity was observed after 6 cycles of reuse.

Additionally, immobilisation of enzyme using nanomaterials has gained increasing interest. It is found that the support in nanoparticle sizes provides a higher surface area for enzyme loading, stronger mechanical strength and enhanced enzyme stability and activity as compared to the conventional support used in immobilisation methods. Tran et al. (2012) investigated the performance of *Burkholderia* sp. lipase immobilised on ferric silica nanocomposite. They found that over 90% of FAME conversion was obtained in 30 h and the immobilised enzyme can be reused up to 10 cycles without a significant loss of enzyme activity. Sarno and Iuliano (2019) immobilised the *Thermomyces lanuginosus* lipase on magnetic ferrosoferric oxide/gold (Fe_3O_4/Au) nanoparticles. This immobilised lipase could convert waste tomato seed oil to 98.5% of FAME. More than 84% of the initial enzyme activity was retained after three cycles due to the stabilising effect of gold in the support. Even though the immobilised enzyme on nanocomposites has shown an outstanding performance in converting lipid feedstock to biodiesel, the support materials investigated are expensive, thus limiting their application in industry.

Table 15.1 Summary of lipase used for the enzymatic biodiesel production

Form of Enzyme	Trade Name	Source of Lipase	Biodiesel Feedstock	Temperature (°C)	Catalyst Dosage (wt%)	Water (wt%)	Alcohol Type; Alcohol-to-Oil Molar Ratio	Time (hr)	Biodiesel Conversion (%)	Reference
Immobilised	Novozym 435	*Candida antarctica*	Palm fatty acid distillates	45	5	5	Ethanol; 2:1	8	90.1	(Mulalee et al. 2015)
	Novozym 435	*Candida antarctica*	Fish oil	35	50	–	Ethanol; 4:1	8	82.91	(Marín-Suárez et al. 2019)
	Novozym 435	*Candida antarctica*	Lard	50	0.04	–	Methanol; 5.12:1	20	97.2	(Huang et al. 2010)
	Novozym 435	*Candida antarctica*	Soybean oil	60	–	–	Methanol; 4.5:1	8	95.3	(Tian et al. 2018)
	Novozym 435	*Candida antarctica*	Karanj oil	50	10		Ethyl acetate; 11:1	12	90	(Modi et al. 2007)
	Novozym 435	*Candida antarctica*	Degummed Soybean oil	30	4	–	Methanol; 3:1	24	93.8	(Watanabe et al. 2002)
	Eversa® Transform 2.0	*Genetically modified aspergillus orzyae*	Sunflower oil	40	10	–	Ethanol; 4:1	3	99	(Remonatto et al. 2018)
	CALB	*Candida antarctica lipase B*	Chlorella vulgaris var. L3	30		–	Methanol	3	90	(Pico et al. 2018)
	–	*Rhizopus oryzae*	Calophyllum inophyllum oil	35	20	15	Methanol; 12:1	72	92	(Arumugam and Ponnusami 2014)
	–	*Recombinant Rhizopus oryzae*	Alperujo oil	30	–	–	Methanol	6	28.62	(Bonet-Ragel et al. 2015)
	Lipozyme TL IM	*Thermomyces lanuginosus*	Fish oil	35	50	–	Ethanol; 4:1	8	68.88	(Marín-Suárez et al. 2019)

(continued)

Table 15.1 (continued)

Form of Enzyme	Trade Name	Source of Lipase	Biodiesel Feedstock	Temperature (°C)	Catalyst Dosage (wt%)	Water (wt%)	Alcohol Type; Alcohol-to-Oil Molar Ratio	Time (hr)	Biodiesel Conversion (%)	Reference
	–	*Thermomyces lanuginosus*	Tomato seeds oil	45	20	–	Methanol; 6:1	24	98.5	(Sarno and Iuliano 2019)
	Lipozyme RM IM	*Rhizomucor miehei*	Fish oil	35	50	–	Ethanol; 4:1	8	89.28	(Marín-Suárez et al. 2019)
	–	*Pseudomonas cepacia*	Jatropha seed oil	50	0.75	5	Ethanol; 4:1	8	98	(Shah and Gupta 2007)
Liquid	NS40116	*Thermomyces lanuginosus*	Chicken fat	30	0.3	2	Methanol; 4.5:1	24	77	(da Silva et al. 2018)
	NS40116	*Thermomyces lanuginosus*	Soybean oil	35	0.5	15	Methanol; 4.5:1	12	94.6	(Rosset et al. 2019)
	NS81006	*Genetically modified aspergillus Niger*	Soybean oil	45	1.5	20	Ethanol; 5.5:1	8	90	(Ren et al. (2011)
	NS81006	*Genetically modified aspergillus Niger*	Soybean oil	40	1.5	10	Methanol; 4.65:1	7	90	(Lv et al. 2010)
	Eversa® transform	*Genetically modified Thermomyces lanuginosus*	Deacidified Beef tallow	35	1	6	Methanol; 4.5:1	8	85.08	(Wancura et al. 2019)
	Eversa® transform	*Genetically modified Thermomyces lanuginosus*	Oleic acid	35.25	11.98	–	Methanol; 3.44:1	2.5	96.73	(Nguyen et al. 2018)

	Eversa® transform	*Genetically modified Thermomyces lanuginosus*	WCO	–	1	2.5	Methanol; 4.5:1	16	97	(Remonatto et al. 2016)
	Eversa® Transform 2.0	*Genetically modified aspergillus orzyae*	Distillate from Soybean refinery	45	0.2	2	Methanol; 6:1	16	96.6	(Mibielli et al. 2019)
	Callera trans L	*Thermomyces lanuginosus*	Crude Soybean oil	35	1	5	Methanol; 5:1	24	96	(Cesarini et al. 2013)
	Callera Trans L	*Thermomyces lanuginosus*	Deacidified Beef tallow	35	1.45	9	Methanol; 4.5:1	8	84.6	(Wancura et al. 2018)
	–	*Thermomyces lanuginosus*	Rubber seed oil	37	5	–	Methanol; 4:1	24	92.83	(Sebastian et al. 2017)
	–	*Candida cylindracea*	Sludge Palm oil	40	0.1	–	Ethanol; 4: 1	5	54.4	(Nasaruddin et al. 2014)
Whole-cell	–	*Rhizopus orzyae* (*R. orzyae* 262)	WCO	30	10	–	Methanol; 3:1	24	84	(Balasubramaniam et al. 2012)
	–	*Rhizopus orzyae*	Soybean oil	35	–	15	Methanol	72	90	(Ban et al. 2001)
	–	*Aspergillus nomius* ST57	Palm oil	40	–	–	Methanol; 4:1	4	78.23	(Rakchai et al. 2016)
	–	*Pichia pastoris* (*P. pastoris* X-33)	WCO	40	6	–	Methanol; 4:1	84	82	(Yan et al. 2014)
	–	*Aspergillus oryzae* (*A. orzyae* NS4)	Chlamydomonus sp. JSC4	35	–	24	Methanol; 7:1	32	97	(Amoah et al. 2017)
	–	*Aspergillus nomius* (*A. nomius PDA-6*)	Palm oil	40	10	–	Methanol; 5:1	40	95.3	(Talukder et al. 2013)

(continued)

Table 15.1 (continued)

Form of Enzyme	Trade Name	Source of Lipase	Biodiesel Feedstock	Temperature (°C)	Catalyst Dosage (wt%)	Water (wt%)	Alcohol Type; Alcohol-to-Oil Molar Ratio	Time (hr)	Biodiesel Conversion (%)	Reference
	–	*Pichia pastoris (P. pastoris* GS115*)*	Soybean oil	55	40	–	Methanol; 4.5:1	72	83.14	(Huang et al. 2012)
	–	*Candida antarctica lipase B and Thermomyces lanuginosus*	Waste grease	30	4	–	Methanol; 1:1	48	95	(Yan et al. 2012)

Table 15.1 summarises the immobilised enzyme that has been studied in the literatures. It can be seen that majority of the studies are revolving around the use of Novozym 435 due to its versatility in converting various types of feedstock and abilily to achieve high biodiesel conversion. Despite the enhancement in reusability of the immobilised enzyme, it still has several drawbacks that need to be addressed. Firstly, the cost of immobilised enzymes is many times higher than the free enzyme. Besides, the supporting structure of enzymes and the adhesion of products onto the support could cause mass transfer barrier between the substrates and enzymes, thus reducing the enzymatic catalytic activity. Moreover, the repeated use of immobilised enzymes may cause the enzymes to detach from the support material. Additionally, the regeneration of enzyme with a solvent may be required after each use. Hence this may further increase the operational complexity and operating cost for biodiesel production. In recent years, there has been growing interest in the use of free lipase in liquid form due to low preparation cost. The application of liquid lipase in biodiesel production will be discussed in the following section.

15.5.2 Liquid Lipase

Free lipase in liquid form has recently been studied for biodiesel production and it has shown promising results for converting low-quality feedstocks to biodiesel. Liquid lipases are liquid formulations that generally consist of enzymes, stabilisers and preservatives to maintain the enzyme stability (Aguieiras et al. 2015). Liquid lipases are significantly cheaper (30–50 times lower) than the immobilised enzymes since it does not require extensive purification process or immobilisation process during manufacturing (Cesarini et al. 2013). The liquid lipase can also be reused by recovering the lipase from the glycerol phase when the reaction is complete, which make it more cost-effective than the immobilised enzyme. Furthermore, the operation involving liquid lipase is much more straightforward than the immobilised enzyme system because simple process equipment such as a stirred-tank reactor (STR) would be sufficient for the process. However, there has been limited research conducted using liquid lipase since liquid lipase was introduced to the market only after 2010.

Examples of liquid lipases which are commercially available include NS40116, Callera Trans L, Eversa® Transform and NS81006. Cesarini et al. (2013) investigated the effect of water content on the soluble lipase Callera Trans L for biodiesel production using crude soybean oil that was not been degummed. They found out that a FAME conversion of 96% was successfully achieved in 24 h. In another work, Wancura et al. (2018) used the same enzyme to convert beef tallow to biodiesel. After 8 h of reaction, 84.6% of beef tallow was successfully converted to biodiesel with a methanol-to-oil molar ratio of 4.5:1. They also noticed that with an addition of 9% of water to the reaction mixture, the inhibitory effect of methanol on lipase was reduced, achieving a higher biodiesel yield.

Rosset et al. (2019) also studied the effect of water content on the liquid lipase NS40116 (genetically modified *Thermomyces lanuginosus*). A range of water content (0–20%) was added to the reaction mixture and the degummed soybean oil was allowed to hydrolyse to produce FFA. Then, the FFA generated was converted to FAME through esterification. The sequential reactions of hydrolysis and esterification were known as hydroesterification. In this case, they found out that the lipase NS40116 was more efficient at high water content (15%) compared to low water content (0–6%) and a high FAME yield of 95.8% was achieved after 12 h of reaction. The results suggested that the water not only provides an interfacial area for biocatalytic action, but also needed for maintaining the structure of the enzyme and minimizing the methanol inhibition effects on the enzyme.

On the other hand, the reuse of liquid lipase NS81006 from genetically modified *Aspergillus niger* was studied by Ren et al. (2011). In their work, soybean oil and ethanol were used to produce biodiesel. After 16 h of reaction, most of the lipase was found to accumulate at the bottom phase (glycerol-rich phase) when the reaction mixture was allowed to separate gravitationally. The entire bottom phase was directly reused in the subsequent batches of reaction. They found out that the biodiesel conversion was maintained around 90% after 5 cycles of reuse. These results demonstrated that liquid lipase could be easily reused using a straightforward approach. The potential of reusing the Eversa® Transform lipase was also investigated by Wancura et al. (2019). The deacidified beef tallow was reacted with methanol under the process condition of 35 °C and 1 wt% lipase for 8 h. After the reaction, the reaction mixture was subjected to gravity settling in a separating funnel. The intermediate lipase-rich phase was subsequently concentrated by centrifugation and the recovered lipase was directly reused for 4 cycles without any pre-treatment. It was observed that the FAME yield reduced tremendously from 85.08% in the first cycle to 35.54% in the fourth cycle. However, considering that liquid lipase has a much lower price than immobilised enzyme, single-use of the liquid lipase is still acceptable.

Several researchers also investigated the synergetic effect from the combination of enzymes from different lipase sources. For instance, Zeng et al. (2017) combined the lipases from *Rhizopus oryzae* and *Candida rugosa* in different ratios to catalyse the conversion of biodiesel from rapeseed oil deodoriser distillates containing an initial FFA content of 52.93%. They observed that with a *Rhizopus oryzae*: *Candida rugosa* ratio of 4:1, a 96.4% of biodiesel conversion was obtained in 6 h of reaction. Besides, the mixed lipases could tolerate a water content of 50% while achieving a high biodiesel conversion. On the other hand, Guan et al. (2010) studied the synergetic effect of *Rhizomucor miehei* lipase (1–3, specific) and *Penicillium cyclopium* lipase (non-specific mono- and diacylglycerol) for the conversion of soybean oil to biodiesel. With a volume ratio of 4:1 (*Rhizomucor miehei* to *Penicillium cyclopium*), 99.7% of biodiesel conversion was achieved in 24 hr of reaction. The results suggested that the combination of two lipases of different specificity can effectively catalyse the methanolysis of soybean oil to yield high FAME conversion. Meanwhile, Eversa Transform lipase was used by Nielsen et al. (2016) to catalyse the transesterification of refined soybean oil. The residual FFA in the biodiesel product was further reduced by esterification that catalysed by a second

enzyme that is known as CALB. The results showed that CALB was as efficient as a normal caustic wash by NaOH and the residual FFA was successfully reduced from 2.3% to below 0.25%.

The summary of liquid lipase investigated in the literature is presented in Table 15.1. In short, high biodiesel conversion using liquid lipase is observed for most studies. The enhanced biodiesel conversion is primarily due to the absence of the immobilisation support in liquid lipase formulation. Hence, the substrates have direct access to the active sites of the enzymes, which reduces the mass transfer resistance significantly. Furthermore, the issues of enzyme leaching or breaking of support due to high shear stress that usually occur in an immobilised enzyme system are avoided when liquid lipases are used in the process. Additionally, the catalyst dosage used for liquid lipases is significantly lower than immobilised enzymes because more enzymes are needed to fill up the porous support materials.

15.5.3 Whole-Cell Lipase

Whole-cell lipase has recently emerged as a biocatalyst for biodiesel production. The major advantage of using whole-cell lipase is that the production cost of the enzyme is low. This is because the process does not require lipase extraction and purification (Guldhe et al. 2016). There are three different forms of whole-cell biocatalyst that have been studied in the literatures. Among all, the fungal cells of *Rhizopus* sp. and *Aspergillus* sp. that produce intracellular or cell-bound lipases are the most common form of the whole-cell biocatalyst. Although a portion of the lipase is secreted to the extracellular space, most of the lipases are still retained within the cell. The intracellular lipase strains are often immobilised on the biomass support particles (BSPs) or cross-linking with glutaraldehyde to enhance the enzyme stability and activity. Besides, by immobilising the lipase strain on the BSPs, the separation of the biocatalyst from the product stream will be much easier.

For instance, Amoah et al. (2016) cultured the intracellular *Aspergillus oryzae* strain on a Czapek-Dox agar plate. Then, the harvested spores were inoculated in a culture medium containing reticulated polyurethane foam BSPs. The cells produced were naturally immobilised in the pores of BSPs during cultivation. After a simple filtration from the culture medium, the whole-cell lipase was ready to use for biodiesel production without the need of purification process. In this case, soybean oil with high phospholipid content was used as the biodiesel feedstock. A FAME content of 90% was achieved in 100 h. On the other hand, Talukder et al. (2013) used WCO as a carbon source instead of glucose or glycerol for the cultivation of *Aspergillus nomius*, and they found that WCO could enhance the catalytic activity of the whole-cell biocatalyst. A biodiesel content of 95.3% was obtained in 40 h, using palm oil as the substrate and 88.2% of activity was retained after 10 cycles of reuse, which proved the reusability of this newly formulated whole-cell biocatalyst. Furthermore, in the study conducted by Tamalampudi et al. (2008), the whole cells of *Rhizopus oryzae* were immobilised on the reticulated polyurethane foam particles

BSPs during the cell-growth stage in the culture medium. Then, the whole-cell biocatalyst was further stabilised by cross-linking with glutaraldehyde. The whole-cell biocatalyst produced was used to catalyse the biodiesel conversion of non-edible *Jatropha curcas* oil. A FAME content of 80 wt% was obtained after 60 h of reaction, and 90% of the initial enzyme activity was retained after 5 cycles of reuse. Although the whole-cell biocatalyst demonstrated the possibility of catalysing the enzymatic biodiesel production, long reaction time was required to achieve high biodiesel conversion when intracellular lipase was used as the whole-cell biocatalyst. The long reaction time may be due to a low mass transfer rate of the high molecular weight substrates to the active site of the cell-bound lipase.

The mass transfer limitation can be reduced by cell-surface display technology, which is an alternative method to prepare a whole-cell biocatalyst. This technology allows the expression of targeted enzymes or recombinant strain on the microbial cell surface by fusing the recombinant strains with anchoring motifs (Han and Lee 2015). In brief, anchoring motifs are carrier proteins such as the outer membrane of protein, lipoproteins and sub-unit of appendages. This allows the displayed enzyme to be easily accessible by the substrates. Besides, the microbial cell surface acts as physical support to the displayed lipase, which enhances the enzyme stability and eases the reusability (Gai and Wittrup 2007). Huang et al. (2012) have successfully displayed *Rhizomucor miehei* on the cell surface of *Pichia pastoris*. The whole-cell lipase produced was subsequently used as the biocatalyst to convert refined soybean oil to biodiesel. A high biodiesel conversion was obtained after 72 h in the presence of iso-octane as the co-solvent. Besides, 80.46% of the initial enzyme activity was maintained after 10 cycles of reuse. On the other hand, the synergistic effect of two different lipases displayed on Pichia pastoris, namely CALB and *Rhizomucor miehei* lipase, was studied by Jin et al. (2013). In the presence of the co-solvent tert-butanol and iso-octane, the soybean oil was successfully converted to biodiesel with a yield of 90% in 12 h. They noticed that the biodiesel yield was higher when combined lipase was used, instead of using the displayed lipase alone. The combination of the displayed lipases also demonstrated a remarkable reusability as 85% of biodiesel yield was achieved even after 20 batches of reaction.

The third method of preparing the whole-cell biocatalyst is by overexpressing the intracellular lipases inside a heterologous host (Macauley-Patrick et al. 2005). *Escherichia coli (E. coli), Saccharomyces cerevisiae* and *Pichia pastoris*, are the primary yeast hosts used for intracellular overexpression method. In this method, the intracellular enzymes will accumulate to the extent that they become "self-immobilised" biocatalyst. At the same time, most of the active enzymes are retained within the cells. For example, Yan et al. (2014) used recombinant P. pastoris for the intracellular expression of *Thermomyces lanuginosus* lipase. The whole-cell was used as a biocatalyst to convert WCO to biodiesel. A biodiesel yield of 82% was obtained in 84 h. In another work, Yan et al. (2012) combined two lipases, namely CALB and *Thermomyces lanuginosus* lipase, and expressed them intracellularly using recombinant *E. coli*. The *E. coli* was subsequently used to convert waste grease

with an initial FFA content of 21.7% to biodiesel. After 24 h of reaction, 95% of biodiesel yield was successfully obtained.

Table 15.1 summarised different type of whole-cell lipases that have been studied in the literature for biodiesel production. Although whole-cell lipase may offer a cheaper route to produce immobilised lipase, the time required to achieve a high conversion is longer than that of immobilised enzyme and liquid lipase. The long reaction time is caused by the mass transfer resistance created by the cell wall and cell membrane of the intracellular lipase, which limits the accessibility of substrates to the enzyme. Moreover, the operation of the whole-cell biocatalytic reactor is more complex than liquid lipase and immobilised enzyme system. Therefore, the application of whole-cell biocatalyst system has so far been limited to lab-scale exploration.

15.6 Industry Application

Until now, a number of companies have already adopted enzymatic technology to produce biodiesel. In 2006, Hainabaichuan Co. Ltd. from China launched an enzymatic biodiesel industrial plant with a capacity of 20,000 t/y, which was then doubled to 40,000 t/y in 2008. This plant uses a combination of Lipozyme® TL IM and Novozym® 435 immobilised enzymes in a STR to convert waste palm oil into biofuel in the presence of tert-butanol solvent (Valero 2017).

In 2007, another Chinese company known as Lvming Co. Ltd. established a biodiesel plant with a capacity of 10,000 t/y. It uses immobilised *Candida* sp. lipase supported on textile membranes as a biocatalyst in STR. WCO is used as the raw material (Valero 2017). In 2012, a continuous enzymatic biodiesel production using immobilised liquid lipase was established by an American company, Piedmount Biofuels, which is located in North Carolina (Valero 2017). A year later, EnzymoCore collaborated with M-Energy (South Korea) to produce 30,000 t/y of biodiesel from brown grease using immobilized enzyme (EnzymoCore 2013).

In 2014, Viesel Fuel LLC partnered with Novozyme and Tactical Fabrication LLC to built an enzymatic biodiesel production plant that produces 16,556 tonnes of biodiesel per year (Rebecca 2014). In the same year, Appa-lachian Biofuels LLC built an enzymatic biodiesel facility in Russell County, Virginia. This company uses immobilized enzymes produced by TransBiodiesel to manufacture 92,710 t/y of biodiesel from waste oil and brown grease (Kotrba 2014).

Meanwhile in 2017, a new pilot plant was built for EnzymoCore in the water treatment centre of Peel near Jerusalem. The plant uses brown grease as feedstock to produce 1500 t/y of biodiesel using EnzymoCore's immobilized enzyme as catalyst (EnzymoCore 2017). On the other hand, Beijing Cenway Bio-Energy Technology Co., Ltd. built two enzymatic plants in Hunan and Hubei Province to convert WCO, trap grease or gutter oil to biodiesel. Each plant has a capacity of 50,000 t/y. Cenway Bio-Energy built another plant with 200,000 t/y biodiesel production in Guangdong

Province in 2017, which increased the total production to 300,000 t/y. In Laurens County, Smisson-Mathis Energy (SME) LLC retrofitted an existing but idled biodiesel production facility with a 3311 t/y capacity enzymatic biodiesel plant in 2017. This plant converts brown grease into biodiesel using a liquid enzyme provided by Novozymes (Kotrba 2017). Furthermore, a California-based biorefinery company, Aemetis Inc. established an advanced enzymatic biodiesel plant in Kakinada, India. The plant uses a patent-pending pre-treatment process technology developed by Aemetis which enables an effective enzymatic biodiesel production. The biodiesel plant has a capacity of 165,555 t/y using one million pounds of low-cost waste feedstock. Additionally, this plant produces refined glycerin which is suitable for pharmaceutical and industrial customers (Kelly 2017).

Nonetheless, it is worth-noting that the number and the production capacity of enzymatic biodiesel plant are still significantly smaller than chemical-catalysed biodiesel plant. The low adoption by industry could be associated with the limitations and challenges of the enzymatic process. The limitations and challenges of the enzymatic process are discussed in the following section.

15.7 Limitations and Challenges

Although lipase offers numerous advantages over chemical catalysts, industry adoption of enzymatic technology for biodiesel production still remains a challenge. The first challenge is associated with the high cost of lipase. Although the cost of some commercial lipases has drastically been reduced in the recent years, the cost of these lipases are still many times higher than the conventional chemical catalysts. Therefore, continuous effort to lower the cost of lipase is essential to widen the use of enzymatic technology in the industry.

Besides, enzymatic process typically requires a long reaction time to achieve a high biodiesel conversion, thus resulting in low productivity. Even though this limitation could be overcome by increasing the reactor size, this approach increases the capital cost of a biodiesel plant. Recently, a notable amount of effort has been made to increase the reaction rate of enzymatic biodiesel production via process intensification. For instance, Gharat and Rathod (2013) applied ultrasonication in converting WCO to biodiesel using Novozym 435. The biodiesel conversion was found to improve from 38.7% to 86.6% in 4 h of reaction time.

Apart from that, the reaction rate could also be increased by improving the homogeneity of the reaction medium using organic solvents (Peña et al. 2008; Lam 2010; Tan et al. 2010; Batistella et al. 2012; Muppaneni et al. 2012; Nasaruddin et al. 2014). Tert-butanol and n-hexane were found to effectively increase the miscibility of lipid feedstock and alcohol, thus increasing the diffusion rate between the substrates. Nevertheless, the use of organic solvents is undesirable from safety viewpoint because they are usually highly toxic and flammable.

Another challenge is the intolerance of lipase to alcohol. Alcohol could have a significant effect on protein structure. It has a tendency to strip off the water layer from a lipase, which is responsible for maintaining an optimal conformation of the

lipase (Fjerbaek et al. 2009). When the alcohol dosage has exceeded the tolerance limit of a lipase, the protein structure of the enzyme would start to unfold, which causes enzyme deactivation. Therefore, alcohol is commonly added stepwise into the reaction mixture to reduce enzyme inhibition. Consequently, this may complicate the process operation. Hence, there has been a keen interest to develop lipase which has high tolerance for alcohol. For instance, the methanol tolerance limit of *Thermomyces lanuginosus* has been enhanced through direct evolution by iterative saturation mutagenesis (Tian et al. 2017). The methanol tolerance limit of lipase produced by mutated *T. lanuginosus* was found to be 30% greater than that produced by the native species.

Another drawback of the enzymatic process is the high residual FFA in the biodiesel after reaction. Typically, the residual FFA content is approximately 2 to 3 wt% (Amoah et al. 2016; Nielsen et al. 2016; Andrade et al. 2017a, b; Mibielli et al. 2020). FFA could promote oxidation and thus causing degradation to the biodiesel. Therefore, an additional post-reaction purification step is usually required to reduce the FFA content. This would result in higher operating cost and waste generation. Therefore, effort needs to be devoted to the development of a low-cost enzyme which can produce biodiesel with low FFA content.

15.8 Conclusion

A shift towards low-quality feedstock will increase the use of enzyme for biodiesel production because enzyme shows great versatility in converting these low-cost feedstocks to biodiesel regardless of the feedstock compositions. However, the cost of the enzyme is prohibitive. Therefore, the development of immobilisation technology and cost-effective enzymes have been of key interest to lower the production cost of biodiesel. Both free and immobilised enzyme systems have been adopted by industry. However, the adoption rate is slow, and the production capacity of the enzymatic plant is significantly smaller than the chemical catalysed biodiesel plant. Overcoming the limitations of the current enzymatic process such as reducing enzyme cost, improving storage stability, enhancing alcohol tolerance and reducing the residual FFA content in biodiesel product is crucial to increase the utilization of enzymes in large scale biodiesel production.

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Chapter 16
Sustainability and Traceability in the Malaysian Oil Palm Industry

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Abstract Over the past century, Malaysia's oil palm industry has developed into one of the world's most efficient and productive agricultural operations. Compared to the other main oilseeds, i.e., soybean, rapeseed and sunflower, oil palm is the highest-yielding oil crop. Proving their contribution to the sustainable development of the oil palm industry is one of the most daunting issues facing the industry players. From the beginning, the industry has adhered to the practices of sustainability, and current activities remain committed to the three pillars of sustainability, namely people, planet and prosperity. The Malaysian oil palm industry endeavours to find an equilibrium between addressing the need to improve well-being (social development) and safeguarding the health and safety of consumers; protecting and managing biodiversity and the environment; and ensuring the nation's economic growth. This can be achieved by embracing the use of blockchain technology and traceability to increase the level of trust of customers by demonstrating the industry's contribution towards sustainable and transparent supply chains.

16.1 Introduction

Our future generations are entitled to appreciate and use the resources we have access to now. These include clean air and water, a stable climate, biodiversity and habitat diversity, untouched or virgin forests, and natural resources that are productive. Sustainable development means that all these resources are still at the disposal of future generations for use. Proper natural resource management will help secure their biological production potential and help retain possible opportunities for their use. One of the major objectives of sustainable land management as defined by Erick et al. (2006) is the harmonisation of agricultural priorities including land, water, biodiversity and environment to meet growing food demands while sustaining ecosystem services and livelihoods. Indeed, the balancing of agricultural goals

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with economic and environmental issues is also a major goal of sustainable land management (Dumanski and Smyth 1993). There are three (3) sustainability areas of concern: environmental, economic and social, which are the fundamental concerns of man. Three main elements are included in sustainability, also referred to as 3Ps - people, planet and prosperity. These key elements are derived from the 'Brundtland Commission' (United Nations [UN] 1987) which states that "sustainable development seeks to meet the needs and aspirations of the present generation without compromising the ability to meet the needs of those in the future." Based on this concept, sustainability is the efficient management of existing resources to meet future needs. Sustainability in the context of the Malaysian oil palm industry is all about reducing the environmental impacts of the supply chain of palm oil, while at the same time improving the economic and social value of the oil palm sector.

The issue of sustainability will be key to the expansion agenda of the oil palm industry. The implementation of the Malaysian Standard (MS) for Malaysian Sustainable Palm Oil (MSPO) together with other existing sustainability certification schemes is expected to propel the industry towards producing a more sustainable palm oil as well as to support the branding of Malaysian palm oil. These sustainability certification schemes are expected to link growers, processors, investors, traders, retailers and non-governmental organisations (NGOs) together so that more realistic, sustainable and responsible palm oil production will evolve with time (Alain et al. 2016).

16.1.1 The Global Oils and Fats Market

The production volume of vegetable oils worldwide reached 234.48 million tonnes in the 2019 crop year. Among the major vegetable oils, palm oil had the highest production volume, estimated at 75.03 million tonnes in that period as shown in Fig. 16.1.

Palm oil is one of the 17 major oils and fats that are being traded in the world market. It is a common vegetable oil used in foods and consumer goods. The volume of palm oil production worldwide has steadily increased over the last few years, and there is a large and growing demand for the export of palm oil. Palm oil accounted for 32% and 57% of oils and fats produced and exported worldwide respectively, maintaining its position as the top edible oil produced and traded. Owing to its special properties and flexible uses, as well as the favourable price over other vegetable oils, palm oil has now gained global popularity, especially among manufacturers. The position of palm oil as the world's leading edible oil traded is likely to continue to be a tremendous force on the markets for oils and fats in the years to come.

In line with the rise in human consumption, the need for the world's edible oil is expected to reach 361 million tonnes by 2050. Additional commodity production options are required to satisfy the potential demand for vegetable oil, and palm oil is

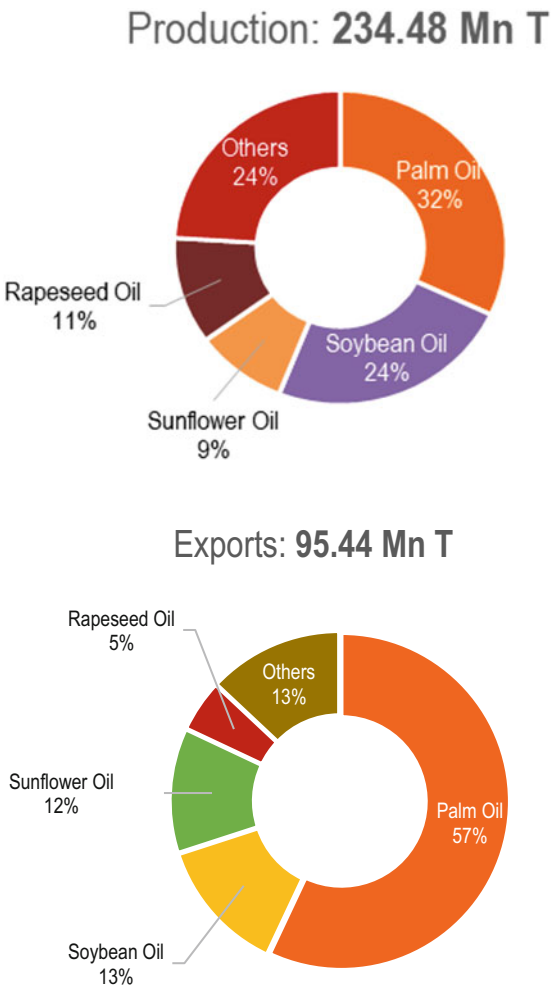

Fig. 16.1 Production and exports of major oils and fats 2019. (Adapted from Oil World 2020)

the most feasible commodity to fulfil this need since it requires the least additional land area (Ministry of Economic Affairs of Indonesia [MEA] 2019).

Malaysia and Indonesia continue to be the major producers and exporters of palm oil globally. Indonesia remained as the major producer and exporter of palm oil with 57% and 55% share of global palm oil production and export, respectively in 2019 due to increase in oil palm acreage and yield. Out of 75.55 million tonnes of palm oil produced, 26% (or 19.64 million tonnes) were produced by Malaysia in 2019. In terms of export, Malaysia's share was 34% (or 18.36 million tonnes) of global palm oil export (Oil World 2020). OECD (2018) foresees that over the next decade, the two main industriously export-driven palm oil producers, Indonesia and Malaysia, which export 70% and 80% of their production, respectively, will continue to control more than two-thirds of the world market for oils and fats.

16.1.2 Overview of the Malaysian Oil Palm Industry

The Malaysian oil palm industry has experienced considerable development since the crop was originally brought to the country from West Africa in the late 1870s. The oil palm species, *Elaeis guineensis*, was initially cultivated as an ornamental plant for decorative purposes. The oil palm industry has since grown to be one of the greatest economic triumphs in Malaysia. According to to Kushairi and Parveez (2017), Tennamaram Estate located in Batang Berjuntai, Selangor (now known as Bestari Jaya) began the first commercial oil palm plantation in 1917 where Henri Fauconnier, a French businessman and author planted dura oil palm seeds he bought from an Indonesian friend. This first commercial oil palm plantation has laid the foundation of industrial growth.

From its meagre beginnings, the crop grew to almost 55,000 hectares (ha) in 1960 (Nambiappan et al. 2018). From now on, growth has been dramatic, reaching 1.023 million ha in the 1980s and 2.030 million ha in the 1990s (Malaysian Palm Oil Board [MPOB] 2017). According to the MPOB estimates, this figure escalated to 3.376 million ha in 2000 and to 5.90 million ha in 2019 (Malaysian Palm Oil Board [MPOB] 2020a). The oil palm industry remains as one of the most important export earners for Malaysia. Total export revenue for oil palm products multiplied significantly from a mere RM11.6 billion in 1996 (Zainal and Deepak 2008) to RM64.84 billion in 2019 (Malaysian Palm Oil Board [MPOB] 2020a).

The advancement of the Malaysian oil palm industry can be attributed to a convergence of good government programmes and policies, and initiatives and dogmas in the private sector. In the 1960s, the Agricultural Diversification Programme was the cause of the change from rubber to oil palm planting. This was followed by land settlement schemes led by the Federal Land Development Authority (FELDA). In the change of crop, privately owned plantations followed suit, leading to a drastic rise in planted hectarage (Nambiappan et al. 2018). The distribution of oil palm planted area by ownership is shown in Table 16.1. Almost 40% of the oil palm planted areas in Malaysia are owned by independent and organised smallholders under government schemes, e.g., FELDA, Federal Land

Table 16.1 The distribution of oil palm planted area by ownership, 2019

	2019	
Categories	hectares	%
Private Estates	3605.436	61.1
Independent smallholders*	986,331	16.7
FELDA	723,545	12.3
FELCRA	185,005	3.1
RISDA	72.444	1.2
State schemes/government agencies	327,396	5.5

Note:*Oil palm smallholder is defined as farmer who owns land or in aggregate of less than 40.46 hectares (100 acres). Average size is 3.9 hectares. Adapted from Parveez et al. (2020)

Consolidation and Rehabilitation Authority (FELCRA), and Rubber Industry Smallholders Development Authority (RISDA).

16.2 Sustainability of the Oil Palm Industry in Malaysia

The fact that the oil palm is a perennial crop with a productive lifespan of about 25 years vouches for its high sustainability standing. In Malaysia, oil palm has largely been cultivated on legitimate agricultural land or on land previously occupied by other tree crops such as rubber, cocoa or coconut. With the increasing shortage of suitable land for oil palm cultivation, a policy was implemented to stop the opening of new forest land for agriculture. The Cabinet of Malaysia on 22 March 2019 had endorsed the following vital policies (Ministry of Plantation Industries and Commodities of Malaysia [MPIC] 2019), namely a. imposition of a limit of 6.5 million hectares on the total area cultivated for oil palms; b. cessation of the planting of oil palm on peatland and further tightening of regulations relating to the current cultivation of oil palm on peatland; c. prohibition on the conversion of reserved forest areas for oil palm cultivation; and d. generating maps of oil palm plantations open for public access. With the policy in place, only logged-over land zoned for agricultural development can be planted with oil palm or other crops. Hence, Malaysia still boasts of a forest cover of over 55.6% (Ministry of Energy and Natural Resources [KeTSA] 2018) since 1917, the year when the oil palm was first commercially planted. At the international level, Malaysia also made a pledge at the United Nations Earth Summit in Rio de Janeiro in 1992 that it would retain at least 50% of its land area as forests, thus assuring that the rich biodiversity in Malaysia's rainforests are protected and preserved in Malaysia's quest for economic development. This pledge has been honoured to this day.

The basic challenge confronting the Malaysian oil palm industry is how to sustain development and global competitiveness in the face of stagnating productivity, increasing production costs and the scarcity of environmentally suitable land for expanding oil palm cultivation. In addition, the industry now has to fulfil expectations of palm oil importing countries regarding environmental performance. To this end, the Malaysian oil palm industry strives to strike a balance between social, environmental and economic development of the industry. Accordingly, within the oil palm industry, the current consensus on sustainability is about taking care of the 3Ps by addressing the need for improving the well-being (social development) and safeguarding the consumer's health and safety; conserving and managing the environment and biodiversity; and ensuring the economic progress of the nation.

16.2.1 Improving the Well-being (Social Development) and Ensuring the Consumer's Health and Safety

One important aspect of the concept of sustainability by the Brundtland Commission is that it is crucial to meet the needs of the poor in all nations, so sustainability initiatives should address the eradication of poverty. The phenomenal growth of the oil palm industry and its position in poverty reduction is, in this regard, exemplary. As of September 2020, the industry provides jobs directly to 421,937 individuals, of which 76.7% or 323,505 are immigrant workers and the remaining 98,432 individuals are locally recruited staff (Ismail 2020). In 2019, there were a total of 492,259 oil palm smallholders in Malaysian with a total area of 1,667,553 ha (Table 16.2).

The Government of Malaysia realised early that the only way for people to break free from the vicious cycle of poverty was for organised and structured development to ensure that economic development goes hand in hand with social development. FELDA has carried out land development and landless resettlement in the country. Under a regulated system for planting economic crops such as cocoa, rubber and oil palm, thousands of rural landless farmers were granted land as a means of earning a living, providing rural jobs and increasing income levels.

In 2011, the income for FELDA's settlers was almost 6 times higher than the national poverty level (Table 16.3). The average income of FELDA settlers and smallholders has consistently been well above the poverty line in Malaysia. This is proof of how extensive the people (social) component has been successfully addressed in Malaysia through the establishment of FELDA. In addition, the FELDA programme has also been recognised by the World Bank and the United Nations as a successful development model for providing social and economic benefits, as reported by the New Straits Times on 19 May 2017 (New Straits Times [NST] 2017).

Over the years, the oil palm industry has consistently contributed towards poverty eradication and narrowing of the income gap between rural and urban folks by creating rural townships where residents enjoy a good quality of life with adequate social infrastructure, e.g., housing, health, religious facilities) (Bronkhorst et al. 2017; Mohammed et al. 2000; Nor Ermawati et al. 2014; Siwar 2016).

Table 16.2 Smallholders participation in oil palm sector, 2019

Type of Smallholders	No. of Smallholders (individuals)	Planted Area of Smallholders (hectares)
Independent	258,657	986,002
Organised	233,602	681,531
FELDA	99,894	432,721
FELCRA	89,440	138,816
RISDA	14,962	25,770
Others	29,306	84,224
Total	492,259	1,667,553

Adapted from Ministry of Plantation Industries and Commodities of Malaysia [MPIC] (2020)

Table 16.3 Malaysia: Average income of organized and independent smallholders

Year	Average Income (RM)/Month		
	FELDA Settler	Independent Smallholders	National Poverty Line Income*
2007	2221	1209	740
2008	3278	1094	691
2009	2459	944	800
2010	3419	1259	720
2011	4648	1413	800
2012	3926	1868	763
2013	3787	1520	860
2014	3874	1952	930
2015	2689	1175	930

Note:*Poverty Line Income (PLI) is a measure of absolute poverty based on the minimum requirement of foods and non-foods for household members to live healthily and energetically (Siwar 2016). Adapted from Nik Ibrahim (2020)

16.2.1.1 Well-being of Native Communities

Malaysia is dedicated not only to forest and biodiversity protection, but also to the well-being of native communities dependent on the forest for their livelihoods (World Resources Institute [WRI] 2013). Indigenous and native customary rights (NCRs) over land are clearly acknowledged. The Federal Constitution guarantees rights that are important to preserving the unique relationship between native peoples and their lands (Human Rights Commission of Malaysia [SUHAKAM] 2008). In Sarawak and Sabah, customary laws passed by the British during their colonial rule acknowledging the indigenous peoples' customary land rights are still in force, and the laws acknowledge that indigenous peoples have indigenous customary rights over the lands they inhabit and cultivate (Julia et al. 2016). For example, under Article 5 of the Sarawak Land Code, NCRs are provided, and under Article 6, Native Communal Reserves are provided. Similarly, the NCR for Sabah is spelled out in Sect. 15 of the State of Sabah Land Ordinance. The truth is that the development of customary lands takes into account the needs of the indigenous people, where the government's goal is to place them at the forefront of economic development. Local communities are encouraged to participate in the development of oil palm smallholdings, as well as in government and private sector development projects for better income (Ferdous Alam et al. 2015).

Oil palms are cultivated on state lands that are legally owned and designated for development by the states. Often, these lands have been disputed by natives as NCR land. There are claims, on the other hand, that these are simply unlawful intrusions by some local native villagers, mostly motivated by the prospect of compensation from the palm oil companies. These locals are often reluctant to be hired in the plantations. These cases are being settled in the courts, and if there is evidence of a breach of NCR, the courts can have reasonable remedies. As such, the presence of such pending cases cannot be used as the gospel truth that indigenous people's land rights are widely violated. This is because, first, the occurrence of conflicts is

minimal relative to the entire oil palm industry, and, second, the country's legal system provides the avenue for adjudication and redress.

In addition, indigenous peoples have organisations that protect their rights, either connected to the government or to NGOs. For example, the Jabatan Kemajuan Orang Asli, JAKOA (Department of Orang Asli Development) is the government agency tasked with overseeing the development of the Aborigines (Orang Asli). This body is part of the Ministry of Rural Development and was established in 1954. Its goals include eradicating Orang Asli's poverty, improving their welfare, encouraging education and improving their general livelihoods. The National Land Code 1965, the Land Conservation Act 1960, the Wildlife Protection Act 1972, the National Parks Act 1980, and, most notably, the Aboriginal Peoples Act 1954 are some laws that affect the Orang Asli. The establishment of the Orang Asli Reserve Land is provided for by this Act.

16.2.1.2 Feeding the World with Healthy and Nutritious Food

Palm oil has been a safe and nutritious edible oil for human for over 5000 years. However, palm oil which feeds more than 3 billion people all over the world each year, is often perceived as a being unhealthy simply based on its fatty acid composition which contains almost equal amounts of saturated and unsaturated fatty acids.

In order to address these concerns, numerous nutritional studies to compare the effects of consumption of palm oil and other oils on various health parameters such as cholesterol and inflammation have been undertaken and these studies have shown that palm oil is comparable to other oils such as olive oil, peanut oil and rapeseed oil among others, on the effects on lipid parameters in the blood. A palm olein diet is also less cholesterol raising compared to the detrimental trans fatty acids (TFA). On the other hand, palm oil has been shown to increase the high-density lipoprotein (HDL) cholesterol which is needed by the body. Palm oil itself is free from cholesterol.

In addition, palm oil is most suitable for high temperature cooking due to its high oxidative stability as compared to most unsaturated oils. Due to its balanced amount of saturated and unsaturated fatty acids, palm oil does not have to be subjected to hydrogenation process which is the cause for the formation of TFA. TFA arising from partial hydrogenation of oils have been proven for their negative effects on health and are currently being banned in most countries. Alternatively, palm oil does raise low-density lipoproteins/high density lipoprotein LDL/HDL ratio as compared to TFA and serves as the best replacer for TFA (Sundram et al. 2007).

The nutritional effects of palm oil have been studied since the 1980's especially on its effects on blood lipid parameters. Although palm oil has almost 50% of saturated fatty acids (SFA) content, palm oil behaves more like a monounsaturated oils such as olive, rapeseed and groundnut oils on their effects on blood lipid markers (Choudhury et al. 1995; Lucci et al. 2016; Ng et al. 1992; Voon et al. 2011) Palm oil has also been reported to be comparable to a number of polyunsaturated oils such as soybean and canola oils on lipid parameters (Sundram et al. 1995; Zhang et al.

1997). From another study carried out in Adelaide, Australia by the Commonwealth Scientific and Industrial Research Organization (CSIRO), the researchers have shown that palm olein in comparison to olive oil, does not affect endothelial function in overweight/obese men after consuming a high protein meal (Stonehouse et al. 2015). Measurement of endothelial functions is used to detect early events that lead to cardiovascular disease. In this regard, palm oil clearly does not have detrimental effects on health especially on cardiovascular diseases due to its SFA content as generally perceived.

Moreover, in recent years the evidence against saturated fats as being a cardiovascular health risk has shifted with increasing evidence from a number of systematic reviews and meta-analyses (Chowdhury et al. 2014; de Souza et al. 2015; Mozaffarian 2011; Siri-Tarino et al. 2010; Skeaff and Miller 2009) which have reported that there is no significant evidence to conclude that dietary SFA is associated with cardiovascular diseases or coronary heart diseases (CHD).

A recent Prospective Urban Rural Epidemiology (PURE) study which is a prospective cohort study from 18 countries in five continents (Dehghan et al. 2017), published in The Lancet dated 4 November 2017, has reported that saturated fat consumption shows no association with cardiovascular disease, myocardial infarction, or cardiovascular disease mortality, instead saturated fat has an inverse association with stroke. In addition, new publications are continuously being churned out negating the negative association of SFA with health. These recent studies include a review by Gershuni (2018) who has reported that there appears to be no consistent benefit to all-cause or CVD mortality from the reduction of dietary saturated fat. In another review, Hamley (2017) has reported from a meta-analysis of available evidence from randomised controlled trials (RCTs) evaluating effect of replacing SFA with n-6 polyunsaturated fatty acids (PUFA) on CHD events, CHD mortality, and total mortality, revealed that recommendations to replace SFA to n-6 PUFA (vegetable oils) are not supported by the literature. These reviews reinforce the conclusions of multiple other recent systematic reviews that have challenged the traditional diet-heart hypothesis.

The additional beneficial effects that can be obtained from red palm oil, on the other hand set a platform for the exploration of a wholesome nutritious oil as part of our daily diet. The red palm oil which is bright orange in colour is rich in tocotrienols, tocopherols, carotenoids, phytosterols, squalene, and coenzyme Q10 and is one of the richest sources of carotenoids. Red palm oil contains around 41% β-carotene and 41.3% α-carotene which exhibit pro-vitamin A activity. Red palm oil is known for its various health effects especially in addressing vitamin A deficiency (Loganathan et al. 2017).

It is important to note that palm oil is also rich in phytonutrients, e.g., carotenoids, tocopherols, tocotrienols, sterols, squalene, coenzyme Q10, phospholipids, and polyphenols that constitute 1% of its weight. These phytonutrients provide additional health benefits due to their antioxidative and non-antioxidative properties.

Amongst these phytonutrients, tocotrienols are the most studied and some of the potential therapeutic effects exhibited by this group of phytonutrients include cardioprotection, neuroprotection, immune-modulatory effects, and anti-cancer

(Aggarwal and Nesaretnam 2012; Meganathan and Fu 2016; Nesaretnam 2008). In a recent review by Subramaniam et al. (2019), tocotrienols have been shown to possess anti-tumour properties in prostate, breast, skin, colon, stomach, pancreatic, liver, and lung cancers through apoptosis, anti-angiogenesis, anti-proliferative, and immunoregulation. The role of tocotrienols in improving the immune response in healthy subjects who were immuno-challenged with tetanus toxoid has been reported in a study by Mahalingam et al. (2011). Tocotrienols are known to exhibit anti-inflammatory effects which are important in modulating various disease conditions (Nesaretnam 2008; Nesaretnam et al. 2012). Tocotrienols have been extensively explored for their neuroprotective effects in numerous studies ranging from in vitro cell culture to in vivo pre-clinical and clinical settings (Khanna et al. 2005). In a study by Gopalan et al. (2014), a significant reduction has been observed in the volume of white matter lesions in tocotrienol supplemented subjects against placebo. These studies have consistently shown the potentials of tocotrienols as neuroprotective agents worthy of large-scale Phase 3 clinical trials as future directives. Another striking observation on the effects of tocotrienol supplementation is in improving end stage liver disease by reducing the model for end stage liver disease (MELD) score (Patel et al. 2012). A larger scale Phase 2 clinical study is currently underway to further explore these potentials. Similarly, other phytonutrients in palm oil are consistently being explored for their potentials in improving human health.

The nutritional research programmes on palm oil and its phytonutrients are being progressively carried out to provide the latest emerging evidence on the health beneficial effects of both the oil and its constituents. The evidences presented in the above are hoped to address the concerns around the perception on the effects of palm oil on health and nutrition.

16.2.1.3 Ensuring Food Safety and Quality

Palm oil is one of the most important revenue earners for Malaysia. This is witnessed by high dependency to the palm oil export of nearly 90% from the total production (Parveez 2020). Being one of the major producers and exporters of palm oil, it is crucial to ensure that Malaysian oil palm industry complies with the food safety legislations and quality standards of destined countries. Food safety generally refers to food that will not impose any health hazards for human consumption (Haghiri 2016) while food quality reflects the consumer perception to value the food products (FAO and WHO XE "World Health Organization (WHO)" 2003). If adulterants are presence in edible oils, they will not only cause health risks (food safety) but also shorten their shelf-life stability (food quality). In Malaysia, the quality and safety aspects of food products are manifested under the Food Act 1983 and Food Regulations 1985. As such, food safety and quality should not be compromised especially 85% of the overall palm oil production is used for food applications. Non-compliance in meeting food safety and quality requirements will not only tarnish the country's reputation but could also jeopardise the economic performance.

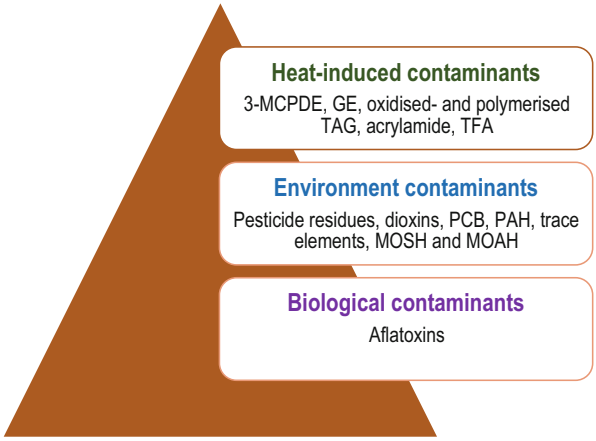

Fig. 16.2 List of potential contaminants in edible oils and fats [3-MCPDE - 3-monochloropropane-1,2-diol esters, GE - glycidyl esters, TAG - triacylglycerols, TFA - trans-fatty acids, PCB - polychlorinated biphenyls, PAH -polycyclic aromatic hydrocarbons, MOSH - mineral oil saturated hydrocarbons, MOAH - mineral oil aromatic hydrocarbons]

Food safety is often associated with the occurrence of contamination in food products including palm oil. In principle, contaminants can be clustered into four groups, namely heat-induced contaminants, environment contaminants, and biological contaminants. The manifestation of potential contamination incidences in edible oils and fats is illustrated in Fig. 16.2.

In this section, the discussion will emphasise on the current food safety issues in the edible oils and fats, especially in palm oil known as 3-monochloropropane-1,2-diol esters (3-MCPDE) and glycidyl esters (GE). The prevalence of 3-MCPDE was first raised by the Federal German Institute for Risk Assessment (BfR) in December 2007. This constituent principally falls under a group of chemical contaminants known as chloropropanols. In March 2008, the European Food Safety Authority (EFSA) informed that the Scientific Panel on Contaminants in the Food Chain (CONTAM) Panel concurred with the BfR hypothesis of fully conversion of 3-MCPDE into free or unbounded form (3-MCPD) considering no scientific evidence was available to dispute the assumption (Fiebig 2011). International Life Science Institute (ILSI) concludes that 3-MCPDE could be detected in all refined vegetable oils and thermally processed foods (International Life Sciences Institute [ILSI] 2009).

In September 2013, EFSA published a report on the occurrence of 3-MCPD in food products in Europe from 2009 to 2011 (European Food Safety Authority [EFSA] 2013). The tolerable daily intake (TDI) was established at 2 μg for every kg of body weight (bw). Food safety concerns have heightened since May 2016 when the EFSA issued a second report related to 3-MCPDE and GE (European Food Safety Authority [EFSA] 2016). The report states that 3-MCPDE and GE are presence in most vegetable oils and fats, with significant levels of these compounds are detected in palm oil.

In February 2018, the European Commission prescribed the GE limit of 1 ppm in refined oils, and this regulation has entered into force starting from 19 March 2018 (Table 16.4). The GE limit of below 0.5 ppm is imposed to those refined oils that are

Table 16.4 Legislative limits for GE in edible oil and fats

Item 4.2	Foodstuffs	Maximum level of GE
4.2.1	Vegetable oils and fats placed on the market for the final consumer or for use as an ingredient in food with the exception of the foods referred to item 4.2.2	1 ppm
4.2.2	Vegetable oils and fats destined for the production of baby food and processed cereal based food for infants and young children	0.5 ppm
4.2.3	Infant formula, follow-on formula and foods for special medical purposes intended for infants and young children (powder)	0.075 ppm until 30 June 2019 0.050 ppm as from 1 July 2019
4.2.4	Infant formula, follow-on formula and foods for special medical purposes intended for infants and young children (liquid)	0.010 ppm until 30 June 2019 0.006 ppm as from 1 July 2019

used to process food for young children and infants. More stringent limits of GE have been enforced on those refined oils used in foods for special medical purposes, either in the form of powder or liquid, for young children and infants (0.006 to 0.075 ppm). After four months of the implementation, the EU Rapid Alert System for Food and Feed (RASSF) refuses to accept a consignment of frying fat containing exceeded GE level from entering the EU countries (EU Rapid Alert System for Food and Feed [RASSF] 2020).

In early October 2018, the European Commission had presented a proposal for two maximum levels of 3-MCPDE and free 3-MCPD in refined oils (Food Standards Agency 2020). Palm oil falls under the category of "other vegetable oils and fish oil" with a maximum level of 2.5 ppm while oils like coconut, maize, rapeseed, sunflower, soybean, and palm kernel have a lower maximum limit of 1.25 ppm. The proposal on two maximum limits seems to imply the perception of "safer oils" for the lower limit and "harmful oils" for the higher limit. The EU will impose the legislative limits for 3-MCPDE starting from 1 January 2021 (Table 16.5).

In regard to palm oil, the maximum level of 2.5 ppm will be enacted on the final or traded products. It is important to note that 3-MCPDE will be partitioned towards the liquid fraction than that of the solid counterpart (Fig. 16.3 and Table 16.6). In order to ensure that palm olein contains 3-MCPDE of 2.5 ppm or 1.25 ppm, the levels of 3-MCPDE in palm oil should be within 2 ppm and 1 ppm, respectively. Similarly, double-fractionated palm olein requires much lower 3-MCPDE content in palm oil to produce superolein with 1.25 to 2.5 ppm of 3-MCPDE.

Realising the urgency of addressing the food safety issues related to palm oil, the Malaysian Government through its agency, namely MPOB is fully involved in mitigating the presence of 3-MCPDE in palm oil since 2009. To date, MPOB has successfully conducted extensive pilot plant trials over 66 runs to investigate numerous mitigation measures to minimise the occurrence of these unwanted contaminants. In fact, MPOB has taken a proactive step to collaborate with commercial

Table 16.5 Legislative limits for 3-MCPDE in edible oils and fats

Item 4.3	Foodstuffs	Maximum level of 3-MCPDE
4.3.1	Vegetable oils and fats, fish oils and oils from other marine organisms placed on the market for the final consumer or for use as an ingredient in food falling within the following categories, with the exception of the foods referred to in [2] and of virgin olive oils and fats from coconut, maize, rapeseed, sunflower, soybean, palm kernel and olive oils (composed of refined olive oil and virgin olive oil) (and mixtures of oils and fats with oils and fats only from this category)	1.25 ppm
	Other vegetable oils (including pomace olive oils), fish oils and oils from other marine organisms and mixtures of oils and fats with oils and fats only from this category	2.5 ppm
	Mixtures of oils and fats from the two above mentioned categories	2.5 ppm
4.3.2	Vegetable oils and fats, fish oils and oils from other marine organisms destined for the production of baby food and processed cereal based food for infants and young children	0.75 ppm
4.3.3	Infant formula, follow-on formula and foods for special medical purposes intended for infants and young children (powder)	0.125 ppm
4.3.4	Infant formula, follow-on formula and foods for special medical purposes intended for infants and young children (liquid)	0.015 ppm

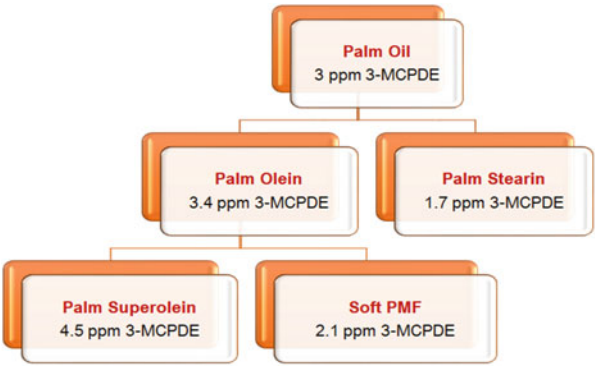

Fig. 16.3 Partitioning of 3-MCPDE in palm oil. PMF, palm-mid fraction. Adapted from Hinrechsen (2015)

Table 16.6 The requirement of 3-MCPDE in palm oil to produce palm oil fractions containing 2.5 ppm and 1.25 ppm in 3-MCPDE

Palm Oil	Palm Olein	Superolein
1.00 ppm	1.16 ppm	1.53 ppm
0.65 ppm	0.76 ppm	1.00 ppm
0.81 ppm	0.95 ppm	1.25 ppm
1.63 ppm	1.90 ppm	2.50 ppm
1.08 ppm	1.25 ppm	–
2.16 ppm	2.50 ppm	–

Adapted from Ahmad Tarmizi (2020)

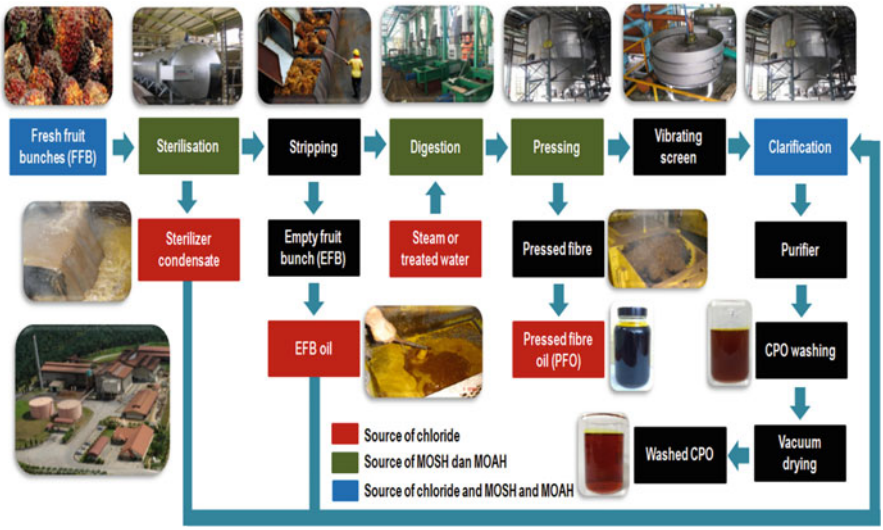

Fig. 16.4 Potential source of chloride and mineral oils at the palm oil mill. Adapted from Ahmad Tarmizi (2020)

mills and refineries in scaling up our pilot plant findings to commercial scale (Ramli et al. 2020).

Investigation done by MPOB demonstrates that the development of 3-MCPDE can be detected as early as at degumming and bleaching stages, and not necessary only at deodorisation step (Ramli et al. 2011). This can be explained from the basis of phosphoric acid used during degumming which catalyses the formation of 3-MCPDE. Moreover, the acidity of bleaching earth is significantly impacting the presence of 3-MCPDE. Similar study has even shown that the reduction of 3-MCPDE is attainable when calcium oxide is used as neutralising agent. On the other hand, chemical refining lessens the level of 3-MCPDE in refined palm oil. Acidity of crude palm oil contributes to higher amount of 3-MCPDE in processed palm oil (PPO) (Ramli et al. 2015). MPOB has also explored the feasibility of substituting phosphoric acid with water at the degumming stage (Ramli et al. 2011). It was found that water degumming successfully decreases the occurrence of 3-MCPDE, but it is not able to yield refined palm oil with acceptable colour.

It is well established that the presence of chloride in crude palm oil (CPO) is the precursor for the formation of 3-MCPDE in PPO. It was recently reported that the natural organochlorines present in CPO and other vegetable oils at different concentrations are responsible for 3-MCPDE formation when the oils are subjected to refining process (Tiong et al. 2018). The potential sources of chloride are mainly from various stages of mill operation (Fig. 16.4) while the possible source of chloride at the refinery is the bleaching stage (Fig. 16.5). Secondary oils such as empty fruit bunch (EFB) oil and especially palm fibre oil (PFO) contain higher level of chloride content (Table 16.7). It is also noted from Table 16.7 that free fatty acids

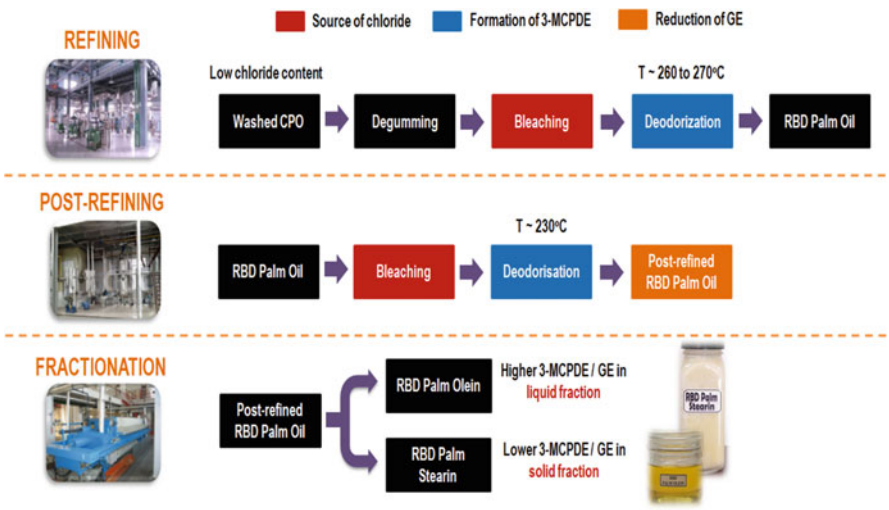

Fig. 16.5 Potential source of 3-MCPDE and GE at the palm oil refinery

Table 16.7 Selected quality parameters in CPO and secondary oils

Samples	FFA (%)	DOBI	Carotenes (ppm)	Phosphorus (ppm)	Chloride (ppm)
CPO	2.48 to 3.63	2.32 to 2.45	547 to 668	7.3 to 35	215 ± 0.21
EFB oil	6.32 to 13.73	1.18 to 1.68	638 to 733	30 to 45	4.59 ± 0.13
PFO	5.80 to 8.26	1.61 to 2.02	1258 to 1800	217 to 1063	69.41 ± 0.68

Adapted from Parveez et al. (2019)

(FFA) and phosphorus contents in both secondary oils are considerably high. Moreover, the latter decreases the deterioration of bleachability index (DOBI) and thus requires excessive usage of bleaching earth to remove the remaining phospholipids in CPO.

On that note, it is imperative that recycling of secondary oils into fresh CPO would not only increase the chloride content in CPO but also adversely impact other quality parameters. Recovery of secondary oils at the mills is primarily aimed to minimise oil loss at the sterilisation stage and increase the oil extraction rate (OER). It is plausible that secondary oils are added back into the processing stream to meet the national average OER of 23% (Malaysia's Performance Management and Delivery Unit [PEMANDU] 2010). Furthermore, the selection of steriliser systems, i.e., horizontal, continuous, vertical and tilting – not only affects the rate of oil loss (Table 16.8) but also CPO quality (Table 16.9). A study shows that CPO quality deterioration upon addition of EFB oil at different ratios influences the efficiency of refining process (Ramli 2018).

Table 16.8 Oil loss from different sterilizer system

Oil loss (%)	Type of sterilisers			
	Horizontal	Continuous	Vertical	Tilting
Steriliser condensate (SC)	0.07 to 0.77	13.95 to 16.30	0.97 to 1.73	0.69 to 1.54
Empty fruit bunch (EFB)	1.82 to 1.95	2.32 to 2.42	1.42 to 1.74	1.31 to 1.82

Adapted from Parveez et al. (2019)

Table 16.9 Effect of steriliser system on selected quality parameters in CPO

Steriliser condensate (SC)	Type of sterilisers			
	Horizontal	Continuous	Vertical	Tilting
FFA (%)	20 to 30	9.33	6.95	6.72
DOBI	1.2 to 15	1.9	2.0	2.1

Adapted from Parveez et al. (2019)

Table 16.10 Selected quality parameters of CPO with different FFA ranges

Range of FFA contents	Less than 2.5% (n = 22) [Average 2.31 ± 0.17%]	3.6 to 4.4% (n = 392) [Average 4.00 ± 0.24%]	5.5 to 6.0% [Average 6.10 ± 0.34%]
Phosphorus (ppm)	14.80 ± 5.50	21.95 ± 7.75	29.20 ± 1.31
Iron (ppm)	6.94 ± 1.32	11.35 ± 5.01	22.07 ± 3.67
DOBI	2.98 ± 0.47	2.56 ± 0.37	2.54 ± 0.37
M&I (%)	0.11 ± 0.05	0.14 ± 0.17	0.24 ± 0.13
PV (meq O_2 kg^{-1})	0.85 ± 0.98	1.68 ± 1.43	1.79 ± 1.33
AnV (unit)	1.19 ± 0.59	1.36 ± 1.18	1.43 ± 0.69

M&I - moisture and impurities, PV - peroxide value, AnV - p-anisidine value. Adapted from Ahmad Bustamam (2018)

A survey conducted in 2015/2016 reveals a drop in some of the quality parameters in CPO collected across Malaysia when compared to the data collected from the previous surveys carried out in 1997/1998 and 1986/1987 (Ahmad Bustamam 2018). Table 16.10 concludes that the increase in FFA is significantly impacting the amounts of phosphorus and iron presence in CPO. In addition, the hydrolytic behaviour (expressed as FFA) in CPO is positively correlated with its oxidative state as evidenced by both peroxide value (PV) (denotes as primary oxidation) and p-anisidine value (AnV) (denotes as secondary oxidation).

From the experience in addressing the issues on 3-MCPDE and GE, it is now the right time to revisit and revise the current Malaysian Standard (MS) related to palm oil covering CPO and palm oil fractions. The MS revision should be followed by strengthening the trade specifications of CPO and palm oil products since both have not been reviewed for many years. The oil palm industry should start to revolutionise and ready to move forward for the enhancement of palm oil quality and safety instead of solely focussing on commercial value and profit. All palm oil sectors are

accountable to ensure the sustainability of palm oil production and trade through food safety and quality.

16.2.2 Conserving and Managing the Environment and Biodiversity

During COP 15, the Prime Minister of Malaysia announced Malaysia's commitment to reducing greenhouse gases (GHG) by adopting a voluntary reduction of up to 40% of gross domestic product (GDP) of the intensity of emissions in 2020 compared to 2005 levels subject of the receipt of the transfer of technology and adequate as well as effective funding. Based on data released by Olivier and Peters (2020), Malaysia achieved a 27.45% reduction in GDP emission intensity in 2018 relative to 2005.

Malaysia practises a sustainable land use policy, taking into account the need to balance developmental needs and conservation of its biodiversity. In 1992, at the United Nations Rio Earth Summit, Malaysia pledged to retain at least 50% of its total land area under forest and that plantation crops would only be permitted on the land set aside for agriculture. Thus, the government halted the conversion of new forest land for agriculture and oil palm cultivation and current oil palm planted area resulted from expansion on designated agriculture land and land previously planted with other crops (Fig. 16.6). Malaysia is also a signatory to several international conventions, including the Convention on Biodiversity 1992 (CBD), the International Tropical Timber Agreement, and the Charter of the Indigenous-Tribal Peoples of Tropical Forests.

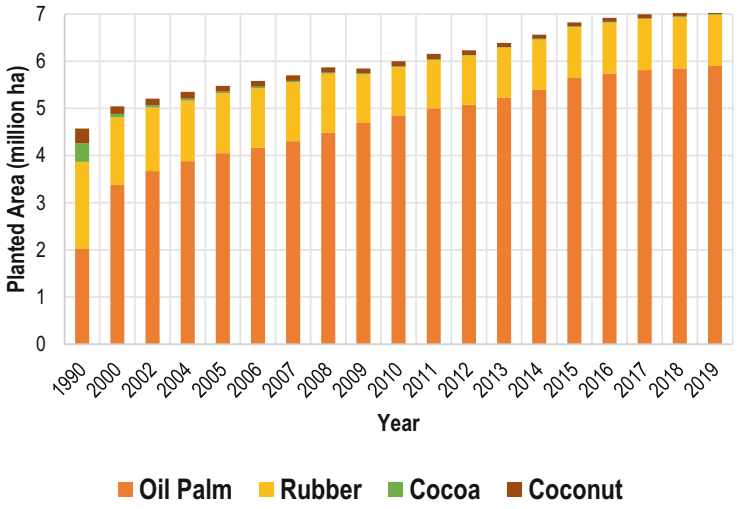

Fig. 16.6 Conversion of other crops to oil palm plantation. Adapted from: Malaysia Open Data Portal (2020) and Ministry of Plantation Industries and Commodities of Malaysia [MPIC] (2020)

16.2.2.1 Land Use

From 1990 to 2019, a total of 3.9 million hectares were converted to oil palm plantations. Malaysia is currently nearing the limits of agricultural land availability for oil palm conversion and expansion. Oil palm cultivation in Malaysia is on legally designated agricultural land. In addition, lands which are encumbered by customary rights of indigenous people are protected by law. Therefore, oil palm is not cultivated on land gazetted as forest reserves, national parks, and wildlife or game reserves. Permanent forests, covering 55.6% of Malaysia's land remain devoted to wildlife habitat and biodiversity conservation.

It is often claimed that oil palm is the cause of deforestation, despite the fact that it is occupying less than 5% of the total vegetable oil producing land and 0.4% of agricultural land in total (Jackson et al. 2019). It should also be noted that the oil palm is the most productive oil crop and produces more than 40% of the worlds edible oil. Hence, it produces more oil per planted hectare than any other oil crops. On a land area basis, it can offer 4 to 7 times more yield than the other seasonal annual oil crops, such as soybean, rapeseed, and sunflower (Table 16.11 and Fig. 16.7). Compared to oils such as soybean and rapeseed, the benefit of palm oil is it produces high output per hectare, combined with the relatively small amount of fertilisers and pesticides required (Fig. 16.8).

Table 16.11 Productivity of the oil palm vis-à-vis other major oil crops

Oil Crops	Production (million tonnes)	% of total production	Total area (million ha)	% of total area
Oil palm*	76.06	42.64	23.45	11.44
Soybean	56.82	31.86	122.78	59.91
Rapeseed	24.94	13.98	31.36	15.30
Sunflower	20.54	11.52	27.34	13.34
TOTAL	178.36	100.00	204.93	100.00

Note:* Combined tonnage of palm oil and palm kernel oil. Adapted from Oil World (2020)
Adapted from *Choo et al. (2011), ‡ Mortimer et al. (2010)

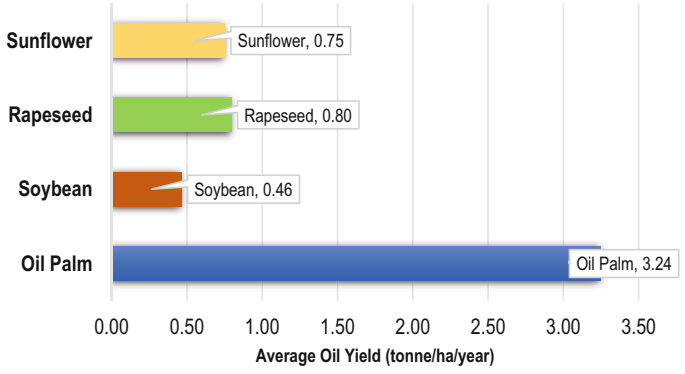

Fig. 16.7 Average oil yield for major oil crops. Adapted from Oil World (2020)

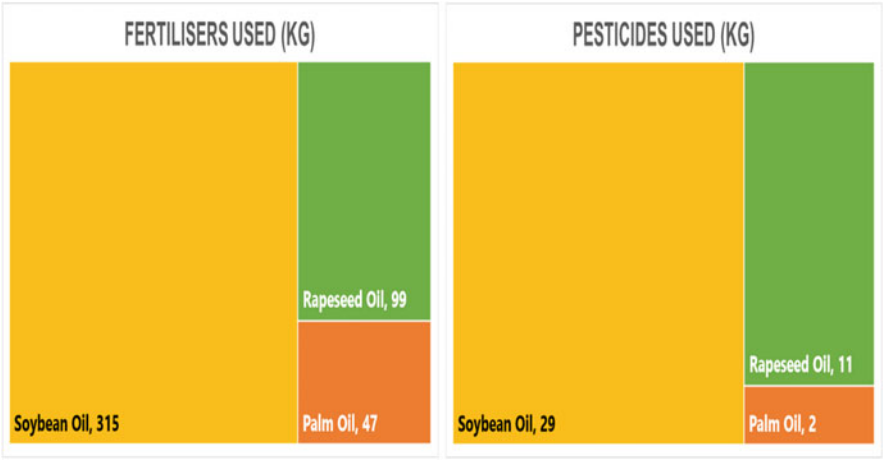

Fig. 16.8 Fertilisers and pesticides (kg) used to produce 1 tonne of vegetable oil. Adapted from The Guardian (2014)

Based on a cross-commodity survey conducted in 2017, it was found that the oil palm industry is the most committed to no deforestation among four global deforestation-related supply chains of commodities (cattle, timber, soy, and palm oil). In addition, 41 out of 50 producers of palm oil with the largest land area and highest market capitalisation have pledged to addressing deforestation, with 29 committing to pursue zero deforestation practices (Meijaard et al. 2020).

16.2.2.2 Research and Development to Increase Yield

Research efforts to further improve the productivity and value of the oil palm in the entire value chain have been catalysed by rising pressure for sustainable palm oil production coupled with decreasing availability of arable land. In addition to the pressure to remain sustainable, in order to increase crop yields, productivity, and production, the industry has continually implemented improvements to mechanised agricultural practices in the plantations (Kushairi et al. 2019). In recent decades, the industry's research and development (R&D) activities have centred on leveraging biotechnological advances to increase oil palm yields (Parveez et al. 2020).

The successful mapping of the oil palm genome, which revealed a single gene responsible for regulating yield, is one of the most important R&D efforts by MPOB (Singh et al. 2013b). This breakthrough provides the potential for new variants to be bred for increased production. In two separate papers in Nature (Singh et al. 2013a, 2013b), the discovery of the single gene, the shell gene, which is essential for oil palm yield, is recorded along with the oil palm genomic sequence. This important finding would resolve the issue of opening new oil palm areas, as it is a direct way to increase palm production without land expansion.

16.2.2.3 Reduction in Greenhouse Gas (GHG) Emissions

New instruments and methods are needed in order to measure environmental efficiency. One of the instruments that can be used by the industry to measure environmental impacts associated with the supply chain of oil palm/palm oil is the life cycle assessment (LCA). LCA is a step-by-step framework for determining the environmental repercussions associated with an operation, product, or process.

The method discerns and quantifies the energy and materials used as well as the waste released into the atmosphere, depending on the life cycle of an operation, product, or process, and thus measures the effect of those energy and materials used and wastes on the environment (Arnold 1993). The LCA also offers the knowledge required to facilitate the implementation of sustainability policies. Specific policies for the responsible use of resources can be formulated by improvement analyses focused on LCA so that sustainability concerns are handled accordingly.

Malaysia considers mitigation using the life cycle method as an investment to ascertain the sustainable production of palm oil, as well as the long-term competitiveness of the oil in the global market of oils and fats. The MPOB launched a national LCA study on the supply chain of palm oil in 2006, acknowledging the significance of LCA for sustainability. The LCA study, starting from the nursery to the production and use of palm biodiesel for oil palm grown on mineral soils, was completed by MPOB. An international panel reviewed and accepted the LCA results and the findings were published in peer-reviewed journals (Choo et al. 2011; Halimah et al. 2010; Puah et al. 2010; Tan et al. 2010; Vijaya et al. 2010; Zulkifli et al. 2010).

The LCA results were used to compute the GHG emissions for the production of refined palm oil compared to those for production of refined rapeseed oil and refined soybean oil. Table 16.12 shows that emissions of refined palm oil were much lower than those of the other two oils, especially when biogas trapping at the palm oil mill was involved in the production pathway.

Notwithstanding the above, the Government of Malaysia is determined in urging palm oil mills to trap biogas from palm oil mill effluent (POME) as fuel. This initiative allows the mills to earn extra income and lowers the carbon footprint. In addition, with emerging global environmental issues and more stringent sustainability requirements inflected by palm oil consumer countries, biogas capture would resolve the current stigma and warrant the Malaysian palm oil players to get continued access to these markets (Loh et al. 2017).

Table 16.12 Total GHG emissions associated with the production of refined vegetable oils

Refined vegetable oil	Total GHG emissions (tonne CO_2eq/tonne oil)
Palm Oil*	1.11 0.63 (with biogas capture)
Rapeseed Oil‡	1.35
Soybean Oil‡	1.70

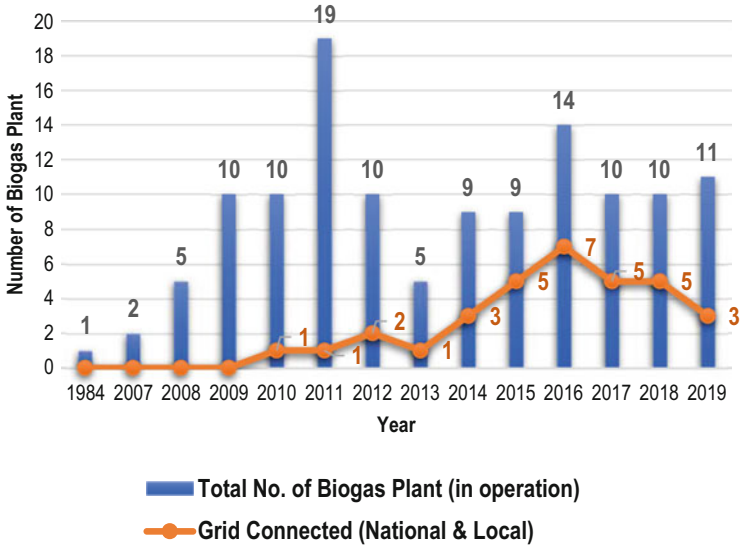

Fig. 16.9 Biogas capturing facilities in palm oil mills. Adapted from Loh et al. (2019)

By the end of 2019, 125 biogas facilities have been installed at palm oil mills nationwide, of which 30 are connected to the national grid, while three are connected to the local grid. (Fig. 16.9). Moreover, Loh et al. (2019) report that under the methane avoidance programme, there are 76 palm oil mills with composting plants. In total, it was estimated that 5.52 million tonnes of carbon dioxide equivalent have been abated annually by the capture and use of biogas.

Another effort undertaken by the Government of Malaysia towards reducing GHG emissions for combating climate change as well as carbon footprint is the implementation of the national biodiesel mandate in both the transport sector (B10 – blending of 10% of biodiesel with 90% of petroleum diesel) since February 2019 and the industrial sectors (B7 – blending of 7% of biodiesel with 93% of petroleum diesel) since July 2019. The Government is compelled to growing the domestic demand for a cleaner environment through a higher blend of biodiesel. Hence, the B20 programme for the transport sector has been implemented in stages beginning with Langkawi on 1 January 2020. This programme requires nearly 1.06 million tonnes of palm oil annually. It is also expected that the B20 mandate will reduce the GHG emission of about 3.2 million tonnes of carbon dioxide-equivalents (CO_2eq) yearly (The Malaysian Reserve 2020).

16.2.2.4 Conservation of Biodiversity

The oil palm industry has been unjustifiably described in the last ten years or so as to be associated with the deforestation and the dwindling of orangutan population. It is

wrongly perceived that the continued use of palm oil, due to habitat destruction, would eventually result in the extinction of orangutan. Due to these misperceptions, a brutal image of the oil palm industry has been drawn. In innumerable anti-palm oil campaigns in recent years, this is evident.

Several conservation projects have been carried out for a long time by the Government of Malaysia and the Malaysian oil palm industry to support populations of endemic wildlife species. Sabah has been the focus of these initiatives, as it has the largest orangutan population in North Borneo (Ancrenaz et al. 2004). There is an incredible diversity and an abundance of wildlife in the forests of the Kinabatangan in Sabah, which includes some iconic and endemic wildlife species such as proboscis monkey, orangutan, Borneo pygmy elephant, Bornean gibbon, and clouded leopard amongst others. The Lower Kinabatangan has been recognised as one of the four 'major elephant ranges' in Sabah (Estes et al. 2012) and a 'high priority area' for Bornean orangutan (Sabah Wildlife Department 2011).

One of the greatest threats to the population of orangutans is that most of them live outside of the protected area. National Geographic (2018) suggests that the key reasons for the decrease in the population of orangutans are logging and hunting. Although only 40% of the habitat of orangutans were found within the protected area in 2004, this figure rose sharply to 85% in 2016 (The Star 2016). Remarkably, the population of orangutans in Sabah had increased from 8041 individuals in 2003 to 10,358 orangutans reported in 2017, according to a study conducted by Simon et al. (2019) and sponsored by World Wide Fund (WWF). This population increase is partly due to the creation of "ecological corridors" that have enhanced connectivity within forest areas, allowing orangutans to migrate from areas adjacent to plantations to protected forests. More than 10,000 orangutans are still protected in today's plantation landscape, which makes the larger oil palm landscape important for the future survival of orangutan and, indeed, species conservation in general. The vast private-owned land-banks provide unexplored windows for conservation. It was reported by Meijaard et al. (2020) that oil palm plantations in East Malaysia provide support for 22 out of 63 species of mammal found in forest ecosystems and 31 out of 130 species of birds. There are numerous species including orangutans entering plantations to feed on palm fruits. Apart from that, the requirements to conserve areas within estates such as biodiversity hotspots and riparian reserves considered as high conservation value (HCV) and reforestation plots have been embedded as part of the Malaysian Sustainable Palm Oil (MSPO) Certification Scheme (Jackson et al. 2019).

At the national and regional levels, Malaysia has two main forestry programmes. The Central Forest Spine Initiative covers 5.3 million ha of area in Peninsular Malaysia (de la Torre et al. 2019) and the Heart of Borneo Initiative, a trilateral partnership between Brunei, Indonesia and Malaysia, protects an area of over 20 million ha (Sloan et al. 2019). These organised efforts help to boost the planet's resilience to climate change by retaining carbon sinks and building huge green lungs, while maintaining the livelihoods of forest-dependent communities. In addition, Malaysia underpins its pledge to reforestation by committing to the planting of

Table 16.13 Parameters limits for discharge into watercourse under the environmental quality (prescribed premises) (crude palm oil) regulations 1977

Parameters	Limits According to Period of Discharge					
	1978 to 1979	1979 to 1980	1980 to 1981	1981 to 1982	1982 to 1983	1984 and thereafter
Biochemical oxygen demand (BOD) 3-day, 30 °C, (ppm)	5000	2000	1000	500	250	100

Adapted from Department of Environment [DOE] (1977)

one million forest trees in Sabah over the next ten years which is funded primarily by the Malaysian oil palm industry players (Borneo Today 2019).

As part of the efforts to conserve biodiversity, the Ministry of Plantation Industries and Commodities has formed the Malaysian Palm Oil Green Conservation Foundation (MPOGCF) through the Malaysian Palm Oil Council (MPOC) to signify the responsibilities of the Malaysian Government and oil palm industry to the conservation of our environment. The foundation incorporates the current Malaysian Palm Oil Wildlife Conservation Fund (MPOWCF) and its projects on wildlife, biodiversity and environmental conservation in the proximity of oil palm plantations such as establishment of the country's first Wildlife Rescue Centre (WRC) in collaboration with the Sabah Wildlife Department, inventory of Sabah's orangutan population, establishment of the Borneo Elephant Wildlife Sanctuary (BEWS), and Sarawak Orangutan Conservation Programme (Sundram 2020).

The Malaysian oil palm industry has proven that conservation and development are two complementary priorities that can be balanced through sustainable resource management, supported by a regulatory structure. Indeed, the oil palm industry is well governed by more than 60 laws and regulations that cover land, environment, wildlife, labour, and employee matters as well as the use of pesticides. On top of that, there are 25 license categories regulated by MPOB to ensure healthy growth of the Malaysian oil palm industry (Malaysian Palm Oil Board [MPOB] 2020b). When technologies become available to increase the effectiveness of environmental protection, laws are also constantly improved. For instance, the regulatory limit for watercourse discharge for palm oil mills has gradually been rendered more stringent from 5000 parts per million (ppm) biochemical oxygen demand (BOD) in 1978 to 100 ppm BOD in 1984 (Table 16.13). A 20 ppm BOD requirement is enforced in many environment-sensitive areas. There are also a few areas where a zero-discharge condition for new mills has been administered by the Department of Environment (DOE).

16.2.3 Ensuring the Economic Progress of the Nation

The oil palm industry remains as a significant backbone of Malaysia's economic growth, contributing 4.5% to the Malaysian GDP in 2018. At 37.9%, oil palm was the largest contributor to GDP in the agricultural sector (total of RM99.5 billion), followed by other agricultural sub-sectors: livestock (14.9%), fishing (12.5%), forestry & logging (6.9%), rubber (2.8%), and other agriculture (25.1%) (Department of Statistics Malaysia [DOSM] 2019). Trade in commodities continues to play an important role in the Malaysia's economy. In line with the increase in global demand and higher output, Malaysia's trade in commodities remains high, supported by the growth of infrastructure projects and programmes. Palm oil has been dominating the export of commodities (palm oil, rubber, timber, cocoa, pepper, and kenaf) under the Ministry of Plantation Industries and Commodities (MPIC). In 2019, palm oil contributed 50.5% (or RM64.84 billion) of the overall export value of commodities (RM128.5 billion) (Fig. 16.10).

The significance of palm oil trading to the Malaysian economy has been attested with the establishment of the Bursa Malaysia for price setting, hedging, and communicating market intelligence to minimise market risk in palm oil trading. Bursa Malaysia Derivatives Berhad (BMD), one of the subsidiaries of Bursa Malaysia is Malaysia's sole operator for the futures and options exchange. It offers the world's most liquid and lucrative crude palm oil futures (FCPO) contract, integrating the role of Malaysia as the global discovery centre for the price of palm oil (Choo 2012).

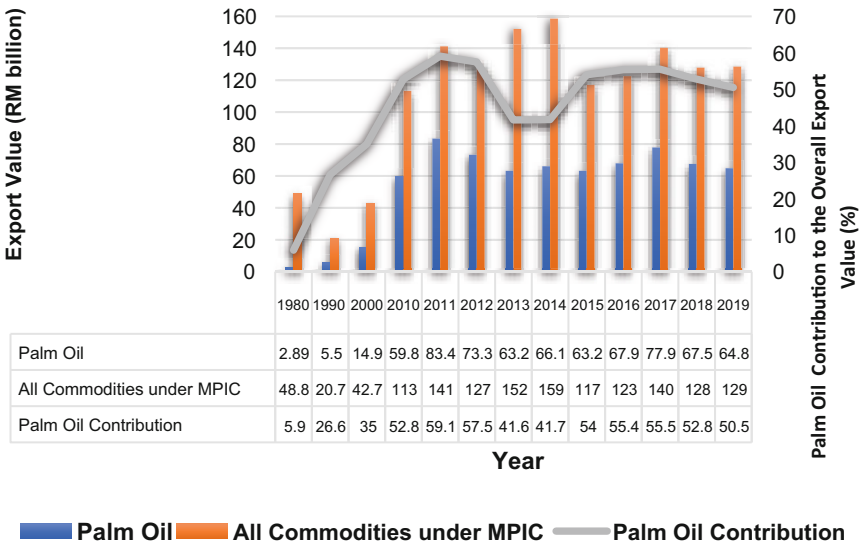

Fig. 16.10 Palm oil contribution to the overall export value of all commodities under MPIC. Adapted from Ministry of Plantation Industries and Commodities of Malaysia [MPIC] (2020)

16.3 Tracebility in the Oil Palm Industry

In the oil palm industry, the drivers for traceability are food safety and sustainability because palm oil is one of the raw ingredients used in food for human consumption and the two criteria are for palm oil to be traded in the global market. Traceability is an important tool in monitoring food safety in the food chains to reduce food safety incidents and ensuring the food raw ingredients are procured from sustainable sources. In the food safety sector, traceability is used as an assurance for the safe consumption of the food. In the EU, Regulation No.(EC) 178/2002 or commonly referred to EU General Food Law defines traceability as "the ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing, and distribution" (European Union [EU] 2002). Meanwhile, CODEX Alimentarius Commission (CAC) defines traceability/product tracing as 'the ability to follow the movement of a food through specified stage(s) of production, processing and distribution" (CODEX Alimentarius Commission [CAC] 2015). According to Olsen and Borit (2013), traceability is "the ability to access any information relating to what is under consideration, throughout the entire life cycle, by means of recorded identifications". Hence, it is important to establish a traceability system to collect and record data on all the ingredients and operations during food production. It can also be used to optimise the performance of a company. In the oil palm industry, the supply chain started from the nursery sector until its point of usage in the food products. Figure 16.11 is a typical oil palm supply chain in Malaysia. Currently, the oil palm industry is using the electronic traceability system, however, some companies are considering using blockchain.

16.3.1 Electronic Traceability System

In Malaysia, at each point of the supply chain, codes of practice have been established to ensure the quality and food safety of the palm and palm kernel oils produced. Figure 16.12 is the list of codes of practice to monitor the safe supply of palm oil products. Under the licenses conditions of MPOB, there are 25 licenses the industry has to apply before they can operate an oil palm premises (Malaysian Palm Oil Board [MPOB] 2020b) and the list of licenses is shown in Table 16.14. Each sector has to submit data on their monthly production to MPOB. Compilation of this data is used to forecast production of palm products. This database can also be converted into traceability system by adding a few features to the current submission system to ensure the connectivity between sectors, such that any incident on violation of food safety can be traced to the source. Currently, MPOB can trace any violation of the food safety to the sector and the company through its regulations.

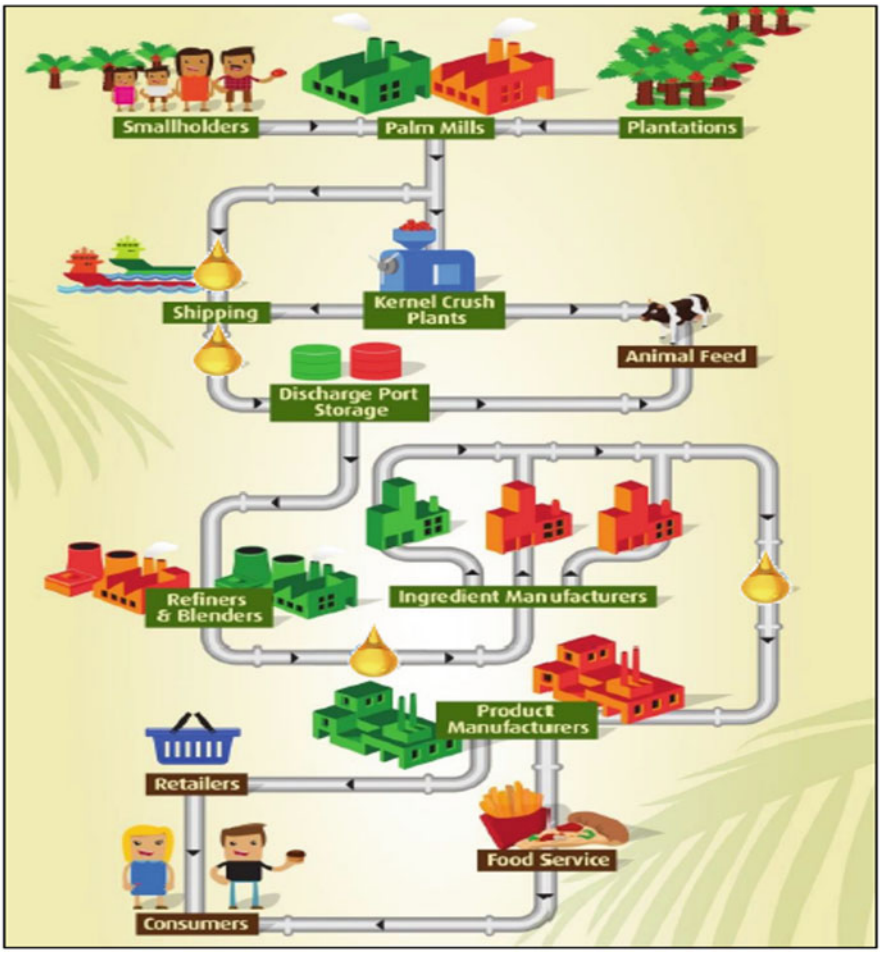

Fig. 16.11 Palm oil supply chain. Adapted from GreenPalm (2013)

In terms of sustainability, the same licensing principle applies throughout the oil palm supply chain. The oil palm industry together with MPOB has developed the Malaysian Sustainable Palm Oil (MSPO) standards for the supply chain from the plantations and smallholdings to the mills. The standards have been gazetted in 2013 and implemented voluntarily in 2015. Figure 16.13 shows the timelines of MSPO implementation in Malaysia for the oil palm industry. In the year 2020, the implementation of MSPO scheme has been made mandatory and MSPO certification is part of the licensing conditions. Hence all members of the oil palm sector have to register for implementation of MSPO and to show evidence of certification, which will be incorporated in the MPOB big data system. This system will be able to trace the transaction for the whole supply chain of oil palm products (seeds, seedlings, fresh fruit bunches (FFB), CPO, kernel) from producer to refinery and downstream

Fig. 16.12 MPOB codes of practice along the oil palm supply chain

user of palm products. Malaysian Palm Oil Certification Council (MPOCC) launched MSPO e-Trace in 2019. Through this e-Trace, the sustainability of the supply chain can be traced to the source and it is transparent. Any violation of the sustainability requirements can lead to withdrawal of the certification and may be even the license revocation.

Under MSPO, traceability of the supply chain certification can be achieved through mass balance (MB) or segregation system (MPOB 2013). This is done by means of declarations on all delivery orders to assure the origin and amount of the products and these products are uniquely identified. The amount of product withdrawn at the respective sector in the oil palm supply chain does not exceed the amount supplied. The requirements of traceability are applicable to all companies handling MSPO certified products in the supply chain. The supply chain starts from the smallholdings or plantations producing FFB, dealers of fruits (DF), palm oil mills (POM) where the FFB are converted into CPO and palm kernels (PK), palm kernel crushing plants where the kernels are crushed into crude palm kernel oil (CPKO) and palm kernel cake (PKC), palm oil refineries where both CPO and CPKO are refined and further converted to downstream palm products. The palm

Table 16.14 Licenses for oil palm supply chain

No	Type of licenses
1	Oil Palm Seed Producer (21)
2	Plants from Oil Palm Tissues Producer (6)
3	Nursery (913)
4	Estate (4999)
5	Small holding (221,903)
6	Palm Fatty Acids Dealer (26)
7	Oil Palm Fruit Dealer (2992)
8	Palm Kernel Dealer (43)
9	Palm Oil Dealer (1678)
10	Mixed Palm Oil Dealer (504)
11	Palm Oleochemicals Dealer (50)
12	Oil Palm Seeds and Seedlings Dealer (15)
13	Plants from Oil Palm Tissue Dealer (1)
14	Commencement of Construction of Oil Palm Mill (13)
15	Oil Palm Mill (456)
16	Palm Kernel Crushing Factory (64)
17	Palm Oleochemicals Plants (53)
18	Refinery (63)
19	Transporter (446)
20	Commencement of Construction of Bulking Facilities (3)
21	Bulking Facilities (42)
22	Laboratory (30)
23	Surveying of Oil Palm Products (57)
24	Oil Palm Products Exporter (102)
25	Oil Palm Products Importer (129)

Adapted from Malaysian Palm Oil Board [MPOB] (2020b)

products are converted into palm biofuel and palm biomass at the biofuel plant. These palm products are stored in storage tanks, bulking installations. or warehouses and are transported using truck, road tankers, train, containers, barges, or vessels. Hence, information on sustainability compliance at the first point, in this case the plantations or smallholders must be made available to the next link which is the mill. The same principle applies to the mill, palm kernel crusher, refinery, and end users of palm products through the whole supply chain and there should not be any break in the link.

The palm products will go through many processes (conversion and transportation) between the smallholders or plantations and the end users. Any individual batch of certified palm products can be traded through one of the two traceability systems approved by MSPO - MB and segregation or identity preserved (IP). MB system allows the mixing of certified and non-certified palm products at every stage of the oil palm supply chain and the amount of certified palm products must be closely monitored to assure that the number of certified products is not exceeded.

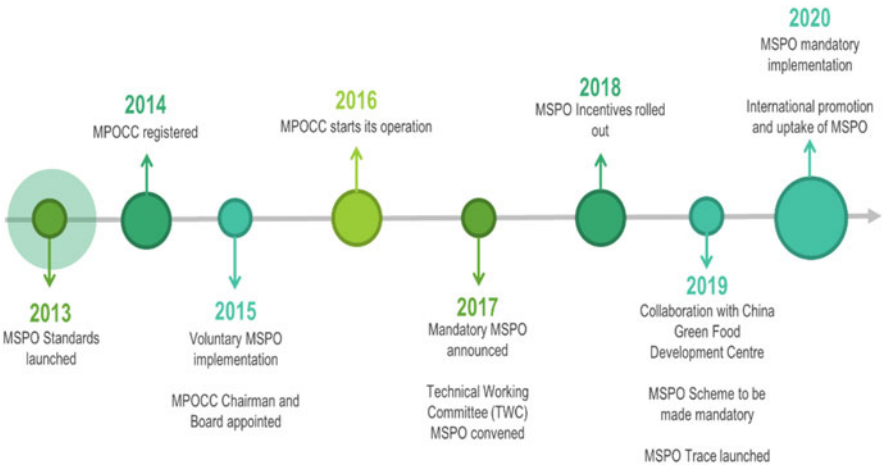

Fig. 16.13 Timelines of the development of MSPO

Segregation is much better because there is a complete separation of certified products from non-certified products, however, IP is the best. Henceforth, it important to maintain chain of custody (COC) to prevent mixing of MSPO certified with uncertified products and to safeguard the purity of the certified products.

Under MSPO, the management of the oil palm company must be committed to implement and maintain the traceability system in line with either the MB or segregation system. All sourcing of oil palm products must be identified especially the origin with respect to MB or segregation traceability system. During the processing, conversion of the palm products must be closely monitored with respect to the amount of certified product especially for MB system. Delivery, storage, sales, and transport of these products must follow the traceability of choice. Documentations for all transaction must be recorded and these documents are evidence of keeping the purity of the certified MSPO products during audits. Like any system, the company has to establish the standard operating procedures (SOP) for the traceability system, which include procedures, description of product flow, organisational structure, responsibilities, and authorities of person responsible of the system. The most important aspect of traceability is records of all sources of origin, processing of palm products, and transaction during sale-purchase of the products. Based on the records and documents required under MSPO, it is convenient to trace any violations of the requirements. The same is also applicable to the food safety and quality of palm products.

16.3.2 Blockchain

Blockchain is a form of digitalised traceability system which is more transparent to the members of blockchain network. This system records all transactions that occurred, and the information is distributed across a network of computers and these are shared among the participants of the blockchain network (Olsen et al. 2019). An example of a blockchain system app that can be applied to oil palm industry is DIBIZ, which uses cloud-based platform to connect among all the stakeholders in a supply chain and accelerates the digital transformation (DIBIZ 2019).

The oil palm growers comprise 40% of smallholders, whose holdings are now being certified under MSPO. The Malaysian Government provides the fund for the MSPO certification of smallholders. Most of these smallholders are not connected with the buyer of sustainable palm oil. By participating in this blockchain app, the smallholders will have access to the buyers as part of the sustainable supply chain. It is more transparent as the information of each step in the supply chain is available to the smallholders and they know the acceptance of their products, in this case FFB. The amount paid to the smallholders will be based on the quality of their FFB and graded using the ripeness standard. Thus, implementation of blockchain is advantageous to the smallholders.

16.4 Conclusion

Sustainable palm oil will be the future for the industry and consumers around the world are clamouring for sustainably produced palm oil. As such, Malaysia will continue to play a direct and proactive role in ensuring the oil palm industry is suitably ready and aligned in producing sustainably produced palm oil.

The Malaysian oil palm industry's commitment to sustainability is evidenced in its participation in sustainable practices including ensuring food safety and quality, providing nutritious and healthy food, sustainability certification, good agricultural practices, trapping of biogas from palm oil mill effluent, use of milling residues as renewable energy and the results of a life cycle assessment of palm oil for improving environmental performance. Efforts in sustainability include a concerted effort by the industry to shift from pollution control to that of pollution prevention, minimisation of pollution from palm oil production and all environmental impacts associated with the full life cycle of oil palm, and investment in, as well as the adoption of new environmentally sustainable technologies. Malaysia's efforts on sustainability are a continuous improvement process because palm oil is a ubiquitous source of feedstock for global industries as well as an important source of employment and economic power for Malaysia.

For Malaysia, sustainability of palm oil is a foremost importance in which the mandatory Malaysian Sustainable Palm Oil (MSPO) Certification Scheme has been

enforced nationwide. In addition to an available supply chain certification that links the sustainable palm oil from the plantation to the final product, Malaysia is seeking to use blockchain technology and traceability to lift the level of confidence in its palm oil sector by demonstrating its commitment to sustainable and transparent supply chains. The blockchain technology will bridge the gap between the issues of the Malaysian oil palm industry's expectations, allegations and realities.

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Chapter 17
Adulteration in Oils and Fats Industry

J. M. Nazrim Marikkar

Abstract Adulterations in oils and fats is a major food safety issue that affects consumers and food industries. Monitoring the purity of oils and fats has therefore become an integral part of quality assurance in industries. As the unscrupulous seeks to adopt subtle ways of adulterations, the detection of fraud becomes more difficult and challenging. A great deal of effort has been undertaken in the past to develop detection strategies using chromatographic techniques, thermal analysis techniques, spectroscopic techniques, etc. Chromatographic techniques with their wide array of column materials and detectors have emerged as the most popular authentication tools for food lipids. They can help generate data bases for compositional profiles for authentic samples of individual oils. However, compositional variations due to varietal difference and different geographic origins should be accommodated. In a number of cases, DSC thermal analysis approach has been proven to be more useful and convenient since no sample pre-treatment is required. Spectroscopic techniques such as FTIR, NIR, and FT-Raman seemed to be very useful analytical tools for detection of adulterations in general since they are rapid and can highlight subtle deviations. However, detection of adulteration using these techniques demand the application of chemometrics tool such as principal component analysis of multivariate data.

17.1 Introduction

Oils and fats form an important part of man's healthy diet. Apart from being a source of energy, they act as important structural and functional constituents of cells in biological systems. In food formulations, oils and fats contribute many desirable quality attributes such as taste, texture, structure, mouthfeel, flavour, and colour. In a competitive market-based economy, maintaining the authenticity of oils and fats in terms of their quality attributes is of paramount importance. The general quality

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attributes of most fatty substances are affected due to various reasons; quality deterioration by adulteration is often cited as an important problem of the oils and fats industry. Expensive vegetable oils and fats such as extra virgin olive oil (Patrícia et al. 2019; Vanstone et al. 2018), cocoa butter (Lipp and Anklam 1998), dietary supplement oils namely cod liver oil (Rohman and Che Man 2009), evening primrose oil, flaxseed oil (Ozen et al. 2003), camellia oil (Xinjing Dou et al. 2018), grape seed oil, and pumpkin seed oil (Butinar et al. 2010) are said to be vulnerable for adulteration. In order to safeguard the consumers from fraudulent practices, considerable amount of research effort has been taken by researchers across the world to develop analytical methodologies to authenticate oils and fats.

Owing to ever increasing public awareness on food quality and safety issues, authentication of oils and fats has become a rapidly advancing field of study. Scientific community, law enforcement authorities, food-manufacturing companies, and personnel involved with supply chain management are interested to keep an update of the developments taking place in this arena (Johnson 2014). A rapid glance at the literature would show how many different analytical approaches were investigated by researchers across the world to cross–check the authenticity of oils and fats. Chromatographic techniques such as gas liquid chromatography (GLC) and high performance liquid chromatography (HPLC), with their wide array of column materials and detectors have emerged as the most popular food authentication tools. Nevertheless, thermal techniques such as differential scanning calorimetry (DSC) also found several uses in food research leading to quality assurance. Thermal analysis of oils and fats by DSC has drawn the attention of researchers due to ease of operation and sample handling. In recent times, scientists focus their attention towards spectroscopic techniques in food analysis because of their potential uses in authentication of oils and fats. Nuclear magnetic resonance (NMR), Fourier transform infrared (FTIR), Fourier transform near infrared (FT-NIR), FT-Raman spectroscopic methods are some of those employed frequently to detect fraud and misbranding of oils and fats from different sources. As the volume of research going into authentication of oils and fat has increased tremendously, it is timely to bring an updated review of the authentication methodologies during the past decade and so on. Our main purpose is to undertake a critical analysis of the analytical techniques, which are employed for authenticity assessment, explaining how and why they give plausible solutions.

17.2 Different Analytical Approaches

17.2.1 Chromatographic Analyses

17.2.1.1 Fatty Acid Analysis

Fatty acids are the basic building blocks of oils and fats occurring in plants, land animals and marine species. They are usually joined together in groups of three,

Table 17.1 Fatty acid composition of selected plant oils

Fatty acid	[1]Coconut Oil	[1]Palm Kernel Oil	[2]Olive Oil	[2]Canola Oil	[3]Cocoa Butter	[3]Engkabang Fat
C6:0	0.4	0.3	–	–	–	–
C8:0	7.3	4.4	–	–	–	–
C10:0	6.6	3.7	–	–	–	–
C12:0	47.8	48.7	–	–	–	–
C14:0	18.1	15.6	–	–	–	–
C16:0	8.9	7.5	14.0	5.6	26.34	16.58
C16:1	–	–	–	–	–	–
C18:0	2.7	1.8	2.8	2.5	37.91	47.83
C18:1	6.4	14.8	72.5	61.8	31.83	32.49
C18:2	1.6	2.6	8.4	20.0	2.75	1.00
C18:3	0.1	0.1	0.7	8.1	–	–
C20:0	0.1	0.2	0.4	0.8	1.17	2.10
C20:1	–	–	1.2	1.3	–	–

[1]Pantzaris and Ahmad (2001); [2]Tan and Che Man (2000); [3]Yanty et al. (2013)

forming a molecule called a triacylglycerol. In a conventional set up, the triacylglycerols are needed to be saponified prior to determine the fatty acid profiles using GLC system equipped with polar capillary column and flame ionization detector. According to the classical approach of authentication, the fatty acid profile of a given sample is cross-checked with reference to an authentic fatty acid data base to see if its values deviate significantly from the range found in the reference (Codex Alimentarius Commission 2001). This approach has been successful in a number of cases as certain fatty acids were found to be remarkably sensitive for adulterations. For instance, lauric acid became the marker to detect adulteration of soybean oil, cottonseed oil and tallow since it did not occur in significant proportions in these oils. Occurrence of castor oil as contaminant in some vegetable oils was ascertained by means of ricinoleic or hydroxystearic acid content (Norris 1982). When making a conclusion using fatty acid profiles, however, one must need to be cautious, as there are variations in the proportions of individual fatty acid based on varietal differences and geographical origins.

While applying fatty acid (FA) analysis approach, getting a positive indication of adulteration would become more difficult when the FA compositions of the contaminant and the original oil were closely similar. As shown in Table 17.1, coconut and palm kernel oils are examples of lauric oils displaying close similarity in fatty acid profiles. Olive oil and canola oil were similar in their fatty acid compositions despite some variations due to country of origin (Tan and Che Man 2000). Likewise, fatty acid profile similarities were noticed in cocoa butter and engkabang fats (Yanty et al. 2013). In such cases, alternative strategies such as fractional crystallization of lipids, regio-specific analysis of FA using pancreatic lipolysis, fatty acid ratio calculations and discriminant analysis of FA data have been adopted by investigators (Dourtoglou et al. 2003). When extra-virgin olive oil was adulterated either by

refined olive oil or olive pomace oil, detection of adulteration especially at lower levels became quite difficult by mere comparison of the overall fatty acid data (Firestone et al. 1988). Suspected adulteration of olive oil with other seed oils (cottonseed, sesame, corn, and soybean) (Norris 1982), and butter fat (ghee) adulteration with lard (LD) were resolved by adopting fractional crystallization as an alternative strategy (Farag et al. 1983). However, due to laborious and time-consuming procedures, this type of methodology might not be suitable for today's rapid routine quality assessments.

Researchers also investigated the potential use of taking certain fatty acid ratios to detect adulterations in fats and oil. Gamazo-Vazquez et al. (2003) proposed a method to take oleic and linoleic acid ratio as diagnostic parameter in the case of detection of olive oil adulteration by other seed oils below 5% became an extreme difficulty. This approach enabled the detection of contamination of olive oil by sunflower oil at the lowest of 1% level. Likewise, Seo et al. (2010) suggested to use stearic acid content in combination with C18:2/C18:1 ratio to detect sesame oil adulteration by corn oil at 5% level. For detection of milk fat adulteration with foreign fats such as LD, tallow and palm oil, FA ratios C14:0/C18:2 and C18:2/C18:0 were used as parameters. For successful discrimination between pure milk fat and admixtures containing more than 3% LD could be achieved by the application of linear-discrimination analysis to FA data (Ulberth 1994).

In these days, researchers pay more attention to use chemometrics techniques such PCA to fatty acid data to discriminate authentic oil samples from adulterated ones. For instance, Dourtoglou et al. (2003) applied principal component analysis (PCA) to the total and regio-specific fatty acid data to discriminate pure olive oil from those adulterated with corn, soybean, sunflower and cottonseed oils. According to this study, even samples adulterated at 5% level could be discriminated along with the possibility of knowing the type of the adulterant. Researchers also spent considerable amount of their effort for authentication of virgin coconut oil (VCO) since it is a premium product that commands higher prices in the edible oil market. Palm olein, the liquid fraction of palm oil has been considered as one of the potential adulterant due to its low cost of production. As shown in Fig. 17.1, application of PCA to fatty acid compositional data helped to discriminate adulterated VCO samples from authentic VCO. As a noteworthy feature, all VCO adulterated with palm olein in the range of 5 to 30% were clustered into one block (Marikkar and Yanty 2018).

As conventional GC systems coupled with FID might not have the capacity to resolve the isomeric forms of different unsaturated fatty acids, effort was made to employ advanced version of GLC systems. While GLC hyphenated with mass spectrometer has been found to give greater details of FA composition (Philips and Beens 1999), comprehensive two-dimensional gas chromatography (GCxGC) was able to unravel the entire spectrum of component fatty acids, including those which occur in lower abundance or in different isomeric forms (Indrasti et al. 2010). According to Chin et al. (2009), these advanced forms of GC systems were found to be able to distinguish LD from other animal fats. However, these were preliminary studies to distinguish LD from other animal fats; hence, further investigation would

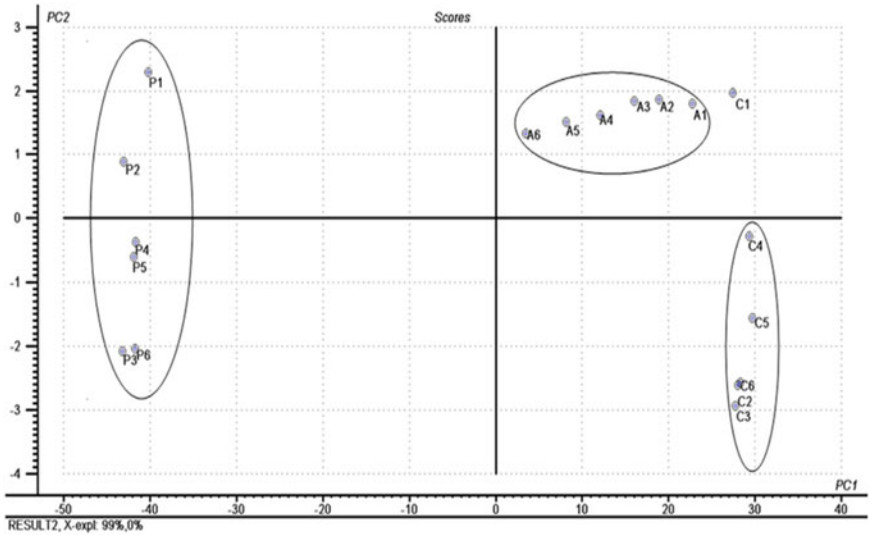

Fig. 17.1 Score plot of principal component analysis applied to fatty acid composition data. A1-A6, adulterated VCO sample; C1-C6, pure VCO samples; P1-P6 plm olein samples (Reprinted with permission from International Coconut Community)

be necessary to show their potential applications in detecting LD as an adulterant in lipid mixtures.

17.2.1.2 Triacylglycerols

Adulterations in oils and fats detected by means of TAG compositional analyses has been explored extensively. In the early days, TAG composition analysis using packed column GC was adopted to detect adulterations of milk fat and cocoa butter. In both of these fats, equations incorporated with the major TAG peaks were developed to differentiate authentic milk fat and cocoa butter samples from the adulterated ones. As these equations were meant to define either pure milk fat or pure cocoa butter, their use might help to detect non-milk fat components at 5% level with 99% confidence in milk products (Timms 1980) while cocoa butter equivalents at 15% level with 95% confidence in cocoa butter (Padley and Timms 1980).

With advancements in technology, packed column GC system were replaced with high-temperature capillary column GC system due to high efficiency and rapidity in profiling the TAG data. The capillary column GC was employed to detect non-milk fat components in goat milk fat (Fontecha et al. 1998) as well as lard in ewe's milk fat (Goudjil et al. 2003). Fontecha et al. (1998) managed to gather a large pool of goat milk samples collected from five different herds of goats to develop a formula in defining pure goat milk fat using TAG composition. Adulteration of goat milk by either palm oil or tallow at 3 to 5% was able to be detected using this formula. A

multiple linear regression equation build based on the TAG composition of pure ewe's milk fat and its admixtures analysed by capillary column GC system was able to detect the adulteration of milk fat exceeding 5% (Goudjil et al. 2003). In another study, Simoneau et al. (1999) used the difference between TAG compositions generated by GC column system to identify adulteration of cocoa butter by plant based cocoa butter equivalents (CBE) in confectionery products (Lipp and Anklam 1998).

In the next phase of development, reversed-phase (RP) HPLC systems became available for determination of TAG composition of oils and fats. The use of RP-HPLC received much attention of researchers for detection of oil adulteration due to two advantages; the sample preparation in RP-HPLC was easy and the characteristic TAG profiles of many oils do not change by the natural variations in fatty acid composition (Antoniosi et al. 1993). RP-HPLC was particularly useful for detection of adulteration in several instances including occurrence of lard in vegetable oils such as palm oil, palm kernel oil and canola oil (Marikkar et al. 2005). Kapoulas and Andrikopoulos (1986) utilised the HPLC-method to detect adulteration of olive oil by other vegetable oils such as sunflower, soybean, cottonseed and corn oils. In this method, detection was carried out using marker peak known as tri-linoleoylglycerol (LLL) TAG peak which is commonly found in other soft oils but not in olive oil. This method was further validated by Antoniosi et al. (1993) to determine the adulteration of olive oil by soybean oil and showed to be useful in detecting the adulteration of as low as 4%.

Detection of olive oil adulteration by HPLC could become more difficult, if TAG compositions of the major oil and the adulterant had become almost similar. For instance, coconut and palm kernel oils, being lauric oils, display close similarity in TAG composition. According to Salivaras and McCurdy (1992), canola oil and olive oil, being known for high level of monounsaturated TAG molecules, showed close similarities in their TAG composition. Likewise, similarities in TAG profiles were noticed in cocoa butter and engkabang fats. In such cases, alternative strategies are essentially required to resolve the adulteration issues. Flor et al. (1993) managed to discover a linear correlation between LOO/LOP and OOO/POO from the database of TAG composition obtained through different grades of commercial olive oil produced from the major olive oil producing countries. According to this study, deviations due to adulteration could be found easily using a straight-line graph since inadequate adherence to this straight line would mean that the virgin olive oil sample was adulterated with other sub-branded olive oils.

PCA build from the TAG compositional data is increasingly be used to resolve adulteration issues in oils and fats. For instance, Tsimidou et al. (1987) demonstrated that the PCA application to TAG compositional data was found to be an effective way to detect adulteration of pure olive oil with maize, rapeseed, cottonseed, sunflower, and soybean oils. Using this approach, it could be possible to classify samples adulterated at 10% level and above from authentic olive oil samples. In another study, Marikkar et al. (2005) demonstrated the use of discriminant analysis to distinctly classify lard in admixtures of vegetable oils such as palm oil, palm

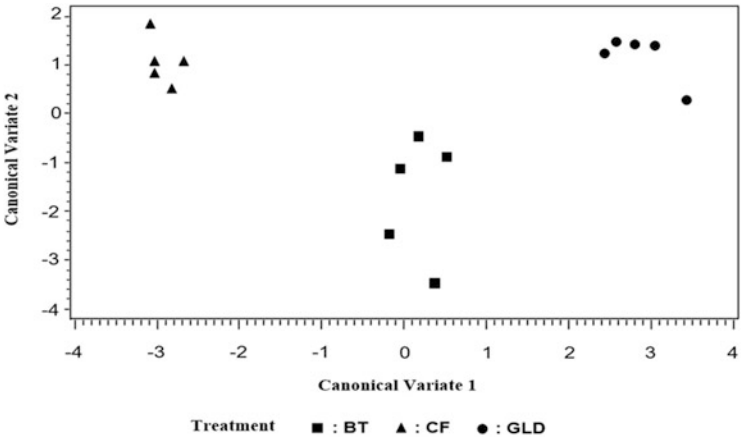

Fig. 17.2 CANDISC plot for Canonical Variate 2 vs. Canonical Variate 1 values of palm kernel oil samples adulterated with lard (GLD), beef tallow (BT), and chicken fat (CF (Reprinted with permission from Elsevier)

kernel oil, and canola. As shown in Fig. 17.2, palm kernel oil adulterated with lard, chicken fat and beef fat were distinctly classified into three different clusters.

17.2.1.3 Minor Constituents

Occurrence of minor constituents as unsaponifiable matter is common among most of the vegetable oils and fats. Sterols, triterpene alcohols, or hydrocarbons such as n-eicosane, n-docosane, squalene, carrotinoids, etc. are some of the minor constituents frequently encountered in most oils and fats. Animal body fats and milk fats are known to possess cholesterol as a minor constituent (Bragagnolo and Rodriguez-Amaya 2002). In the past few years, minor components in plant oils received escalating research interest from researchers due to their multiple touted health benefits (Boskou 2009). Aside this, determination of minor components in oils and fats would be useful to resolve some of adulteration issues. For instance, analysing the sterol content allows the detection of adulterants in milk fat, butter, olive oil, and vegetable fats (Alonso et al. 1997). In his review, Azadmard-Damirchi (2010) highlighted the benefits of phytosterols to detect adulteration of olive oil by hazelnut oil, or other sub-branded olive products.

Conventionally, the unsaponifiable matter of lipids where majority of minor components reside can be determined directly using GC systems. For instance, the analysis of total sterols using this approach has greatly helped in solving the olive oil adulteration. In his study, Al-Ismail et al. (2010) mentioned the use of thermo-stable polar columns in GLC system for sterols determination to detect olive oil adulteration with other seed oils. In majority of olive oils, 95% of the total sterol constituents is mainly composed of campesterol, stigmasterol, β- Sitosterol and Δ 5-avenasterol.

Interestingly, the relative proportions of these four sterols fit well into the equation as given below (Eq. 17.1). When calculated, majority of the olive oils conferred the Af value in the range of 19.7–25.45. Whilst, other seed oils such as corn, sunflower, soybean and cottonseed oils were found to have Af values in between 2.04 and 2.9. As such, adulteration of olive oil with other seed oils can be detected through the reduction of the Af value. Nevertheless, it is impossible to use this approach to detection of adulteration for hazelnut and lampante oils due to the relatively low proportions of campesterol and stigmasterol in them.

$$\mathrm{Af} = [100 - (\mathrm{Campesterol}\% + \mathrm{stigmasterol}\%)]/(\mathrm{Campesterol}\% + \mathrm{stigmasterol}\%)] \ldots \quad (17.1)$$

Alternatively, the total sterol can be seperated into the individual sterol species by thin layer chromatography or chromatography on a silica gel column prior to GC analysis either in its free or derivatised form using capillary column or a more advanced on-line coupled liquid chromatography-gas chromatography (LC-GC) with a better accuracy (Plank and Lorbeer 1983). Analysis performed using capillary GC does not need saponification, extraction, and derivatization steps. Hence, it can reduce the total time of analysis (Alonso et al. 1997). On-line coupling of LC-GC offers a rapid separation of free sterols in edible oils and fats as separation is integrated in the GC system. Hence, the LC-GC managed to reduce not only the sample preparation time but also increase in the accuracy of detection of sterols in oils and fats (Senorans et al. 1996). A comprehensive development in the analysis of sterols using on-line coupled LC-GC systems has been detailed out by Villen et al. (1998).

The possibility of using cholesterol as a marker for detecting lard in ghees has been discussed by Farag et al. (1983). Since both cow and buffalo ghees possessed with higher cholesterol content than lard, a decreasing trend in the cholesterol concentration in the admixtures indicated the presence of lard adulteration. It allows the detection of as low as 5% of lard content in admixtures of both cow and buffalo gees. Alonso et al. (1997) suggested a more simple method for the direct analysis of sterols after methanolysis without the need to go through the extraction process of the unsaponifiable matter. Initially, this approach was used to evaluate milk fat adulterations with palm oil. As milk fat contains a high cholesterol content whereas palm oil is rich in plant sterols such as campesterol, stigmasterol, and β-sitosterol, a comparison between the chromatograms of the authentic sample and adulterated sample would show a decrease in the cholesterol content and an increase in the plant sterols composition. This would indicate the possibility of adulteration of milk fat with either palm oil or its fractionated components. Besides proving to have a good repeatability, this method also showed to be more rapid in comparison to other methods.

The use of hydrocarbons present in vegetable oils or animal fats such as squalene to detect adulteration has received tremendous interest from the researchers lately, mainly because of the abundance of the hydrocarbon in olive oils when compared to

any other vegetable oil (Nenadis and Tsimidou 2002]. Conventionally, GC was used to detect squalene after going through isolation from the unsaponifiable matter and fractionation into their isomers. European Union Commission (1993) developed an official method to analyse squalene using a short capillary column that is coated with a low-polarity stationary phase alongside with the simultaneous detection of waxes. According to another study, lard or margarine adulteration in cow and buffalo ghees could be detected through the analysis of hydrocarbons from the unsaponifiable matter at the minimum of 5% (w/w) mainly because of the presence of a higher concentration of n-nonacosane in lard than ghees of cow and buffalo as well as the absence of n-nonacosane in ghees originating from both cow and buffalo (Farag et al. 1983).

Carotenoids being a plant pigment is useful for the authentication of oils and fats. They can be classified into (all-E)- α- and (all-E)–β-carotene, (all-E)-lutein, and (all-E)-zeaxanthin and analysed using a simple spectrophotometric method or HPLC with a UV detector. A comparative analysis between photometric and liquid chromatographic approaches was made by Franke et al. (2010). It was found that the total carotenoid contents of the photometric method were significantly higher than those obtained by HPLC method. A possibility in the variation could be attributed to the over-estimation of total carotenoid content by photometric method as the absorbance is measured at maximum at 446 nm representing the whole group of carotenoids. Among other vegetable oils, palm oil is found to contain the highest β-carotene level while coconut oil possesses negligible amount of carotenoids. Hence, Viver (1999) used carotenoid content as a marker to determine the adulteration of coconut oil with palm olein.

Tocols (tocopherols and tocotrienols) known commonly as vitamin E are another group of minor components used to determine authenticity of oils and fats. Vitamin E exist in four different isomers of α, β, γ, and δ. Tocopherols are in abundance in cottonseed, wheat germ, soybean, peanut and canola oils whilst tocotrienols are largely found in palm oil. Determination of the tocopherol content of oils and fats was performed using the HPLC system equipped with either normal-phase stationary column and a florescence detector or a reversed-phase stationary column and UV detector (Andrikopoulos et al. 1991; Coors and Montag 1988). In certain cases, better efficiency in peak separation was achieved by purifying the oils using gel permeation chromatography coupled with evaporative light scattering detector (ELSD) (Chase Jr. et al. 1994). Tocopherol analysis was found to be useful in detecting the adulteration of milk fat with vegetable fats (Alonso et al. 1997), sunflower oil with groundnut oil (Rossell 1998), and butter with margarine. Elham et al. (2008) demonstrated that adulteration of cocoa butter with palm mid fraction was detected through the analysis of the tocotrienols. Authors claimed that palm mid fraction in CB as low as 5% (w/w) could be detected using the method reported.

The use of minor components such as tocopherols and carotenoids for detecting adulteration in fats and oils has to be applied cautiously. Composition of these minor components could be altered during the refining process, particularly in the bleaching and deodorization steps where the bleaching earth absorb most of the plant pigments whereas the deodorization performed under high temperature might

lead to the decomposition of some minor components (Franke et al. 2010). Other than this, tocopherols might undergo auto-oxidation naturally during storage when exposed to light, oxygen, and temperature (Coors and Montag 1988). Hence, when interpreting the results for authentication purposes, it is important to take these factors into consideration.

17.2.2 Thermal Analyses

Thermal analysis by DSC has been a potentially useful approach for food authentication studies. Fats and oils are usually composed of a wide variety of triacylglycerols, which melt or crystallize over a wide range of temperatures. When these fats and oils are subjected to thermal analysis by DSC, the output is primarily associated with melting or crystallization of these TAG molecules. The DSC output coming in the form of melting and cooling curve could serve as a fingerprint to identify different oils and fats. In a pioneering effort, Dyszel and Baish (1992) demonstrated the use of DSC in the identification of various edible vegetable oils. This study triggered the curiosity of several researchers to investigate the use of DSC to establish the standard reference curves of several plant oils (Yanty et al. 2012; Tan and Che Man 2000) and animal fats (Nina Naqiyah et al. 2017; Yilmaz and Karakaya 2009). If any significant deviation is detected in the DSC curve of a particular oil or fat with respect to the standard reference curve, it can be taken as a preliminary evidence of adulteration.

DSC was successfully used to detect adulteration in vegetable oils such as palm oil (PO) (Marikkar et al. 2001), canola oil (CaO) (Marikkar et al. 2002), sunflower oil (SFO) (Marikkar et al. 2012) etc. When PO was adulterated with LD, a characteristic adulteration peak corresponding to LD appeared at −43.9 °C of the low-temperature region of the cooling thermograms (Marikkar et al. 2001). LD adulteration (1 to 20%) into PO was found to cause gradual increase in size of this peak. Likewise, addition of small amounts of LD into either CaO or SFO resulted in the appearance of an extra peak located in the higher temperature region of the DSC heating curve and the peak area was found to correlate linearly with the increasing concentration of LD. When this study was repeated with LD stearin in place of LD as adulterant, results similar to the previous study were obtained (Marikkar and Rana 2014). DSC investigations were also extended to detection of adulterations in lauric oils such as coconut and palm kernel oil. Virgin coconut oil (VCO) is a premium product with a high market value; its authenticity and quality assurance are important to safeguard consumers from fraudulent practices.

Recently, Marikkar et al. (2019) demonstrated the use of DSC curves to differentiate pure VCO from those adulterated with palm olein. As shown in Fig. 17.3, the overlay of DSC curves indicated the gradual deviations in DSC features affecting all DSC parameters associated with the two peaks. According to Pearson correlational analysis, DSC parameters of the heating curves namely peak temperature, onset, and peak height were found as sensitive to adulteration. The changing proportion of

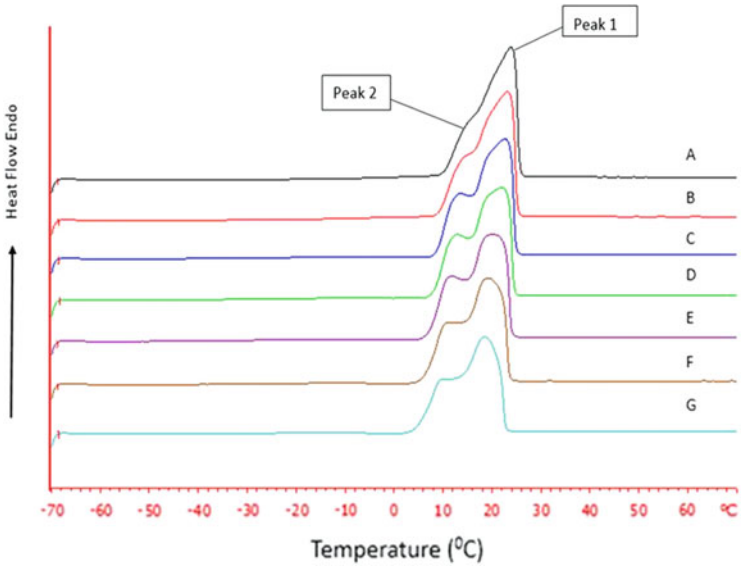

Fig. 17.3 DSC heating curves of (A) virgin coconut oil (VCO), (B) VCO adulterated with 5% palm olein (PO), (C) 10% PO, (D) 15% PO, (E) 20% PO, (F) 25% PO and (G) 30% PO (Reprinted with permission from International Coconut Community)

saturated to unsaturated TAG molecular ratio in the admixtures was seemed to have caused these changes.

17.2.3 Spectroscopic Analyses

In the past few years, utilization of spectroscopic techniques such as UV-visible, FT-IR, FT-NIR, FT-Raman, and NMR has been investigated in a variety of food analyses. They have become a well sought after option because of the high throughput of analysis and ease of handling. To date, a great deal of effort has been made to use them for detection of adulterations in oils and fats. In the early days, characterization of pure vegetable oils, butters fat and margarines was carried out using FTIR spectroscopy (Guillen et al. 2003; Guillen and Cabo 1997a,b; Safar et al. 1994). Comparisons were also made between plant oils with lard (Guillen and Cabo 1997a) and other animal fats (Che Man and Mirgani 2001) to distinguish their spectral features. As shown in Fig. 17.4, majority of oils and fats apparently have similar mid-IR spectra with slight spectral dissimilarities existing in certain frequency regions are useful for authentication of oils and fats. The observed differences in spectral characteristics of oils and fats were mainly due to molecular compositional and structural differences such as degree of unsaturation and carbon chain length, monounsaturated to polyunsaturated acyl group ratio, variations in

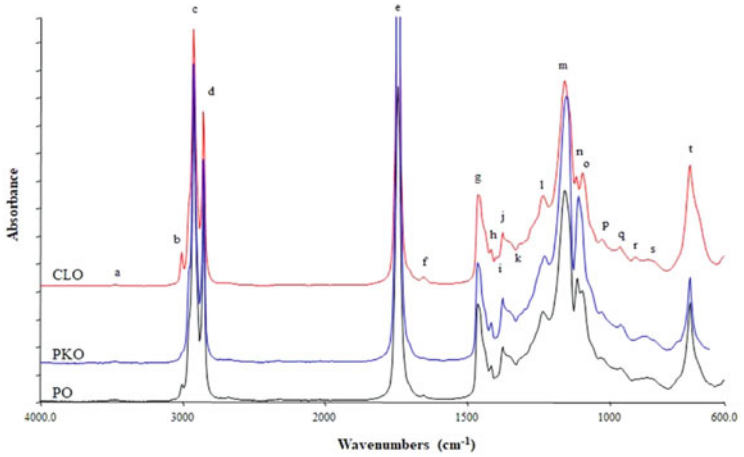

Fig. 17.4 FTIR Spectra of palm oil (PO), palm kernel oil (PKO) and canola oil (CLO)

trans fatty acid content (Guillen and Cabo 1997a,b; Safar et al. 1994). These differences were successfully exploited to check adulterations in oils and fats as reported in several studies.

Mid-IR spectroscopy has been successfully used for identification of adulteration in vegetable oils, namely canola oil, corn oil, extra virgin olive oil, soybean oil, and sunflower oil as well as marine lipids such as cod-liver oil. Chemometric techniques such as discriminant analysis were frequently employed to fully distinguish the subtle differences in the spectra of the major oil and its adulterants. This approach has been found to be useful to differentiate olive oil from other seed oils (Rohman 2017), virgin coconut oil from those adulterated with palm kernel olein (Manaf et al. 2007), cocoa butter from cocoa butter alternative (Goodacre and Anklam 2001), cod-liver oil from cod-liver oil mixed with lard (Rohman and Che Man 2009), and lard from admixtures of meat and poultry fat (Rohman and Che Man 2010). According to the aforementioned works, the adulteration of pure oils can be detected using discriminant analysis through the finger print region, which falls under 1500–1000 cm^{-1} (Rohman et al. 2010).

For estimation of the amount of adulterant quantitatively, in majority of the cases, partial least square (PLS) approach was employed to select the most appropriate spectral regions that gives the best correlation with the concentration of the analyte (s) of interest (Lai et al. 1994, 1995). According to some of the recent studies, PLS regression is applied to the whole finger print region (1500–900 cm^{-1}) for the purpose of quantifying the adulterant (Rohman and Che Man 2010; Goodacre and Anklam 2001). This approach is equally applicable to determine lard content in oil extracted from product such as cake, chocolate (Che Man et al. 2005), biscuits (Che Man et al. 2011), etc. Under certain circumstances, semi-quantitative approaches were used to quantify the adulteration of lard in these products since a steady change in absorbance values could be observed with the increased proportion of lard in

different regions of the spectra. For instance, a reduction in absorbance values was observed in the region of 990–950 cm^{-1} when lard content in cake gradually increases. This could be probably resulted from the type of shortening used in the preparation of cake as it may be high in trans fatty acid content. Hence, addition of LD tended to decrease the trans fatty acid content of the shortening used in cake formulation. Nevertheless, use of this particular frequency region for detection of lard adulteration in chocolate products was found to be not useful since authentic cocoa butter used for chocolate making does not possess any trans fatty acids (Che Man et al. 2005). In such situations, the entire frequency region of 4000–650 cm^{-1} was more appropriate to detect lard adulteration in chocolate. These findings clearly showed that it is not possible to fix a common region to build the PLS regression for quantification of lard content in lipids extracted from different products. Hence, selection of different spectral regions is necessary based on the differences of sample matrix found in different foods.

A review presented by Reid et al. (2006) highlighted the use of other spectroscopic techniques such as UV-Visible, FT-NIR, and FT-Raman, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) for the detection of various types of food adulteration. UV-Visible spectroscopy was studied by researchers for detection of adulteration in extra virgin olive oils. For instance, Jiang et al. (2013) employed UV spectrometry combined with principle component analysis (PCA) and partial least-squares regression (PLSR) for analysis of extra virgin olive oil (EVOO)-vegetable oil (corn oil, soybean oil, and sunflower oil) blends. The results showed that EVOO-vegetable oil blends, oil blends without EVOO, and oil blends with palm oil (PO) can be discriminated using PCA based on the first three principal components. In another study, Oguz and Banu (2019) conducted a comparative study on the use of UV–Visible and fluorescence spectroscopy in combination with chemometrics for detection of adulteration of fresh olive oils with old olive oil. According to these authors, application of orthogonal partial least square-discriminant analysis (OPLS-DA) was successful in differentiating adulterated oils from the pure oils while partial least squares (PLS) regression technique was able to predict the quantitative adulteration levels.

As a vibrational spectroscopic technique, FT-NIR has already received recognition for its uses in the measurement of adulterations in milk fat (Anthony et al. 2016; Kasemsumran et al. 2007; Sato et al. 1990), olive oil (Jiang and Chen 2019; Christy et al. 2004; Wesley et al. 1995), clarified butter (Mabood et al. 2018) etc. Anthony et al. (2016) compared the quality of FT-NIR spectra to detect cow and buffalo ghee adulteration under transmittance and reflectance modes of operation. They recommended that FT-NIR spectroscopy under transmittance mode would be more suitable for detection of adulterations in ghee. In another study, Jiang and Chen (2019) employed a novel variable selection algorithm called bootstrapping soft shrinkage (BOSS) to study the potential use of FT-NIR spectroscopy to detect adulteration in EVOO. This study found that FT-NIR spectroscopy technique was an effective tool for the detection of EVOO adulteration using BOSS as a promising wavenumbers selection algorithm in chemometrics analysis. In an attempt to develop reliable technique to detect and quantify tallow adulteration in butter

samples, Mabood et al. (2018) applied chemometric models including principal components analysis (PCA), partial least-squares discriminant analysis (PLSDA), and partial least-squares regressions (PLSR) to FT-NIR data. A variety of applications of Fourier transform Raman spectroscopy using a near-infrared laser excitation source have now begun to emerge. FT-Raman spectroscopy has been investigated for detection of virgin olive oil adulterations by pomace oil, soybean oil, and corn oil (Duraipandian et al. 2019; Baeten et al. 1996). Duraipandian et al. (2019) attempted to quantify the purity EVOO by applying PLS to a Raman spectral database of oil samples obtained from 11 binary mixtures (EVOO and rapeseed oil),16 ternary mixtures (EVOO, rapeseed and corn oil) and 44 quaternary mixtures (EVOO, rapeseed, corn and soybean oil). These authors claimed that the developed method is not limited to EVOO, but can be applied to refined EVOO.

17.3 Conclusion

Assurance quality of edible oils and fats might play an indispensable role to safeguard the interest of consumers. Owing to the prevalence of adulterations, much effort has been taken to develop methods for their detection. Chromatographic techniques have given emphasis to chemical markers for identification through compilation of comprehensive data bases giving acceptable ranges of various fatty acids and TAG molecules, minor constituents etc. With the advancement of technology, multivariate data analyzing techniques such as PCA and CANDISC are applied to fatty acid and TAG compositional data for faster discrimination of pure samples from the adulterated ones.

In thermal analysis, DSC has been found to be useful for detection of adulterations in a number of cases. As it is highly sensitive for compositional changes caused by adulteration, it clearly indicated deviations thermal curves or emerging extra peaks. Since it can provide results under both cooling and heating modes of operations, there is always a dual option for detection of adulterations. Mid-IR, FT-NIR and FT-Raman spectroscopies are yet another series of attractive options to detect the adulterations in oils and fats due to rapidity in analysis, minimal sample preparation etc. Since adulterations in oils and fats could bring about deviations in different spectral regions of vegetable oils, it would be prudent to exploit them through chemometrics data handling approaches. In majority of the cases, PLS based calibration models can be built for the purpose of quantifying the adulterant. However, a common PLS model might not fit most of the fats and oils. Therefore, it is a must for each of the individual oil to have their own PLS models built using the different spectral region.

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