Chapter 3
Analysis of Food Emulsifiers

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Analytical methods used to measure food emulsifiers are derived from lipid analysis (Firestone, 2001; Otles, 2004; Wood et al., 2004; Byrdwell, 2005a). Test Methods are of several types and are carried out for several reasons. Food additives are regulated by government agencies to ensure health and safety. Specifications may be set for starting materials, products, processing methods, and maximum use levels in foods. Tests may also be necessary to ensure the absence of degradation products, microorganisms and foreign materials. Composition of emulsifiers may be related to their functional performance in finished foods. Nongovernmental specifications for food emulsifiers may be negotiated between the supplier and the customer, usually a processed food producer. Tests nay be carried out in the manufacturer's processing line or control laboratory, after which the manufacturer may issue a certificate of analysis. The customer may check the analyses as part of the receiving process, and accept or reject the shipment. Disputes may be submitted to an independent testing laboratory for resolution. Standardized test methods have been developed by professional societies, such as, the Association of Official Analytical Chemists (AOAC) (Horvitz, 2005), the American Oil Chemists Society (AOCS) (Firestone, 2005a), the International Union of Pure and Applied Chemistry (IUPAC) (Paquot and Hauffen, 1987), Leatherhead Foods Research Association, and the National Academy of Sciences (Food Chemicals Codex) (Codex, 2004).

To determine emulsifiers in intact food products, fats and emulsifiers must first be extracted. Fats and oils are soluble in nonpolar solvents, such as hexane and toluene. However, emulsifiers are amphiphilic and therefore, less soluble, particularly when emulsifier concentration is high compared to total lipid. Chloroform and chloroform/methanol have been effective for extraction of emulsifiers (Flor and Prager, 1980). Because these solvents are classified as hazardous waste, provisions should be made for recycling. In cases where the lipid concentration is high relative to emulsifier concentration, extraction with hot hexane, followed by acetonitrile was reported (Halverson and Qvist, 1974). Solid samples (e.g., cakes or powdered coffee whiteners) may be conveniently extracted in a Soxhlet extraction apparatus. Liquid samples (e.g., milk or ice cream mix) are generally extracted in a separatory funnel or countercurrent distribution apparatus. Another factor complicating extraction is that emulsifiers may be tightly complexed with starches or proteins, or may be encapsulated in a biopolymer matrix. Pretreatment with amylase enzyme may overcome this problem (Jodlbauer, 1976).

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3.1 Thin Layer and Column Chromatography

After lipids have been extracted from the food matrix, emulsifiers may be separated by simple thin layer or column chromatography. For example, on a silica gel column, triacylglycerols may be eluted with hexane. 5% Diethyl ether in hexane may be used to elute diacylglycerols, followed by elution of monoacylglycerols with 10% diethyl ether in hexane (Firestone, 2005b). A silver-impregnated Celite column was reported to accomplish this separation with a single solvent system (Dieffenbacher et al., 1988; Dieffenbacher et al., 1989). The isolated fractions may be quantitated gravimetrically, or may be subjected to further analytical techniques.

Thin layer chromatography (TLC) and paper chromatography have been used to identify food emulsifiers (Wyrziger, 1968; Murohy and Grislet, 1969; Murphy and Hibbert, 1969; Murphy and Scott, 1969). Samples may be spotted on a plate, coated with an adsorbent, such as silica, alumna, or florisil. Spots may be visualized by spraying with dichlorofluorescein and viewing under an ultraviolet light. Plates already containing a fluorescent indicator are commercially available. Spots are identified by their Rf values. A quantitative method has been developed which carries out the chromatographic separation on a coated rod, rather than a plate. The dried rod is placed in a scanning flame-ionization detector and peaks are recorded on an x-y plot. These methods are simple, rapid, economical, and reasonably reliable. One major disadvantage is that molecules having similar Rf values to compounds of interest will obscure the results. Preparative thin-layer chromatography has been used to separate lipids from foods for further analysis by gas-liquid chromatography (Paganuzzi, 1987).

Mono- and diacylglycerols are readily separated on a boric acid-impregnated silica gel plate. A petroleum ether/diethyl ether/acetic acid solution has been used to separate monoacylglycerols from alimentary pastes (Schmid and Ottender, 1976). A chloroform/acetone mixture was used to separate monoacylglycerols from propylene glycol esters of fatty acids (Kanematsu et al., 1972). Quantitative determinations have been achieved using a coated rod and a flame ionization detector (Regula, 1975; Takagi and Itabashi, 1986).

Because of their importance in lipid metabolism, and their functions in membrane structures, phospholipids have received a great deal of attention in lipid analysis. Many TLC methods have been reported for these lipid derivatives from animals and oilseeds (Erdahl et al., 1973; Vyncke and Lagrou, 1973; Kimura et al., 1969; El-Sebaiy et al., 1980; Lendrath, 1990; Biacs et al., 1978). A 2-dimensional procedure on silica gel plates separated phospholipids using acidic and basic solvents (Watanabe et al., 1986; Firestone, 2005c). The method was used to separate constituents of soy and egg lecithins. Detection of compounds in this class may be done with a conventional spray, such as sulfuric acid or dichlorofluorescein. However, phospholipids may be distinguished from other lipids by using selective reagents or spectroscopic detection (Senelt et al., 1986; Duden and Fricker, 1977). Quantitative detection on a silica rod has also been reported (Tanaka et al., 1979). Experimental design has been reported to be a useful tool to optimize separations of phosphorous

containing lipids (Olsson et al., 1990). Since phospholipids occur at low concentrations in biological samples, TLC has largely been replaced by more sensitive methods. High performance liquid chromatography (HPLC), mass spectrometry (MS), and their combination (HPLC/MS) will be discussed later in this chapter.

Monoacylglycerols may be modified by reaction with organic acids (see Chap. 2) to form molecules having unique functionality. TLC can be used to monitor the progress of the reactions and analyze the composition of the final product (Bruemmer, 1971; Yusupoca et al., 1976; Judlbauer, 1981). Specifically, succinylated and lactylated (Shmidt et al., 1976) as well as DATEM surfactants have been analyzed.

Molecules with multiple esterification sites and/or polymeric head groups present a formidable challenge analytical. These tests generally involve a titration. For example, free fatty acid may be titrated with a standard alkali. Kieselgel G TLC plates using a hexane/acetone/acetic acid solvent system (Regula, 1975). Spots were visualized by spraying the plate with bromocresol green. Sucrose esters of fatty acids have been characterized by TLC (Li et al., 2002), and rod-TLC/flame-ionization.

3.2 Wet Chemical Analysis

The earliest methods used for analysis of fats, oils and their derivatives were wet chemical procedures, that is, they involve solvents and chemical reactions. These tests generally rely on a titration or colorimetric determination. For example, free fatty acid may be titrated with a standard alkali in alcohol solution. Wet chemical methods are time-tested, simple, and require relatively inexpensive equipment. On the other hand, they are labor-intensive and require disposal or recycling of large quantities of solvent. A number of these methods are being replaced by instrument tests, which use autosampling, digital data collection, and, much less solvent.

3.2.1 α-Monoacylglycerol (α-Monoglyceride)

Synthesis of monoacylglycerols (see Chap. 2) yields an approximately 90:10 ratio of α- and β-isomers. α-Monoacylglycerol has a single fatty acid esterified to the sn-1 or sn-3 (primary) positions of the glycerol backbone. The β-isomer has the fatty acid esterified at the sn-2 (secondary) position. Therefore, the statistically random distribution theory would predict a 2:1 ratio. The variation may be rationalized by the lower steric repulsion in the α-isomer.

α-Monoacylglycerols have adjacent (vicinal) free hydroxyl groups at the sn-1,2 or sn-2,3 positions of the molecule. Reaction with periodic acid causes cleavage of the chain between the vicinal hydroxyl groups (Fig. 3.1). Standard analytical procedures are based on this reaction (Firestone, 2005d). The surfactant is reacted with an excess of periodic acid in a methanol solution. Potassium iodide is added and the

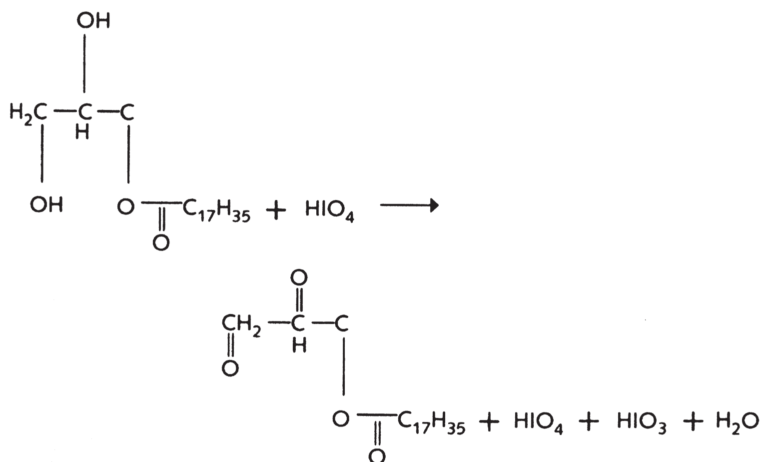

Fig. 3.1 Cleavage of Vicinal diols by periodic acid

liberated iodine is titrated with a standardized arsenite solution. In order to correct for the presence of free glycerol, a sample is extracted and the glycerol is determined (Firestone, 2005e). The wet method is not suitable for samples which contain other molecules with vicinal hydroxyl groups, or when the concentration of monoacylglycerol is $<15\%$.

Since the majority of monoacylglycerol occurs as the α-isomer, this test has been accepted over time as a quality control specification specification. Due to problems with solvent disposal, the method has largely been replaced by gas-liquid chromatography, which provides a measurement for total monoacylglycerol ($\alpha = \beta$).

3.2.2 Acid Value/Free Fatty Acid

Fatty acids are used as starting materials in the preparation of surfactants by direct esterification (see Chap. 2). During interesterification, a small amount of fatty acid may be split off by the catalyst as soap. After neutralization, the resulting free fatty acid is retained in the product. Since fatty acids affect functionality in a number of applications, its concentration must be analyzed.

The acid value is determined by dissolving a weighed sample of the surfactant in a solvent and titrating with standard potassium hydroxide to a phenolphthalein end point (Firestone, 2005f). In cases where the method is used to monitor the reaction of acetic anhydride (DATEM or acetylated monoacylglycerols), an aprotic solvent system must be used to prevent anhydride reaction with alcohol. Potentiometric titration to an equivalence inflection point may also be used.

This approach is particularly useful for dark-colored samples where a visual end point may be difficult to observe. Acid value is defined as the number of milligrams of potassium hydroxide required to neutralize the acid in one gram of sample, and is calculated by the formula:

$$\frac{(A-B)N}{W(56.1)}$$

A = ml KOH solution to neutralize the surfactant sample
B = ml KOH solution to neutralize a blank sample
N = normality of KOH solution
W = wt. of sample in g, and 56.1 is the molecular wt. of KOH

The percentage of free fatty acid is determined by dividing the acid value by a factor, characteristic of the fatty acid present (Firestone, 2005g). For example, C12 (lauric) = 2.81, C16 (palmitic) = 2.19, and C18 (stearic or oleic) = 1.99. The method is not applicable to samples containing other mineral or organic acids.

3.2.3 Iodine Value (IV)

The fatty acids, used to prepare surfactants, may contain saturated or unsaturated alkyl chains. Since unsaturated chains pack differently in crystalline and polymorphic forms, substantial differences in functionality may be observed with variation in unsaturated content. Unsaturated chains are also vulnerable to oxidative degradation.

Reagents, which add across carbon-carbon double bonds, have been used to determine degree of unsaturation since the early years of organic chemistry. Two commonly accepted methods have been developed: (1) The Wijs Method (Firestone, 2005h) reacts iodine monochloride with a surfactant in carbon tetrachloride. Excess reagent is then titrated with standard thiosulfate solution. (2) The Hanus Method is nearly identical but employs iodine monobromide as the reagent. Because of the high toxicity of carbon tetrachloride, a modified method has been developed which uses cyclohexane as the solvent (Firestone, 2005i). Iodine Value is defined as the number of centigrams of iodine absorbed per gram of sample (same as the wt. % of iodine absorbed). The following formula is used to calculate the iodine value:

$$\frac{12.6N}{(S-B_0)W}$$

S = ml solution to titrate the sample
B = ml solution to titrate a blank
N = normality of the thiosulfate solution
W = weight of the sample

When reporting the iodine value, it is important to include the test method which was used. Instrumental methods, such as gas-liquid chromatography and infrared spectroscopy, have been developed to measure the iodine value.

3.2.4 Peroxide Value (PV)

As mentioned in the previous section, surfactants containing unsaturated fatty acids are vulnerable to oxidative degradation (rancidity). The initial stage of the oxidative chain reaction is insertion of oxygen into a carbon-hydrogen bond to form a hydroperoxide. Surfactants which have been bleached, such as sorbitan monostearate or sodium stearoyl lactylate, may contain residual peroxides. These species represent potential oxidative rancidity to finished food products.

Peroxides and hydroperoxide are determined by treating a weighed sample with an excess of potassium iodide in acetic acid/chloroform solution (Firestone, 2005j). Because of the toxicity and carcinogenic potential of chloroform, a method was developed using isooctane as an alternative (Firestone, 2005k); Iodine which is liberated by the reaction, is titrated with standard thiosulfate solution to an endpoint with a starch indicator. Precautions must be taken to ensure that glassware is free from residual oxidizing or reducing agents. Strong ultraviolet light must be avoided because of its tendency to promote photochemical oxidation. Peroxide value is defined as the number of milliequivalents of peroxide (AOAC uses the term "active oxygen") per kg. of sample. It is expressed in the formula:

$$\frac{1000(VT)}{M}$$

V = volume of titrant
T = normality of thiosulfate solution
M = weight of sample in g.

Recently, high performance liquid chromatography (HPLC) was used to determine peroxide.

3.2.5 Saponification Value

As with any other carboxylic acid ester, cleavage of the ester bond may be induced by reaction with alkali and water to produce an alcohol and the salt of the carboxylic acid. This reaction is known as saponification. The saponification value is defined as the number of milligrams of potassium hydroxide required for reaction of one gram of sample (Hummel, 2000a; Firestone, 2005l). A weighed sample is reacted with an alcoholic potassium hydroxide solution and excess alkali is titrated with standard hydrochloric acid solution to a phenolphthalein end point. Alternatively, a potentiometric titration may be used when a visual end point is difficult to observe. Saponification is calculated using the formula:

$$\frac{56.1N(B-S)}{W}$$

B = ml required to titrate a blank
S = ml required to titrate the sample
N = normality of the reagent, 56.1 is the molecular weight of KOH
W = wt of the sample in g

When comparing triacylglycerols, saponification value is a measure of fatty acid chain length. Shorter chains give higher values while longer chains produce lower values. For surfactants, the saponification value is sensitive to both the chain lengths of the fatty acids present and the degree of substitution. Shorter fatty acid chains and higher degrees of substitution produce higher saponification values. Conversely, longer fatty acids and lower degrees of substitution will give lower saponification values.

3.2.6 Hydroxyl Value

When polyols are esterified to produce surfactants, some hydroxyl groups are left unesterified. These groups may be determined by reaction with acetic anhydride in the presence of pyridine. The reacted sample is then treated with water and heated to hydrolyze excess anhydride to acetic acid. The acetic acid is then titrated with standard alkali with an indicator to determine the end point. The hydroxyl value is defined as the number of milligrams of potassium hydroxide equivalent to the hydroxyl content of one gram of sample (Hummel, 2000b; Firestone, 2005m). It is calculated using the formula:

$$\frac{56.1T(V_0-V)}{M}+AV$$

T = normality of KOH titrant, 56.1 = mol. wt. of KOH
V_0 = ml required to titrate a blank
V = ml to titrate the sample
M = wt. of sample in g
AV = acid value of sample

As a measure of hydroxyl groups in a surfactant, the hydroxyl value is an indicator of the hydrophilic character. Higher hydroxyl values are correlated with higher HLB values.

The reaction/titration procedure is time consuming and requires a great deal of skill on the part of the analyst. Minor variations in the method may cause large discrepancies in the results. It is therefore recommended that the hydroxyl value should be determined as an average of duplicate samples. Efforts have been made to correlate hydroxyl values to instrumental methods, such as near infrared reflectance spectroscopy.

3.2.7 Lactic Acid Analyses

Lactic acid is used in the manufacture of surfactants, such as lactylated monoacylglycerols or propylene glycol esters, sodium stearoyl lactylate (SSL), and calcium stearoyl lactylate (CSL). Lactic acid in these products occurs in two forms: free and esterified. Total lactic acid content is the sum of these forms. Lactic acid is a bifunctional molecule which can self-condense to form polylactic acid.

Total lactic acid has been determined by reaction of a weighed sample with alcoholic potassium hydroxide, neutralization with hydrochloric acid, extraction with diethyl ether, and titration with standard potassium hydroxide.

Free and polylactic acid and water insoluble combined lactic acid (WICLA) are determined by dissolving a weighed sample in benzene. The aqueous extract is titrated with potassium hydroxide to determine the free acid. The upper benzene layer is dried and the sample reacted and titrated in the same manner as for total lactic acid. Two problems with the above methods are the laborious extraction/phase separations and the use of carcinogenic benzene. A modification of the method uses a chloroform/petroleum ether solvent for the determination of lactic, citric, and tartaric acids (Franzke, 1977). The same authors demonstrated that enzymatic, rather than potassium hydroxide, could be used for the cleavage reaction in determination of total lactic acid (Franzke and Kroll, 1980).

3.2.8 Reichert-Meisel Value

Some fats and oils, such as butter and coconut, contain short chain fatty acids (C4-C10). The Reichert-Meisel method was developed to determine the content of these acids (Firestone, 2005n). The method has been applied to determination of acetic acid esterified to monoacylglycerols and tartaric acid esters of monoacylglycerols.

A weighed sample is hydrolyzed in alkali solution, followed by neutralization with dilute sulfuric acid. Liberated acetic acid is distilled and titrated with standardized alkali to a phenolphthalein end point. The method is equipment intensive, since the distillation apparatus must be replaced or cleaned between analyses. The distillation step is also time consuming.

3.2.9 Moisture

The presence of moisture in food surfactants is generally undesirable. It affords the opportunity for microbial growth and may cause ester cleavage to produce free fatty acids (hydrolytic rancidity). Surfactants may become contaminated by pumping through inadequately dried lines. In solid products, moisture may be picked up in flaking and spray chilling, especially in high humidity environments. There are two

cases where water is deliberately added: (1) in polysorbates, where a small amount of water is needed to prevent phase separation; (2) surfactant gels, which enhance functionality in specific applications.

Older methods relied on gravimetric techniques to determine water loss from a sample after heating. These methods were unable to distinguish between water and other volatile components. Another method dissolves a large sample in toluene and distills water and toluene into a graduated separation tube. Water is quantitated by volume. The method is best suited to samples where the water content is $>0.5\%$. It is also equipment intensive and time consuming. Titration with Karl Fischer reagent (SO_2/I_2/pyridine/2-methoxyethanol) has been developed for determination of commercial fats and oils (Firestone, 2005o), industrial oil derivatives (Firestone, 2005p), and lecithin (Firestone, 2005q). Autotitrators are available which can process large numbers of samples without the need for cleaning between samples. Some impurities, such as peroxides, can react with Karl Fischer to give high results. Near infrared spectroscopy has been used to determine moisture in raw materials.

3.2.10 Fatty Acid Soaps

Sodium and calcium salts of fatty acids (soaps) are formed in food surfactants by the use of alkaline catalysts in the manufacturing process. Inadequate neutralization at the end of the reaction results in residual soap. Residual alkalinity can result in degradation due to disproportionation reactions, especially during molten storage.

One analytical method consists of dissolving a weighed sample in organic solvent/water mixture and titration with a standard hydrochloric acid solution (Firestone, 2005r). Although the scope of the official method is limited to refined vegetable oils, the procedure may be adapted for surfactants. Bromophenol blue or phenolphthalein may be used as an indicator. The method may also be adapted for potentiometric titration. An alternative method to determine whether a product has been neutralized is to measure the pH. A 5% solution of the surfactant is allowed to equilibrate to ambient temperature and the pH is measured with a standard electrode. Values in the range of 6.5–6.8 indicate the absence of soap and proper neutralization of the product.

3.2.11 Phosphorus and Phospholipids

Soy lecithin is a widely used food surfactant derived from soybean oil refining. Structurally, phosphoric acid is esterified to a diacylglycerol and to an organic base or inositol. Monoacylglycerol phosphate has a similar structure. One way to determine the concentration of these surfactants is to analyze for phosphorous, and then apply a gravimetric factor. A titrimetric method saponifies a sample, followed by precipitation with molybdate solution. The precipitate is washed and dissolved in

an alkali solution. Excess alkali is then titrated with standard acid. Another method involves ashing a sample, dissolving the ash in acid, and determining the phosphorus colorimetrically with molybdate (Firestone, 2005s). A simpler, albeit less precise, approach is to precipitate the phospholipid in acetone. The precipitate is dried and the insoluble content determined by weight. Acetone-insolubles can also be determined by turbidity measurement (Goldstein, 1984).

Because of the importance of phospholipids in lipid metabolism and membrane structure, a great deal of effort has been expended to develop new quantitative methods. Techniques, such as spectrophotometry, thin layer chromatography (TLC), high performance liquid chromatography (HPLC), mass spectrometry (MS), and HPLC/MS, are discussed elsewhere in this chapter.

3.3 Measurement of Physical Properties

Physical properties of food surfactants often play a critical role in the appearance, texture, and flavor release in finished food products. Chapter 6 will discuss the physical properties of food emulsifiers in greater detail. In this section, we will survey some common methods for measuring physical properties.

3.3.1 Color

Although color may be considered a physical property, its origin arises from the chemical composition of the starting lipids. Fats and oils contain minor components such as tocopherols, carotenoids, and chlorophyll. These compounds are removed during processing but may be “locked in” if the fat/oil has been thermally abused. Side reactions during manufacture may also lead to dark colors. For example, carrying out the reaction at high temperature can cause caramelization of sucrose. Dark colors may not only cause a defect in the appearance of foods but may also be an indicator of other problems, such as oxidation. The lightest possible color is therefore a quality goal. Since most colors originate in fat/oil starting materials, strict receiving guidelines must be developed.

Color determination is most often performed by comparison of a sample to a set of standards, such as colored glasses. A widely used method in the oil processing industry is the Lovibond method (Firestone, 2005t), also referred to as the Wesson Method (Firestone, 2005u). A column of liquid (or molten) sample in a glass tube is observed over a white background and compared to a set of colored glasses. Values are determined for red (R) and yellow (Y), which arise from minor constituents in vegetable oils. A related test is the Gardner method (Firestone, 2005v). This procedure is used for lecithin and industrial oils and reports a single number for color. Another comparative test, the FAC method (Firestone, 2005w), is applied to samples

too dark to be read by the other methods. Photometers and spectrophotometers have been used to determine colors in the UV-visible range (Firestone, 2005x). These methods are objective, noncomparative determinations, which are also useful for quantitation of other colors (for example, green arising from chlorophyll.

3.3.2 Refractive Index

Clear liquids refract light because of the differences in the speed of light in different media. Refractive index is the ratio of the speed of light in air to the speed of light in the liquid. Measurements are carried out in a refractometer (Firestone, 2005y). It is commonly used as a rapid method to monitor chemical reactions. The measurement is correlated to a chemical property, such as iodine or hydroxyl value. For example, in the polymerization of glycerol, the refractive index increases with the degree of polymerization. Determination of the end point is quickly determined by refractive index and confirmed later by hydroxyl value.

3.3.3 Melting Point

Fats, oils and their derivatives are heterogeneous compositions and do not display sharp melting points as do pure, homogeneous compounds. Rather, a broad melting range is observed. To further complicate the situation, polymorphic crystals may melt and recrystallize into a different polymorphic form. However, melting behavior is often critical to functionality in foods. For example, the melting point of a peanut butter stabilizer must be matched to the filling temperature to prevent oil separation. This poses a significant challenge of melting behavior as a quality measurement. A number of methods have been developed to describe the melting behavior of fat based ingredients in diverse food applications.

Capillary melting points have been common methods for organic compounds. For fats and surfactants, the melting range needs to be converted to a single number. The definition of capillary melting point has been defined as the temperature at which the sample becomes completely liquid or clear (Firestone, 2005z). This end point is difficult to observe if the sample contains suspended inorganic or dark colored matter. For such samples, the slip point (also known as the softening point) (Firestone, 2005aa) is a more useable method. In this test, a sample in an open capillary tube is heated at a programmed rate and the melting point is defined as the temperature at which the sample slips out of the tube. This method will give a lower value than the standard capillary method because it measures the onset of melting. When reporting melting points, it is critical to report the method used. The dropping point (Firestone, 2005ab) is obtained in an instrument which heats a solid

sample disk at a programmed rate. At the melting point, the sample drops through a detection system and the temperature is recorded. This method does not rely on observation and judgment of an operator.

3.3.4 Viscosity

Viscosity is a physical property of food emulsifiers, which is important to transfer, such as pumping through pipelines. It is generally used as a control measure for viscous liquids, such as polyglycerol esters. The property is temperature dependent: viscosity decreases as temperature increases. Products may need to be heated in order to be pumped through heat-traced pipes.

The viscosity of lecithin and other viscous liquids may be measured by a procedure known as the "bubble-time method." A sample is poured into an ASTM tube in a constant temperature bath (Firestone, 2005ac). The tube is inverted and the time required for the bubble to reach the top is recorded. This value is converted to viscosity by comparison to a calibration curve constructed from authentic standards. Viscosity may also be measured directly with a Brookfield viscometer (Firestone, 2005ad). This technique is preferred when samples are not clear liquids.

3.3.5 Specific Gravity

Specific Gravity is measured for cases where weight and volume need to be converted. For example, a batch recipe may specify a weight of a liquid ingredient. If the ingredient is pumped through a mass flowmeter, the weight must be converted to volume. Specific gravity is also important in specifying the volume of a package required to hold a specified weight of emulsifier. Specific gravity is measured in a pycnometer at 25 °C if the sample is liquid at ambient temperatures. 40 °C or 60 °C may be required for higher melting materials (Firestone, 2005ae). A method is also available for measuring the specific gravity of solids (Firestone, 2005af). Air bubbles must be carefully removed in order to obtain accurate values.

3.4 Instrumental Methods of Analysis

Advances in analytical chemistry have enabled the development of sophisticated instruments that may be applied to analysis of lipids. Instrumental methods have several advantages over wet chemical titrations: (1) More detailed information about composition and structure; (2) Less waste disposal and solvent recovery; (3) Automation of sample introduction and data archiving; in some instances, more

rapid results. The greatest obstacle to widespread adoption of instrumentation in the food industry is the high initial cost of equipment.

3.4.1 Gas-Liquid Chromatography (GLC)

Gas-liquid chromatography separates a stream of vaporized sample in a heated column packed with an absorbent. Detection of eluting peaks may be accomplished using thermal or flame ionization detectors. A mass spectrometer may also be used in combination with GLC to provide structural information for each peak (GC/MS). Application to lipids is difficult because of their low volatility. High temperatures or reaction to prepare volatile derivatives have been used to overcome this problem.

The most common GLC method is the determination of fatty acid composition. Fatty acids are cleaved from their polyol backbone, followed by reaction to form a more volatile derivative, such as a methyl ester (Firestone, 2005ag). The sample is injected into the GLC and separated on a packed or capillary column. The chain length of the fatty acids and the degree of unsaturation determines separation. Retention times of the peaks are recorded and correlated to previously analyzed internal standards. Concentrations are determined by peak height or area, corrected by the response factor for each peak.

Mono- and diacylglycerols are the simplest food emulsifiers compositionally. GLC analysis is accomplished by reaction of a dry sample with chlorotrimethylsilane and hexamethyldisilazane in the presence of pyridine (Nakanishi and Tsuda, 1983; Brueschweiler and Dieffenbacher, 1991; Firestone, 2005ah). GC/MS analysis has also been reported (Lee, 1988). The method may also be used to analyze mixtures of propylene glycol esters and monoacylglycerols. Figure 3.2 shows a

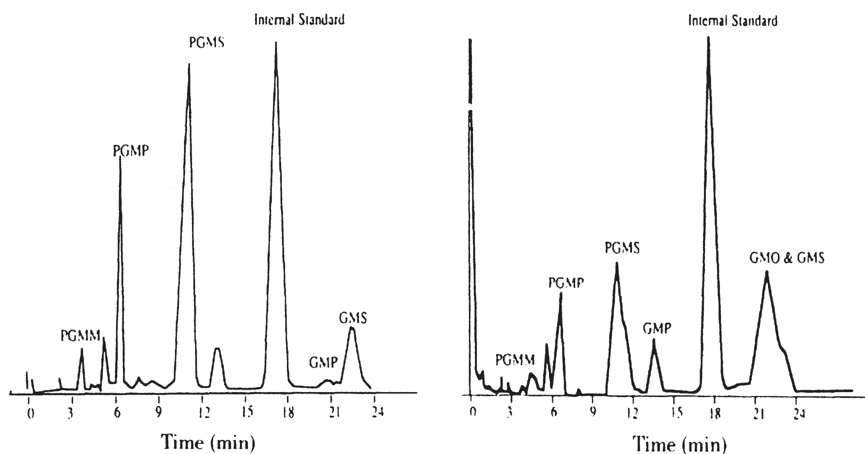

Fig. 3.2 GLC separation of monoglycerides and propylene glycol ester emulsifiers. (**a**) Commercial emulsifier; (**b**) in shortening. (Hasenhuettl et al., 1990.)

separation of such a mixture. Eluted peaks are quantitatively determined by reference to an internal standard. Monoheptadecanoylglycerol (monomargarin) has historically been used as a standard. However, it is expensive, difficult to synthesize and solutions are not stable over time. (±)-Batyl alcohol has been suggested as an alternative (Hasenhuettl et al., 1990). It is a commercially available glyceryl ether having the same molecular weight as monomargarin. The ether linkage makes it stable to disproportionation. Cholesteryl acetate has also been recommended.

Polyol distributions of food surfactants may be determined by cleavage of the fatty acids by saponification, followed by analysis of the polyol fraction. If the polyol is not sufficiently volatile or unstable at high temperatures, they may be converted to trimethylsilyl ethers. For example, sorbitol, sorbitan and isosorbide, cleaved from sorbitan mono- or tristearate, can be determined by GLC (Murphy and Grislett, 1969; Tsuda et al., 1984). Glycerol through dodecaglycerol, obtained from polyglycerol esters, may be determined using their volatile derivatives (Schuetze, 1977). Supercritical fluid chromatography (Macka et al., 1994) and a combined GLC/HPLC method (DeMeulenaer et al., 2000) have been used to obtain polyglycerol distribution. Reaction and high temperature gas chromatography have determined polysorbates

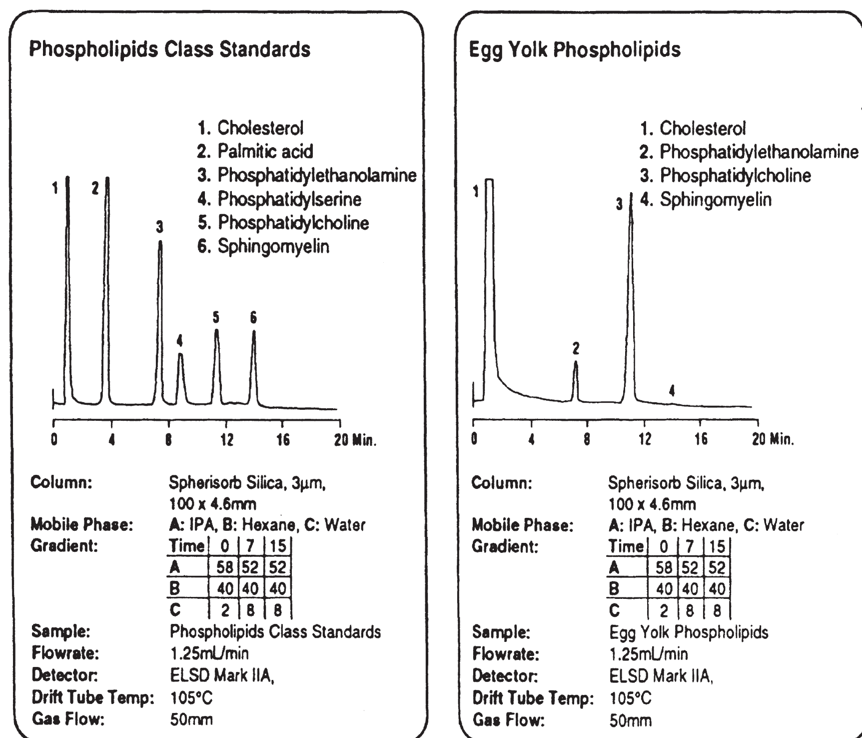

Fig. 3.3 HPLC separation of phospholipids using an evaporative light-scattering detector. (Courtesy of Alltech Associates, Inc.)

(Lundquist and Meloan, 1971; Kato et al., 1989). Although sucrose and fatty acid esters of sucrose decompose at high temperatures, they have been analyzed by GLC (Karrer and Herbertg, 1992). Addition of mass spectroscopy confirms the eluted peaks and is a source of additional information (Uematsu et al., 2001). GLC is also a valuable tool for the detection of contaminants, such as heat exchange fluids (Firestone, 2005ai).

3.4.2 High Performance Liquid Chromatography (HPLC)

HPLC is a logical extension of column chromatography. It is a very useful technique for lipid derivatives, since the sample does not need to be converted to a volatile derivative. A sample is injected onto a column and a carrier solvent carries it through. Recently, column diameters have been made very small to minimize the amount of solvent. The nature of the column determines the mode of separation. A standard column, for example silica gel, separates compounds by adsorption of the polar groups. Nonpolar (reverse phase) columns, such as polystyrene cross-linked with divinylbenzene, adsorb lipophilic regions of the molecule. When both techniques are used in a single sample, complementary information is often obtained. A size exclusion column separates compounds by shape and molecular weight.

One problem encountered with HPLC analysis of lipids is their poor response to conventional detectors. Saturated lipids do not absorb UV light at a unique region of the spectrum. A refractive index (RI) detector may be used, but it is less sensitive and limited to an isocratic (single solvent) system. An evaporative light scattering detector (ELSD) has been developed to overcome these problems (Christie, 1992; Hammond, 1993; Bruns, 1988; Lee et al., 1993). Solvent is flashed off in the detector and the residual nonvolatile matter scatters light and is recognized as a peak. Figure 3.3 shows a separation of phospholipids using this detector.

Perhaps the most commonly reported separations by HPLC have been monoacylglycerols (Filip and Kleunova, 1993; Takagi and Ando, 1994; Ranger and Wenz, 1989; Tajano and Kondoh, 1987; Martin et al., 1989; Rilsom and Hoffmayer, 1978; Brueschweiler, 1977; Firestone, 2005aj) and phospholipids (Christie, 1996; Melton, 1992; Sotirhos et al., 1986; Hurst and Martin, 1984; Huyghebaert and Baert, 1992; Tumanaka and Fujita, 1990; Rhee and Shin, 1982; Hsieh et al., 1981; Kaitaranta and Bessman, 1981 p. 5; Firestone, 2005aj; Luquain et al., 2001). Free glycerine may also be determined by HPLC (Firestone, 2005ak).

Polyglycerol mono- and polyesters have been separated by HPLC on a LiChromasorb column (Garti, 1981; Kumar et al., 1984). Sorbitan esters of fatty acids have also been separated on the same stationary phase (Garti and Ascerin, 1983). Sucrose esters of fatty acids were determined using their 3,5-dinitrobenzyl derivatives (Murakami et al., 1989). Determination of propylene glycol alginate in aqueous systems has been accomplished by high performance anion exchange chromatography (Diepenmaat-Walters et al., 1997). Contamination of lipid derivatives with heat exchange fluids can be detected by HPLC (Firestone, 2005al).

3.4.3 Mass Spectrometry (MS)

Mass spectrometry has been a powerful tool for structural determination of organic molecules. Molecular, or parent ions, indicate the molecular weight of the molecule. Fragmentation of the molecule produces daughter ions, which provide evidence of substructure and functional groups. Tandem mass spectrometry allows both molecular and daughter ions to be resolved in a single determination. Progress in lipid analysis using MS has been hindered by two factors: (1) Lipids are nonvolatile and not amenable to injection into high-vacuum instruments, for example, electron impact MS, (2) Commercial lipids are complex mixtures which produce a bewildering array of molecular and daughter ions. Development of a variety of ionization methods and the combination with HPLC has led to encouraging results. However, the high capital cost of these instruments currently limits their use to research.

Soft atmospheric pressure ionization methods, such as atmospheric pressure chemical ionization (APCI), atmospheric pressure photo ionization (APPI) (Cai and Syage, 2006) electrospray ionization (ESI), and matrix-assisted laser desorption ionization (MALDI), have enabled the characterization of lipids (Byrdwell, 2005b). ESI is useful for polar lipids over a fairly wide range of molecular weights. Low molecular weight nonpolar lipids are more amenable to APPI. In an emerging field, known as lipidomics, a complex mixture of lipids can be directly injected into a mass spectrometer, and characterized by a wide variety of ionization methods (Ham and Gross, 2005). Phospholipids are distinguished from other lipids using their lithium salts and the nitrogen rule.

Fast atom bombardment (FAB) was used to characterize the phospholipids in egg yolk (Trautler and Nikiforov, 1984). Protonated molecular ions (MH+) were easily resolved and identified. Polysorbates in foods were characterized by negative ion MS (Daniels et al., 1985). Two families of peaks were recognized: free polyoxyethylenes and polyoxyethylenes esterified to sorbitans. Fatty acids esterified to sorbitans could also be identified in the spectra. MALDI time-of-flight MS was also reported as a method for analysis of polysorbates (Frison-Norrie, 2001). Sucrose esters of fatty acids were analyzed by ESI/MS (Schuyl and van Platerink, 1994). This technique showed a family of molecular ions corresponding to degree of esterification of fatty acids to sucrose.

3.4.4 High Performance Liquid Chromatography/Mass Spectrometry

Integration of HPLC/MS has been difficult due to the necessity for removal of large volumes of solvent prior to MS analysis. Early efforts consisted of collection of peaks from the HPLC, evaporation of the solvent, and direct injection directly into the MS ionization source. Concurrent development of microbore columns and ionization techniques such as APCI and ESI, allowed the marriage of the two powerful

technologies. When ELSD, a destructive detector, is used, a split stream is diverted to the MS source. Normal phase HPLC, coupled with ESI tandem mass spectrometry (MS/MS), is useful for separation and characterization of complex phospholipid mixtures (Larsen and Hyattumff, 2005). Phospholipids are separated by head group class and molecular weights of class members were determined by MS. Additional detail could be obtained by collecting the fractions from normal phase HPLC, using reverse phase HPLC to separate class members, and find detailed structure by MS. Polyglycerol esters were separated by LC and their structures confirmed by MS (DeMeulenaer et al., 2000). In this case also, additional detail could probably be obtained by a combination of standard and reverse phase HPLC. Glycolipid biosurfactants have been characterized by HPLC/MS (Nunez et al., 2005). Because the head groups of these substances are large and complex, the methodology may be useful for high HLB surfactants, such as polysorbates, polyglycerol esters, and sucrose esters.

Because normal phase and reverse phase HPLC are orthogonal separation methods, coupling them both simultaneously to MS (HPLC-2/MS) has been developed as a useful technique for separation of complex lipid/phospholipid mixtures (Byrdwell, 2005c). APCI and ESI are also complementary techniques. Coupling of all four modalities (HPLC-2/MS-2) is capable of yielding enormous amounts of structural and compositional data simultaneously.

Although HPLC/MS is an extremely powerful tool, it is far too expensive for routine analysis for food surfactants. However, it will likely find use as a research tool in universities and large companies.

3.4.5 Spectroscopic Methods

As previously pointed out in our discussion of HPLC detectors, saturated lipids do not absorb light in any useful region of the UV/VIS spectrum. However, functional groups of surfactant molecules can form colored complexes with a number of reagents. Measurements of absorbance in a spectrophotometer can then be correlated with concentration of the surfactant. Anionic functional groups form complexes with methylene blue, which may be detected at 650nm. Cationic surfactants react with Orange 2 to yield a complex detectable at 485 nm (Lew, 1975). A DATEM/meta-vanadate complex could be measured at 490nm (Shmidt et al., 1979). Phosphatidylcholine in lecithins, can complex with methylene blue (Hartman et al., 1980), dipicrylamine (Mueller, 1977), or Reinecke's salt (Moelering and Bergmeyer, 1974), for spectrophotometric analysis. Total phosphorous can be determined through the phosphomolybdate complex (Firestone, 2005am). Polyoxyethylene chains can form colored complexes, which can then be determined spectrophotometrically (Kato et al., 1989). Polysorbates have been analyzed by this method in a number of food products (Daniels, 1982; Saito et al., 1987; Tonogau et al., 1987).

In contrast to the UV/VIS, the infrared spectrum has a number of wavelengths, which are diagnostic of functional groups found in surfactants. In particular, double

bond, carbonyl, and hydroxyl stretching bands have been used for qualitative and quantitative analysis. Infrared spectroscopy was used to confirm the identity of polysorbates determined by other methods (Kato et al., 1989). Near-infrared (NIR) determines the iodine value by correlation of double bond stretching bands with a calibration curve (Firestone, 2005an). NIR was also used as a rapid determination of hydroxyl value of polyglycerols and polyglycerol esters by measuring the –OH stretch (Ingber, 1986). Spectrophotometry may also be used to detect impurities in food surfactants and lipids. An alternative to peroxide value measures iodine liberated by reaction with peroxides (Yamanaka and Kudo, 1991). Residual dimethylformamide in sucrose esters has been determined by measurement of the absorption peak at $1675\,cm^{-1}$ (Jakubska et al., 1977). However, this technique is not sufficiently sensitive to detect impurities at the ppm level. NIR and Fourier transform (FT-IR) methods have the advantage of rapidly obtaining compositional informational data. This is an opportunity to monitor the progress of chemical and enzymatic reactions, for example, in the esterification of glycerol with fatty acids (Blanco et al., 2004). FT-IR is also useful for the determination of hydration (Pohle et al., 1997) bilayer geometry and metal ion binding strength (Grdadolnik and Hadm, 1993) of phospholipids.

Atomic absorption spectroscopy (AA) is useful for detection of metals in surfactants and lipids. Heavy metal contaminants, such as lead (Firestone, 2005ao), or pro-oxidants (iron, copper, chromium) (Firestone, 2005ap) can be detected. Other metal ions detected are sodium, calcium, magnesium, nickel, silicon, and cadmium (Firestone, 2005aq).

3.4.6 Nuclear Magnetic Resonance

Atoms having an odd atomic number, display a magnetic resonance, which is characteristic of their chemical environment. Measurements may be carried out by placing a dissolved sample in strong electromagnetic and radio frequency fields. The magnetic field is varied (swept) and peaks are recorded by a radio frequency drtector. Peak positions are determined by atoms to which the nucleus is bonded. Splitting patterns are observed which indicate adjacent atoms with a magnetically susceptible nucleus. Wide-line (low resolution) NMR is frequently used to determine the solid fat content (SFC) of a sample (Firestone, 2005ar). This method is limited to shortenings and hard butters, which may contain food surfactants. Chemical shifts have been used to identify mesomorphic phases of surfactants in aqueous systems (Lindblom, 1996). Mesomorphic phases are discussed further in Chap. 6.

Proton (^1H) nuclear magnetic resonance is the oldest method applied to organic molecules. However, because of the large number of protons present on alkyl chains, it has limited utility in lipid analysis. Phosphatidylcholine content has been determined by measuring the choline protons at 3.3 ppm (Press et al., 1981; Kostelnik and Castellano, 1973). Measurement of the vinylic protons at 5.5 ppm has been proposed as an alternative to the titrimetric method for iodine value (Sheeley et al., 1986).

Table 3.1 ^{13}C chemical shifts (ppm) for some food surfactants

Surfactant structure	Gl-1	Gl-2	Gl-3	$N\text{-}CH_3$	$O\text{-}CH_3$
Soy phosphatidylcholine	63.01	70.51	63.33	66.26	59.34
Egg phisphatidylcholine	62.94	70.63	63.78	66.62	50.32
Soy phosphatidylethanolamine	62.81	70.59	64.07	40.69	62.08
Egg phosphatidylethanolamine	62.81	70.55	64.07	40.59	62.13
1-Monoacylglycerol	65.04	70.27	63.47	–	–
1,2-Diacylglycerol	65.04	72.25	61.58	–	–
1-Propylene glycol monoester	69.46	66.13	19.2	–	–
2-Propylene glycol monoester	65.92	71.77	16.25	–	–
Propylene glycol diester	65.42	67.98	16.5	–	–
Monoacetylated monoacylglycerol					
A	62.07	72.89	61.40	–	–
B	63.00	68.19	65.26	–	–
Diacetylated monoacylglycerol	62.00	69.16	62.33	–	–

Chemical shifts of carbon (^{13}C) are sensitive to the presence of functional groups. For example, a carbonyl carbon will have a drastically different shift than a carbon in a methyl group. Since there are many fewer carbon than hydrogen atoms in lipids, spectra are less complex and easier to interpret (Gunstone, 1993). Chemical shifts for glyceryl and carbons were used to measure the levels of monoacylglycerols, diacylglycerols, and free fatty acids in olive oil (Sacchi et al., 1990). Regio- and stereoselectivity of monoacylglycerol, derived from enzymatic reactions, can also be established (Mazur et al., 1991). Chemical shifts for diagnostic carbon atoms for monoacylglycerols, propylene glycol esters, acetylated monoacylglycerols, phosphatidylcholine, and phosphatidylethanolamine are shown in Table 3.1. ^{1}H and ^{13}C NMR have been used to determine multilamellar phospholipids (Everts and Davis, 2000), polyglycerols (Istratov et al., 2003), and polysorbate 60 (Dang et al., 2006).

Phosphorous (^{31}P) NMR is a very useful technique for determining structure and concentration of phospholipids (Glonek and Merchant, 1996; Gillet et al., 1998). Since there is only one phosphorous per molecule, peak assignment is straightforward compared to ^{1}H and ^{13}C NMR. This is somewhat offset by the numerous phosphorous-containing molecules present in nature. Optimization of solvent systems for best resolution was reported (Bosco et al., 1997). Phospholipids in milk fat globule membrane have been characterized by ^{31}P NMR (Murgia et al., 2003).

3.5 Setting Specifications

The practice of setting analytical specifications for food ingredients may be a matter of custom, such as accepting the manufacturer's values, or it may be a carefully reasoned approach based on product functionality. When developing new products, or something similar to existing products, the first approach is usually acceptable, and even time saving. Manufacturers of food surfactants are knowledgeable in applying

their ingredients in a variety of processed foods. Sometimes, however, a food processor may develop a “new to the world” product, which has no analogy to a food in current commerce. In this case, a logical, databased approach is preferable.

The first step in product development is to determine the attributes which are critical to consumer acceptance. This is traditionally done by quality descriptor analysis (QDA), focus groups, and consumer panels. Ingredients which enhance these characteristics can then be tested. Before testing surfactants, the regulatory and label requirements must be examined. For example, does the product need to be “all natural” or Kosher? Is the proposed ingredient permitted in the new food product?

Once the attributes and ingredients have been identified, a statistical design should be developed to optimize desired attributes. Since ingredient interactions are well known (Gaonkar and NcPherson, 2005), the initial design should be full-factorial. Once any two or three factor interactions have been identified, a fractional-factorial design can be drawn up to reduce the number of experiments. Once an optimal surfactant system has been identified, the range of acceptable analytical constants, for example monoacylglycerol content, must be defined. These values, along with analyses for absence of contaminants, are written into a raw material specification.

The food processor and the surfactant supplier should confer to determine whether these specifications can be met consistently. A history of the supplier’s analytical results should fall in the range at least with 95% confidence. Failure to routinely meet these limits could result in returned surfactant shipments, production delays, or even product recalls. It may be necessary to re-visit the product design to develop a more robust product formulation.

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