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# Microwave-assisted Extraction for Bioactive Compounds

## Theory and Practice

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Farid Chemat • Giancarlo Cravotto
Editors

# Microwave-assisted Extraction for Bioactive Compounds

## Theory and Practice

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# Preface

The use of microwave energy in chemical laboratories was first described in 1986 contemporaneously by R. Gedye and R.J. Giguere in organic synthesis and by K. Ganzler in the extraction of biological matrices for the preparation analytical samples. Since then, several laboratories studied the enormous potential of this non-conventional energy source for synthetic, analytical and processing application. So far, the use of dielectric heating in synthesis and extraction is documented by over 3,000 and 1,000 articles respectively.

The field of microwave-assisted extraction of bioactive compounds is quite young. In the last two decades, new investigations have been prompted by an increasing demand of more efficient extraction techniques, amenable to automation. Shorter extraction times, reduced organic solvent consumption, energy and costs saved, were the main tasks pursued. Driven by these goals, advances in microwave extraction have resulted in a number of innovative techniques such as microwave-assisted solvent extraction, vacuum microwave hydro-distillation, microwave Soxhlet extraction, microwave-assisted Clevenger distillation, compressed air microwave distillation, microwave headspace extraction, microwave hydro-diffusion and gravity, and solvent-free microwave extraction. One of the success stories of the twenty-first Century has been the partial replacement of conventional extraction processes, with "green" procedures (reducing energy, time, solvent, and waste) based on microwave irradiation.

Scope of this book is to present a detailed survey on the full potential of microwaves in extraction processes. Following an introduction to microwave theory (Chap. 1), Chap. 2 details mass and heat transfer, induced by microwave, in solid-liquid extraction as a unit operation in chemical and food engineering. Applications in which microwave-assisted-extraction have afforded spectacular results and applications are discussed extensively in term of process and product: essential oils (Chap. 3), fat and oils (Chap. 4), antioxidants and colours (Chap. 5), proteomics (Chap. 6), and pharmaceutical and nutraceutical compounds (Chap. 7). The last Chap. (8) give responses to major questions to convert laboratory innovations into industrial success for microwave-assisted extraction: scale-up, quality and safety consideration….

This book has been prepared by a team of chemists, biochemists, chemical engineers, physicians, and food technologists who have extensive personal experience in research and development of innovative microwave extraction processes and products at laboratory and industrial scale. This book addresses primarily to science graduate students, chemists and biochemists in industry and food quality control, as well as researchers and persons who participate in continuing education and research systems.

We wish to thank sincerely all our colleagues who have collaborated in the writing of this book. We hope to express them our scientific gratitude for agreeing to devote their competence and time to ensure the success of this book.

Avignon, France — Farid Chemat
Torino, Italy — Giancarlo Cravotto

# Editors

**Farid Chemat** is Professor of Chemistry and Director of the laboratory for green extraction techniques of natural products (GREEN) at the Université d'Avignon et des Pays de Vaucluse, France. Born in Blida (1968), he received his engineer diploma (1990) and his Ph.D. (1994) degree in process engineering from the Institut National Polytechnique de Toulouse. After periods of postdoctoral research work with Prolabo-Merck (1995–1997), he spent 2 years (1997–1999) as senior researcher at University of Wageningen (The Netherlands). In 1999, he moved to the University of La Réunion (France) as assistant professor and since 2006 holds the position of Professor of Food Chemistry at the University of Avignon (France). His research activity is documented by more than 100 scientific peer-reviewed papers, and about the same number of communications to scientific meetings, 4 edited books, 25 book chapters and 7 patents. His main research interests have focused on innovative and sustainable extraction techniques (especially microwave, ultrasound and green solvents) for food, pharmaceutical and cosmetic applications. He is co-ordinator of a new group named "France Eco-Extraction" dealing with international dissemination of research and education on green extraction technologies for food cosmetic, pharmaceutical industries.

**Giancarlo Cravotto**, Giancarlo Cravotto (born in Torino, 1961) after a 3-year experience in the pharmaceutical industry, he became a researcher in the Department of Drug Science and Technology (University of Torino). He is currently Full Professor of Organic Chemistry and Department Director since 2007. His research activity is documented by more than 200 scientific peer-reviewed papers, several book chapters and patents. His main research interests are the synthesis of fine chemicals, cyclodextrin derivatives and bioactive compounds. These studies have paved the road to innovative synthetic procedures and the preparation of new catalysts and ionic liquids, exploiting non conventional techniques such as high-intensity ultrasound, microwaves, flow chemistry and ball milling. His research group composed by chemists, pharmacists and engineers, developed a number of hybrid flow-reactors that combine different energy sources and are well suited for process intensification. These non-conventional techniques and equipments have been applied in organic

synthesis, in the degradation of persistent organic pollutants and plants extraction. He collaborates with several industrial partners in the field of phytoextracts, pharmaceutics, food processing and packaging, fine chemicals, cosmetics, petrochemicals and textiles. Prof. Cravotto is Editor of two international journals: *Ultrasonics Sonochemistry* (Elsevier) and *Green Processing and Synthesis* (De Gruyter).

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# Chapter 1
# Microwave-Assisted Extraction: An Introduction to Dielectric Heating

**Cristina Leonelli, Paolo Veronesi, and Giancarlo Cravotto**

## 1.1 Introduction to Dielectric Heating

Microwave (MW) irradiation uses an electromagnetic field at a specific frequency in some way similar to that of photochemical-activated reactions. The MW frequency range is an ample interval that ranges from 300 MHz to 300 GHz. However, only a few frequencies are allowed for industrial, scientific, and medical uses (ISM frequencies), and in general 0.915 and 2.45 GHz are those most used worldwide. A typical MW generator for such frequencies can be found in the magnetron, the same device that equips domestic and laboratory MW ovens. Magnetrons for industrial applications can reach power ratings in the tens of kilowatts (kW); laboratory appliances usually use ratings below 1 kW. Recently, the introduction of solid-state generators has permitted the emission band of the MW generator to be made narrower, allowing the user to vary the frequency of the system within the range of allowed ISM frequencies. This variation can play an important role in chemical synthesis, especially insofar as selectivity and efficiency are concerned. However, the typical power rating for solid-state generators operating at 2.45 GHz is 100 W, which is also often used in medical applications.

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### 1.1.1 Electromagnetic Field–Matter Interaction

At a frequency of a few gigahertz (GHz), namely, at the allowed ISM (industrial, scientific, and medical) frequency of 2.45 GHz, matter interacts with the electromagnetic field mainly via dipole reorientation and induced polarization phenomena. Even though the interaction with the electric field is of principal importance for most of the chemical environment, the fact that the magnetic component accounts for magnetic loss in compounds with high permeability is another mechanism via which heat is generated.

At 2.45 GHz, the energy of a MW photon is close to 0.00001 eV, and hence it is too weak to break even hydrogen bonds. Moreover, it is also much lower than the energy required for Brownian motion. Thus, one has to keep in mind that the efficiency of MW irradiation on chemical syntheses is strictly related to the conversion of electromagnetic energy to heat.

MW radiation is not considered to be effectively ionization radiation, and thus current limitations on MW exposure are purely based on the thermal damage that can occur to body tissues [1].

The degree to which electromagnetic energy is converted into heat in a reaction medium is dependent, in practical terms, on the local strength of the electromagnetic field and on the permittivity and the permeability of the chemical compounds or mixture (two or more reactants for solvent-free synthesis, or reactant plus solvent plus catalyzer for solution chemistry). Practically, this dependency means that both the nature of reactants and the geometry of the MW reactor affect heat generation in the reaction medium.

At this point, a more detailed description of the dielectric and magnetic properties of the compounds is necessary to better evaluate the possible interaction between the reactant molecules and the MW when designing the reaction mixture.

The permittivity $\varepsilon^*$ of a material is a complex number that contains a real component, $\varepsilon'$, and an imaginary component, $\varepsilon''$, as described by Eq. (1.1):

$$\varepsilon^* = \varepsilon' + i\,\varepsilon'' \tag{1.1}$$

In practical terms, $\varepsilon'$, the dielectric constant, represents the ability of a material to be polarized by an external electric field and can be considered a relative measure of the MW energy density [2]: this is often expressed as a relative dielectric constant, which indicates that it is relative to the permittivity of free space, $\varepsilon_0$, as in Eq. (1.2) [3, 4]:

$$\varepsilon' = \varepsilon_r \varepsilon_0 \tag{1.2}$$

$\varepsilon''$ is called dielectric loss, or the loss factor, and it quantifies the efficiency with which the electromagnetic energy is converted to heat [5]. Sometimes this factor also includes the contribution to heat generation caused by the induction of real currents, that is, the electrical conductivity contribution.

**Table 1.1** Dielectric constant (ε′), tangent loss (tan δ), and dielectric loss (ε″) for solvents at 2,450 MHz and room temperature [5]

| Solvent | Dielectric constant (ε′) | Loss tangent (tan δ) | Dielectric loss (ε″) |
|---|---|---|---|
| Water | 80.4 | 9.889 | 12.3 |
| DMSO[a] | 45.0 | 0.825 | 37.125 |
| DMF[b] | 37.7 | 0.161 | 6.079 |
| Ethylene glycol | 37.0 | 1.35 | 49.950 |
| Methanol | 32.6 | 0.856 | 21.483 |
| Ethanol | 24.3 | 0.941 | 22.866 |
| Chloroform | 4.8 | 0.091 | 0.437 |
| Toluene | 2.4 | 0.040 | 0.096 |
| Hexane | 1.9 | 0.020 | 0.038 |

[a]DMSO, dimethyl sulfoxide
[b]DMF, dimethylformamide

It is, however, more common to use the loss tangent, tan δ, a linear combination of dielectric constant and loss factor, to account for these losses. It is defined as in Eq. (1.3) [3, 4]:

$$\tan\delta = \varepsilon'' / \varepsilon' \tag{1.3}$$

The loss tangent is then considered to be the ratio between the dissipative (including electrical conductivity losses) and capacitive behavior of the materials; the higher the value, the better the material will heat under MW irradiation.

A more evident relationship between material heating and dielectric and magnetic properties can be found in the power density, $P_d$ (W/m$^3$), from Poynting's theorem, Eq. (1.4):

$$P_d = \omega\varepsilon_0\varepsilon''_{eff}|E_{rms}|^2 + \omega\mu_0\mu''_{eff}|H_{rms}|^2 \tag{1.4}$$

where ω is the angular frequency, $|E_{rms}|$ is the magnitude of the electric field, $\varepsilon''_{eff}$ is the imaginary part of the permittivity of the dielectric material, $\mu_0$ and $\mu''_{eff}$ are the susceptibility of vacuum and material, respectively, and $|H_{rms}|$ is the intensity of the magnetic field [4].

When these concepts are applied to an ordinary chemical reaction, we can simplify MW heating by considering that polar solvents or compounds will generally heat up better than apolar materials. As a matter of fact, chemists are familiar with the relative dielectric constant, which is used to distinguish between polar and apolar solvents, but additional information on the loss tangent is also necessary, as summarized in Table 1.1.

The dielectric properties, however, are dependent not only on frequency but also on the material temperature. Hence, to completely understand or model MW heating behavior, such temperature dependence must be known. Figure 1.1 shows the permittivity of some selected solvents.

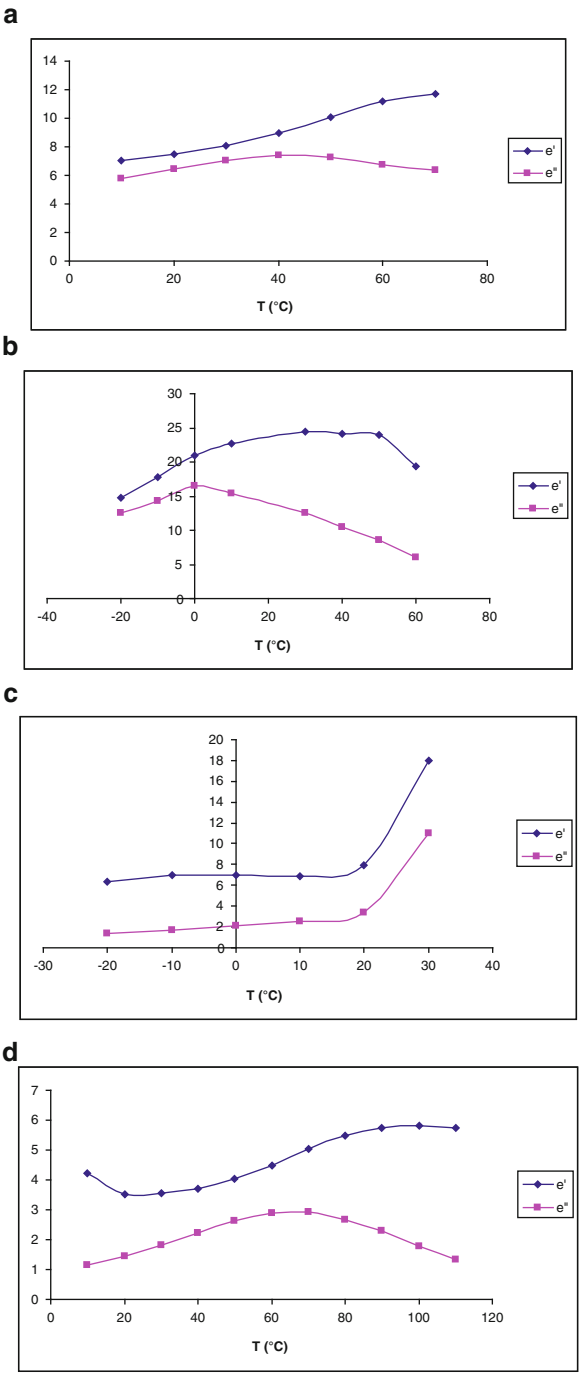


**Fig. 1.1** Temperature-dependent dielectric properties of ethanol (**a**) [6]; methanol (**b**) [7]; propanol (**c**); butan-1-ol (**d**)

Because the frequency of the MW generator is fixed, the chemist can play on two other factors to reach the desired process temperature level:

1. Chose a suitable solvent
2. Use a *suscepting* material.

### 1.1.2 The Temperature Dependence of Material Dielectric Properties

The theory and principle of plant extraction by means of enabling technologies has been reviewed in comprehensive studies [8–10]. However, to summarize briefly, it should be pointed out that the efficiency of the extraction depends on the nature of the sample matrix and the analyte to be extracted as well as its location within the vegetal matrix. The choice of the best solvent naturally depends on the nature of the plant matrix and the class of compounds to be extracted. Strict international rules in the pharmaceutical industry, and in particular in the food industry, restrict the number of solvents that can be used. The major physical parameters that are of importance for MW-assisted extraction include solubility, the dielectric constant, and dissipation factors. Working at 2,45 MHz, the polarity of the solvent is the main factor because solvents with high dielectric constants (e.g., water and alcohols) can absorb more MW energy than nonpolar solvents [11].

### 1.1.3 Peculiarities of Microwave Heating

It is usually accepted that a *suscepting* material is a solid or a liquid that heats up rapidly, when irradiated by MW, in response to a strong interaction with the electrical or magnetic field. The addition of such a material, which is able to absorb MW energy and transform it into heat better than the reaction mixture alone, leads to a faster heating cycle. When a mixture of vegetal particles and solvents of different permittivity values is treated with MW, differentiated heating results. The phenomenon is also known as *selective heating* and continues until thermal equilibrium is reached (Fig. 1.2). This simple consideration, derived from application of Eq. (1.4), suggests that it is also necessary to consider the thermal conductivity of the material as one of the parameters we need to explain overall MW heating.

Before we start the summary of MW ovens that are commercially available for the chemical laboratory, an additional definition is necessary to better understand the heating mechanism and the difficulties of scaling up apparently successful laboratory-scale reactions: the *power penetration depth*.

The distance, $D_p$, from the surface of a semi-infinite dielectric slab at which 1/e (63.2%) of the incident power is dissipated is given by Eq. (1.5):

$$Dp = \frac{\lambda_0 \sqrt{\varepsilon'}}{2\pi\varepsilon''} \tag{1.5}$$

Equation 1.5 [5] allows us to select possible materials for extraction vessels according to their capability to completely attenuate the incident MW power along

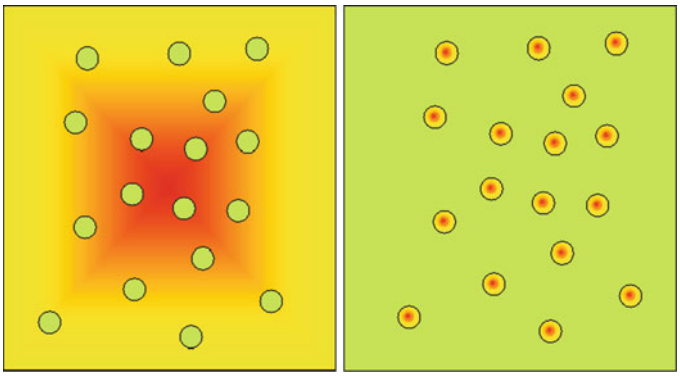

**Fig. 1.2** Features of selective microwave (MW) heating caused by differences in permittivity in a biphasic mixture (*yellow* to *red* indicates heating of the phase with higher losses; *green* indicates low-loss materials)

their thickness (small penetration depth) or, conversely, their ability to transmit the incident MW well (large penetration depth).

A typical extraction vessel is made of *transparent* materials such as polytetrafluoroethylene (PTFE) or quartz through which MW radiation passes without significant attenuation. A second possible type is a vessel made of a *susceptting* material, such as suitable polymers including graphite powder, silicon carbide, or other high-loss materials. In the latter case the container absorbs MW energy, allowing only a small proportion to pass and directly heat the reaction media; in this case, the reactants are heated indirectly. This technique is better known as the "hybrid" heating.

In general, a mechanical or magnetic stirrer is necessary, whereas in the case of liquids time can be sufficient to allow natural convection modes to lead to good homogenization of temperature distribution.

## 1.2 MW Ovens for the Chemical Laboratory

The growing interest in MW-assisted extraction has stimulated new applications and the design of suitable reactor geometries. Laboratory-scale applications often exploit the ovens used for organic and inorganic synthesis [12].

Now let us comment on the general features that characterize these commercial applicators in terms of process intensification. Microwaves are very useful when one needs to efficiently deliver energy into the reaction vessel, but only when the following requirements have been met:

1. The electric field profile needs to be homogenized, either using mode stirrers or via the rotation of the reaction vessel itself
2. The reactor geometry needs to be well designed, taking into account the penetration depth of the MW (as described later)

3. Temperature and pressure within the reaction chamber need to be controlled for continuous monitoring of process parameters
4. Reactor and spare parts costs must be considered
5. Safety issues and MW leakage must be considered

The basic components of a MW applicator, usually identified by the term *oven* or *furnace* (for temperatures higher than 250–300°C), are as follows:

- The MW source, typically a magnetron, which is characterized by a frequency and output power of MW irradiation
- The transmission lines that connect the source to the cavity, generally waveguides and coaxial cables for single-mode and multimode applicators; for radiant geometry, antennas can also be used [5]
- The MW cavity, a metallic box of various shape and size; open structures are also used for radiant applicators

To better evaluate the most recent developments in chemical reactors adapted to MW heating, we now describe the most simple geometries.

### *1.2.1 Single-Mode Cavities*

Single-mode applicators possess various advantages over multimode applicators; not least among these is the availability of analytical solutions and, hence, a precise value for the electromagnetic field distribution in an empty or simply loaded applicator. Moreover, the existence of analytical solutions makes single-mode applicators easier to design and relatively simple to assemble because they use basic components; moreover, they can present higher electromagnetic field homogeneity in precise zones of the applicator. Naturally, there are some drawbacks: these include the usually small dimensions, the ease with which arcing and plasma are generated as a consequence of high field strength, and finally the high cost per processable load volume.

Single-mode applicators are usually the starting point for a process and are often used to assess the feasibility of a MW-assisted process, and in some cases are used in scaling up and the passage to continuous flow processes. Some examples of this type are the commercial reactors of the Discover SP/Explorer SP by CEM series (Fig. 1.3) and the U-guide reactors (Miniflow) by SAIREM (Fig. 1.4).

### *1.2.2 Multimode Cavities*

Domestic ovens, which despite their unsophisticated control systems are still the most often used laboratory kilns, belong to the multimode applicator type. The advantages of these applicators follow:

- Ease of construction, the possibility to homogenize the electromagnetic field with rotating devices (moving the load or perturbing the electromagnetic field with mode stirrers)

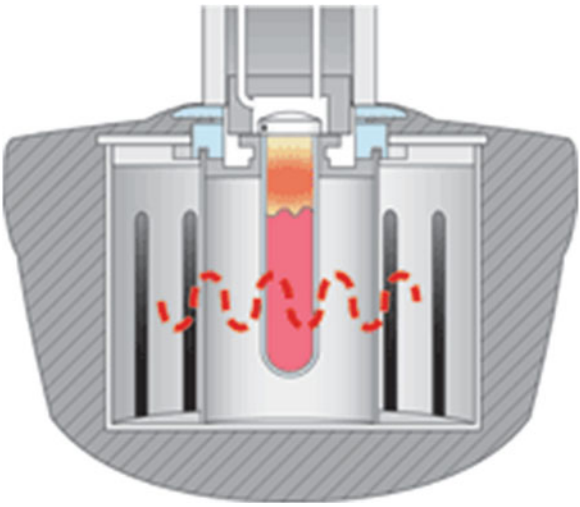

**Fig. 1.3** Schematic view of a cross section of the CEM Discover & Explorer SP MW Synthesizers with the reaction medium in the central position (vial filled with liquid). (Image from producer's commercial website: http://www.cem.com/content656.html)

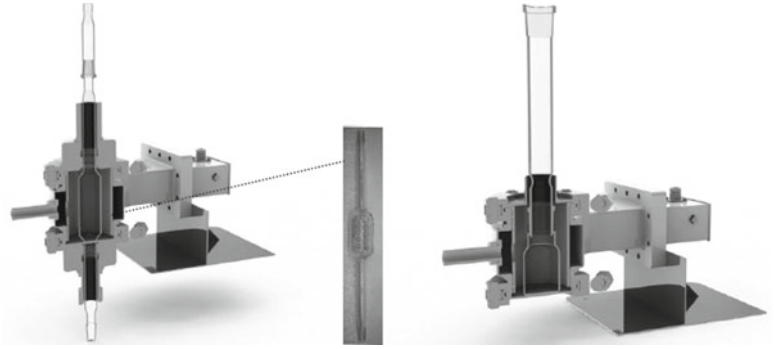

**Fig. 1.4** Rendering of the core of the SAIREM U-guide patented system, in continuous flow configuration. (Courtesy of SAIREM)

- Large dimensions
- Possibility of installing multiple MW inlet ports
- Relative inexpensiveness

Some of the drawbacks are undoubtedly the absence of an analytical solution for the Maxwell equations, which describe the electromagnetic field, in the case of partially loaded applicators (hence rendering it necessary to carry out a numerical simulation to find the electromagnetic field distribution in the load) and the need for expensive thermal insulation over large areas.

The larger dimensions of multimode applicators and the possibility to multifeed using a number of MW generators make scaling up easier, but the limits imposed by the power penetration depth must be taken into account.

**Fig. 1.5** Multimode applicator and reaction vessels of the Synthos 3000 by Anton Paar. (Image from producer's commercial website: http://www.anton-paar.com/Microwave-Synthesis-Synthos-3000/Microwave-Synthesis/60_Corporate_en?product_id=120)

Many producers of laboratory equipment have MW multimode applicators in their catalogues: these include the Synthos 3000 by Anton Paar (Fig. 1.5), the Ethos Ex by Milestone (Fig. 1.6), and the larger Batch-10 reactor by UpScale (Fig. 1.7). The latter provides up to 5 kg/batch of product in static mode or stop-flow mode.

### *1.2.3 Continuous Flow Cavities*

Continuous flow reactors seem to positively fulfill the requirements for process intensification, although technical issues must be addressed for extraction applications. In continuous flow reactors where the fluid in the reactor may be responsible for taking MW radiation outside the vessel and for some distance, MW leakage and safety are highly relevant [3].

Some continuous flow reactors are in reality assemblages of multiple batch reactors, operating in tandem, to provide an apparently continuous flow. Other applicators are adaptations of single- or multimode systems where the original reaction vessel is substituted by pipings or similar devices to have the reactants pass through the MW application zone. Some other reactors are purposely designed for continuous processing and include multiple MW sources and measurement points to achieve proper process control. All the manufacturers of single- or multimode applicators described in the previous paragraph have a continuous flow version of their apparatuses, and in some cases the move from laboratory to industrial scale makes production particularly easy. For example, Oleos has recently developed an eco-extraction strategy, using a U-shaped applicator, which is effective even in a very heterogeneous and viscous system. This system also provides high-density MW power in the processing region: this eases the generation of micropressure inside the cells of the matrix under processing, and favors heat and mass transfer [13].

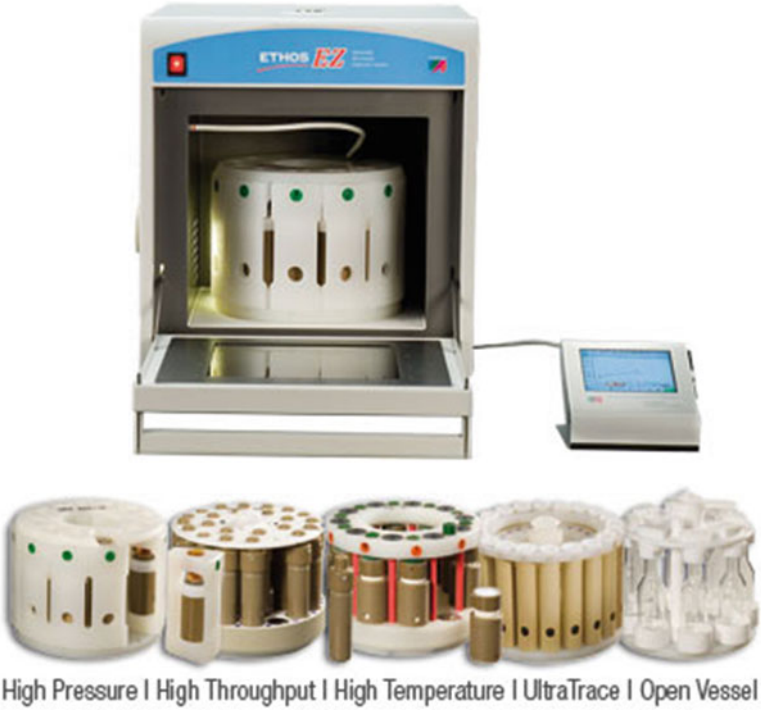


**Fig. 1.6** Milestone multimode applicator with different reaction vessel arrangements. (Image from producer's commercial website: http://www.milestonesrl.com/analytical/products-microwave-digestion-ethos-one.html)

### *1.2.4 Other Applicators*

There is also a large number of emitting structures that can operate at very different output power ratings according to the final application. Their advantages include good heating homogeneity, large dimensions, open termination, and the fact that they are less affected by load variations with respect to closed applicators. The drawbacks are the difficulties in design and in preventing MW leakage.

## 1.3 Process Parameter Controls

To provide the highest degree of reproducibility to a MW-assisted process, the presence of a robust and reliable control system is essential. Typically, this includes one or more controllers and multiple sensors, some of which entirely dedicated to safety whereas others are used to monitor the main process variables such as temperature and pressure. However, other control strategies can be implemented, for instance,

**Fig. 1.7** UpScale Microwave batch-10 multimode reactor, fed by antennas. (Image from producer's commercial website: http://www.upscalemicrowave.com/)

those based on weight variation, degree of advancement of a certain reaction via Fourier transform infrared spectroscopy (FT-IR) or Raman sensors, or even on reflected power variations as the reactants evolve into the final products.

### *1.3.1 Temperature Sensors*

The most common and versatile temperature sensor is probably the thermocouple. However, its use in presence of electromagnetic fields has been debated for a long time. The metallic nature of the thermocouple elements, and of its sheath also, actually induce perturbations in the electromagnetic field distribution. Moreover, the typical needle-like shape of thermocouples is prone to favor electromagnetic field concentration near the tip, hence possibly causing localized overheating of the reaction medium. However, depending on reactor geometry and reactant nature, thermocouples have been successfully used, especially when immersed in a high-loss dielectric fluid. Nevertheless, the use of thermocouples, or of metallic elements in general, should be avoided if possible, and noncontact methods, or the use of nonperturbative optical fibers, should be preferred (Fig. 1.8).

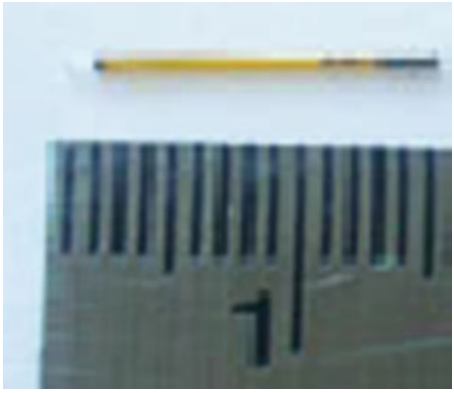

**Fig. 1.8** An optical fiber for temperature measurements in the range −40°C to 250°C, with a response time < 0.5 s. (Image from producer's commercial website: http://www.fiso.com/section.php?p=20)

The use of noncontact methods, such as optical pyrometers, has the disadvantage that only surface temperature is measured and, furthermore, some problems in attaining reliable temperature measurements may be caused by smoky environments. Measuring temperature through a viewing port necessitates a port material (window) that is transparent to the infrared radiation used by the pyrometer: this excludes most of the commonly used window materials, requiring their substitution by more fragile window materials.

Noncontact methods, such as thermal cameras, allow to perform temperature measurements on large areas, but a proper control strategy must then be implemented to feed the system controller with the proper data. Thermal cameras are typically used to check on safety issues, that is, verifying that no part of the load surface exceeds a predetermined temperature level.

If the temperature in the load volume must be known, a possible option is the use of optical fibers, which can also be coated with polytetrafluoroethylene (PTFE) to operate in the most severe environments. Optical fibers are usually available for a wide temperature range, starting below the freezing point of water and reaching 2,000 K. However, as a single optical fiber is not usually able to cover the whole temperature range, the installation of multiple optical fibers and controllers is required. Furthermore, optical fibers must be progressively removed as the reaction temperature surpasses their maximum temperature usage limit. However, for most extraction processes, a single fiberoptic system, such as those manufactured specifically for MW environments, can be used (Figs. 1.8, 1.9). Such systems can also account for load rotation, making multiple point temperature measurement easy.

The drawback of the temperature sensors described is that none provides the complete temperature distribution in the reaction volume: only surface temperature or point information is available. Hence, the selection of the measurement zone must be carefully addressed, possibly with the aid of numerical simulation to foresee which regions will generate more heat.

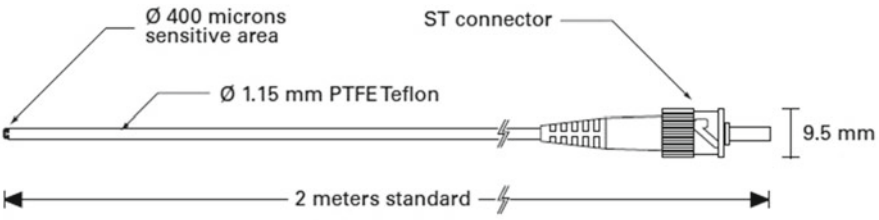


**Fig. 1.9** An optical fiber construction schematic for temperature measurements in the range −270°C to 250°C, with a response time < 0.5 s. (Image from producer's commercial website: http://www.neoptix.com/t1-sensor.asp)

### *1.3.2 Pressure Sensors*

Measuring pressure can often be easier than measuring temperature, because in most cases pressure is almost constant in the entire reaction volume. There are of course many exceptions, especially in the presence of heterogeneous systems of channel-like geometry, but for most extraction processes reaction pressure can be considered a property of the system in a certain status. Hence, the problems of selecting the proper measuring point seem less severe. However, it must be taken into account that using a transducer gas in a closed pipe to measure pressure can induce errors because of the progressive temperature change of the gas temperature as a function of the distance from the reaction medium or the presence of cooling parts. Moreover, in some cases gases can be generated during processing, and this must be taken into account as well when indirect temperature measurements are performed by pressure measurements. Besides classical pressure sensors (such as piezoelectric, membranes, load cells), some devices have been devised specifically for use in presence of high-strength electromagnetic fields. One of these is based on the change in optical properties of a sensing material, such as a glass ring. Using polarized light, the ring causes a change in the colour of the transmitted light depending on the pressure to which the ring is exposed to (usually proportional to the pressure inside the reaction volume). Fiberoptics can also be used to measure pressure by the change in the length of a cavity (a Fabry–Perot cavity, practically a portion of the optical fiber) enclosed between two semitransparent mirrors, induced by the forces acting on one of the cavity walls. Pressure must be monitored in all the reactions occurring in closed environments because the generation of overpressure can be dangerous for equipment and operator. This constraint would make open-vessel reactors intrinsically safer, as they can be operated at atmospheric pressure and the reagents can be added at any time during the treatment. Such reactors also allow large samples to be processed without the requirement of a cooling step before loading or unloading. On the other hand, a closed-vessel system allows higher temperatures to be reached because the higher pressure inside the vessel raises the boiling point of the reaction medium used. Moreover, the risk of airborne contamination is decreased, but safety concerns arise when working with pressurized systems.

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# Chapter 2
# Fundamentals of Microwave Extraction

**Priscilla C. Veggi, Julian Martinez, and M. Angela A. Meireles**

## 2.1 Basic Principles

### *2.1.1 Mechanism of Microwave Extraction*

The fundamentals of the microwave extraction (MAE) process are different from those of conventional methods (solid–liquid or simply extraction) because the extraction occurs as the result of changes in the cell structure caused by electromagnetic waves.

In MAE, the process acceleration and high extraction yield may be the result of a synergistic combination of two transport phenomena: heat and mass gradients working in the same direction [1]. On the other hand, in conventional extractions the mass transfer occurs from inside to the outside, although the heat transfer occurs from the outside to the inside of the substrate (Fig. 2.1). In addition, although in conventional extraction the heat is transferred from the heating medium to the interior of the sample, in MAE the heat is dissipated volumetrically inside the irradiated medium.

During the extraction process, the rate of recovery of the extract is not a linear function of time: the concentration of solute inside the solid varies, leading to a nonstationary or unsteady condition. A series of phenomenological steps must occur during the period of interaction between the solid-containing particle and the solvent effectuating the separation, including (1) penetration of the solvent into the solid matrix; (2) solubilization and/or breakdown of components; (3) transport of the solute out of the solid matrix; (4) migration of the extracted solute from the external surface of the solid into the bulk solution; (5) movement of the

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DOI 10.1007/978-1-4614-4830-3_2, 

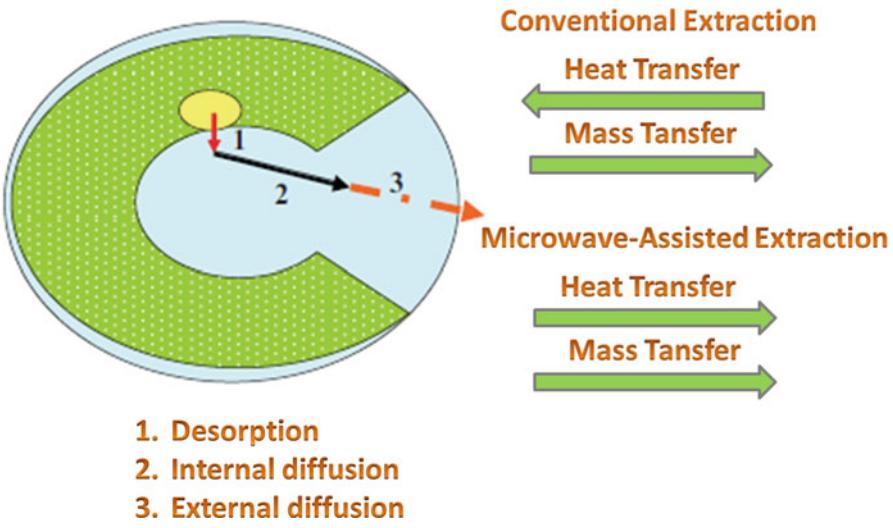


**Fig. 2.1** Basic heat and mass transfer mechanisms in microwave and conventional extraction of natural products. (Adapted from Périno-Issartier et al. [2])

extract with respect to the solid; and (6) separation and discharge of the extract and solid [3].

Therefore, the solvent penetrates into the solid matrix by diffusion (effective), and the solute is dissolved until reaching a concentration limited by the characteristics of the solid. The solution containing the solute diffuses to the surface by effective diffusion. Finally, by natural or forced convection, the solution is transferred from the surface to the bulk solution (Fig. 2.2).

The extraction process takes place in three different steps: an equilibrium phase where the phenomena of solubilization and partition intervene, in which the substrate is removed from the outer surface of the particle at an approximately constant velocity. Then, this stage is followed by an intermediary transition phase to diffusion. The resistance to mass transfer begins to appear in the solid–liquid interface; in this period the mass transfer by convection and diffusion prevails. In the last phase, the solute must overcome the interactions that bind it to the matrix and diffuse into the extracting solvent. The extraction rate in this period is low, characterized by the removal of the extract through the diffusion mechanism. This point is an irreversible step of the extraction process; it is often regarded as the limiting step of the process [5].

Many forces, such as the physicochemical interactions and relationships, can be exposed during the extraction (dispersion forces, interstitial diffusion, driving forces, and chemical interactions), and the persistence and strength of these phenomena may be closely tied to the properties of the solvent (solubilization power, solubility in water, purity, polarity, etc.) [6].

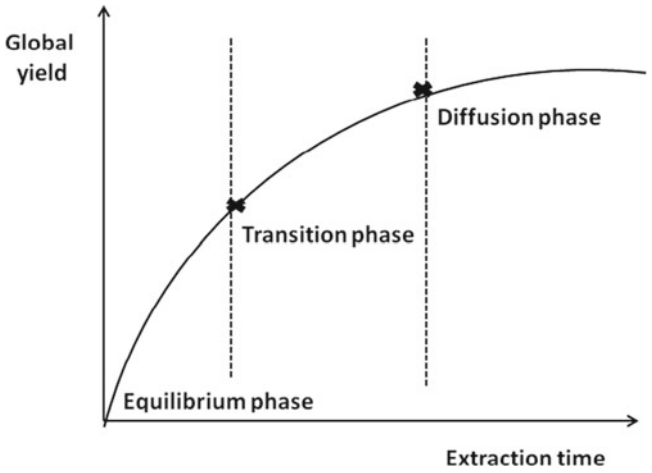


**Fig. 2.2** Schematic representation of yield versus time in extraction processes. (Adapted from Raynie [4])

### *2.1.2 Mechanism of Microwave Heating*

In the microwave heating process, energy transfer occurs by two mechanisms: dipole rotation and ionic conduction through reversals of dipoles and displacement of charged ions present in the solute and the solvent [7, 8]. In many applications these two mechanisms occur simultaneously. Ionic conduction is the electrophoretic migration of ions when an electromagnetic field is applied, and the resistance of the solution to this flow of ions results in friction that heats the solution. Dipole rotation means rearrangement of dipoles with the applied field [8].

Energy transfer is the main characteristic of microwave heating. Traditionally, in heat transfer of the conventional process, the energy is transferred to the material by convection, conduction, and radiation phenomena through the external material surface in the presence of thermal gradients. In contrast, in MAE, the microwave energy is delivered directly to materials through molecular interactions with the electromagnetic field via conversions of electromagnetic energy into thermal energy [9].

The most important properties involved in microwave processing of a dielectric are the complex relative permittivity ( $\varepsilon$ ) and the loss tangent (tan $\delta$) [10, 11]:

$$\varepsilon = \varepsilon' - j\varepsilon'' \tag{2.1}$$

$$\tan\delta = \frac{\varepsilon''}{\varepsilon'} \tag{2.2}$$

where

$$j = \sqrt{-1} \tag{2.3}$$

**Table 2.1** Physical constants and dissipation factors for solvents usually used in microwave-assisted extraction (MAE) [14, 15]

| Solvent | Dieletric constant,[a] $\varepsilon'$ | Dissipator factor tan δ ($\times 10^{-4}$) | Boiling point,[b] (°C) | Viscosity,[c] (cP) |
|---|---|---|---|---|
| Acetone | 20.7 | 5,555 | 56 | 0.30 |
| Acetronitrile | 37.5 | | 82 | |
| Ethanol | 24.3 | 2,500 | 78 | 0.69 |
| Hexane | 1.89 | | 69 | 0.30 |
| Methanol | 32.6 | 6,400 | 65 | 0.54 |
| 2-Propanol | 19.9 | 6,700 | 82 | 0.30 |
| Water | 78.3 | 1,570 | 100 | 0.89 |
| Ethyl acetate | 6.02 | 5,316 | 77 | 0.43 |
| Hexane–acetone (1:1) | | | 52 | |

[a]Determined at 20°C
[b]Determined at 101.4 kPa
[c]Determined at 25°C

The material complex permittivity is related to the ability of the material to interact with electromagnetic energy, whereas $\varepsilon'$ is the real part, or *dielectric constant*, and $\varepsilon''$ is the imaginary part, or *loss factor*. The dielectric constant determines how much of the incident energy is reflected at the air–sample interface and how much enters the sample (for vacuum, $\varepsilon' = 1$); the loss factor measures the efficiency of the absorbed microwave energy to be converted into heat [12]. The loss tangent (tan δ or dielectric loss) is the most important property in microwave processing; it measures the ability of the matrix to absorb microwave energy and dissipate heat to surrounding molecules, being responsible for the efficiency of microwave heating [12, 13] As a result, a material with high loss factor and tan δ combined with a moderate value of $\varepsilon'$ allows converting microwave energy into thermal energy.

The first factor one must consider when selecting microwave physical constants is the solvent to be used. It is important to select a solvent with high extracting power and strong interaction with the matrix and the analyte. Polar molecules and ionic solutions (typically acids) strongly absorb microwave energy because of the permanent dipole moment. On the other hand, when exposed to microwaves, nonpolar solvents such as hexane will not heat up.

The degree of microwave absorption usually increases with the dielectric constant. In Table 2.1, the physical parameters, including dielectric constant and dissipation factors, are shown for commonly used solvents. A simple comparison between water and methanol shows that methanol has a lesser ability to obstruct the microwaves as they pass through but has a greater ability to dissipate the microwave energy into heat [8]. The higher dielectric constant of water implies a significantly lower dissipation factor, which means that the system absorbs more microwave energy than it can dissipate. This phenomenon is called *superheating*: it occurs in the presence of water in the matrix. This strong absorption provides an increase of the temperature inside the sample, leading to the rupture of cells by the in situ water. In some cases it can promote the degradation of the target compound or an "explosion" of solvent, and in

other cases it can increase the diffusivity of the target compound in the matrix [16]. Therefore, the microwave power must be sufficient to reach the boiling point of the water or other solvent, setting the separation temperature.

The second factor to be considered is the solid matrix. Its viscosity affects its ability to absorb microwave energy because it affects molecular rotation. When the molecules are "locked in position" as viscous molecules, molecular mobility is reduced, thus making it difficult for the molecules to align with the microwave field. Therefore, the heat produced by dipole rotation decreases, and considering the higher dissipation factor (δ), the higher is this factor, the faster the heat will be transferred to the solvent [11].

### 2.1.3 Heat Transfer in Microwave Heating

When the system is subjected solely to heating, then Eq. (2.4) can be solved by itself. Thus, the initial condition needed to determine the unique solution of Eq. (2.4) is the initial temperature of the system, given as

$$\mathrm{T(x,\ y,\ z,\ t)}\big|_{\mathrm{t=0}} = \mathrm{T_0}\left(\mathrm{x,\ y,\ z}\right) \tag{2.4}$$

The convective boundary condition at the material surfaces is given by Newton's law of cooling and is used as follows:

$$\mathrm{h}\left(\mathrm{T_a} - \mathrm{T}\big|_{n=a}\right) = \mathrm{k_t}\,\frac{\partial T}{\partial n}_{n=a} \tag{2.5}$$

And, the adiabatic boundary condition applied in the center of the substrate particles is

$$\left.\frac{\partial T}{\partial n}\right|_{n=0} = 0 \tag{2.6}$$

where $n$ is the specific dimension, $a$ is the boundary position, h is the convective heat transfer coefficient, $\mathrm{k_t}$ is the thermal conductivity, and $\mathrm{T_a}$ is the temperature of the surrounding air.

Considering a transient heat transfer in an infinite slab, for one-dimensional flux, the corresponding equation is

$$\frac{\partial^2 T}{\partial x^2} + \frac{q''}{k_t} = \frac{1}{\alpha}\frac{\partial T}{\partial t} \tag{2.7}$$

where $x$ is the heat flux direction, $q''$ is the heat generation, $\mathrm{k_t}$ is the thermal conductivity, and α is the thermal diffusivity.

Food materials are, in general, poor electric insulators. They have ability to store and dissipate electric energy when subjected to an electromagnetic field. Microwave energy in itself is not thermal energy. The heating is a result of the electromagnetic energy generated with the dielectric properties of the material combined with the electromagnetic field applied. Dielectric properties play a critical role in determining the interaction between the electric field and the matrices [17]. The rate of conversion of electrical energy into thermal energy in the material is described by Chen et al. [18]:

$$P = K.f\,\varepsilon' E^2 \tan\delta \tag{2.8}$$

where $P$ is the microwave power dissipation per volume unit, $K$ is a constant, $f$ is the frequency applied, $\varepsilon'$ is the absolute dielectric constant of the material, $E$ is the electric field strength, and tan $\delta$ is the dielectric loss tangent.

The distribution of the electric field depends on the geometry of the irradiated object and its dielectric properties. The depth of penetration of a wave ($Dp$) can also have an important role in the choice of the working frequency and depends on the thickness of the matrix being treated. The energy absorption inside the solid material causes an electric field that decreases with the distance from the material surface. The penetration depth ($Dp$) is the distance from the material surface where the absorbed electric field ($\varepsilon$) is reduced to $1/\varepsilon$ of the electric field at the surface: this corresponds to an energy loss of about 37% [19]. The penetration depth is inversely proportional to the frequency and the dielectric properties of the material, as shown by the following expression [20]:

$$Dp = \frac{c}{2\pi f'\sqrt{2\varepsilon'}\left[\sqrt{1+\tan^2\delta}-1\right]^{1/2}} \tag{2.9}$$

where $c$ is the speed of light (m/s). This equation is approximated by the following (Eq. (2.24)), when tan $\delta << 1$, which is usually the case:

$$d = \frac{\lambda_0\sqrt{\varepsilon_r'}}{2\pi\varepsilon_r''} \tag{2.10}$$

where $\lambda_0$ is the wavelength in vacuum and $d$ the approximate penetration depth.

The depth of penetration varies inversely with the loss factor and is even less when the product is sensitive to microwaves. If the penetration depth of the microwave is much less than the thickness of the material, only the surface is heated, and the rest of the material is heated by conduction. For transparent media, that is, a loss factor <0.01, the depth of penetration is not problematic and will dissipate the energy. The presence of a standing wave will induce the creation of "hot spots" where the power dissipated exceeds the heat transfer to cooler areas of the environment.

## 2.2 Heat and Mass Transfer: Balance Equations and Kinetics

Plant materials can be considered as porous media because of their similarities to solid food that can be treated as hygroscopic and capillary-porous [21]. According to Datta [22] the distinction between porous and capillary-porous is based on the presence and size of pores. Generally, porous materials have pores $\geq 10^{-7}$ m, whereas for capillary-porous materials the pores are $\leq 10^{-7}$ m. The presence of pores makes the water transport in these systems more intricate, because, in addition to the contribution of molecular diffusion, the transport within the pores is also caused by Knudsen diffusion (mean free path of molecules is long compared to the pore size), surface diffusion, and hydrodynamic flow [21].

Considering the extraction process, microwaves are generally used in two situations: (1) MAE that can be treated as a solid–solvent extraction, in which case the equations developed by Takeuchi et al. [23] can be used, and (2) solvent-free MAE (SFMAE), which can be treated as a two-step process in which in the first step, system temperature in any given location is less than that of water evaporation, and in the second step, the temperature at any given location is equal to the boiling temperature; thus, the electromagnetic energy is entirely used to evaporate the water.

### *2.2.1 Heat and Mass Balance Equations for Solid–Liquid MAE*

The mass transfer equations for solid–liquid extraction were presented by Takeuchi et al. [23] for an isothermal process. The factors that control the extraction of a solute from a matrix using MAE are the mass transfer rate of the solute from the matrix to the solution phase and the strength of solute–matrix interactions. Although the solubility of the solute in the solvent is recurrently indicated as a limiting factor, it should not be so because the solvent-to-solid ratio is large enough to assure that the extract–solvent mixture forms an infinite diluted solution.

The rate of dissolution of a solute into the extraction solvent is controlled by the mass transfer rate of the solute from the solid matrix into the liquid. The transfer of the solute inside the solid particle occurs because of the concentration gradient in the solid–liquid interface, and it can be characterized by the effective diffusion. The equation that describes this phenomenon is based on Fick's law:

$$\frac{N_C}{A_T} = -D_{BC}\frac{dC_C}{dz} \tag{2.11}$$

where $N_C$ is the rate of dissolution of the solute $C$ in the solution (kg/s), $A_T$ is the area of the solid–liquid interface (m$^2$), $D_{BC}$ is the diffusivity of the solute in the solvent–inert solid (m$^2$/s), $C_C$ is the concentration of solute $C$ in the solution (kg/m$^3$), and $z$ is the distance inside the porous part of the solid matrix (m). The minus sign gives a positive flux term because the gradient is negative (flow occurs down a concentration gradient, from high to low concentration).

Diffusion coefficient data are necessary to make calculations. Diffusivities may be determined experimentally or predicted. Orders of magnitude of diffusion coefficients ($D_{BC}$) for solids are $10^{-9}$ to $10^{-10}$ m$^2$/s. When concerned with impermeable porous solids with fluid-filled pores, the effective (or apparent) diffusion coefficient is used:

$$D_{CBeff} = \frac{\varepsilon}{\tau} D_{BC} \tag{2.12}$$

where $\varepsilon$ is the void fraction or porosity of the solid and $\tau$ is the tortuousness of the pores.

On the surface of the solid particle, the transfer of the solute occurs simultaneously by molecular and turbulent transport. In this step, the mass transfer rate can be expressed by the following equation:

$$N_C = V \frac{VdC_C}{dt}\bigg|_S = A_T K_L \left(C_{CS} - C_C\right) \tag{2.13}$$

where $K_L$ is the mass transfer coefficient (m/s), $C_{CS}$ is the reference concentration of the solute $C$ in the solid surface (kg/m$^3$), and $C_C$ is the concentration of the solute $C$ in the solution at time $t$ (kg/m$^3$).

Integrating Eq. 2.14 from $t=0$ and $C_C = C_{C0}$ to $t=t$ and $C_C = C_C$, we obtain:

$$\int_{C_{C0}}^{C_C} \frac{dC_C}{C_{CS} - C_C} = \frac{Ak_L}{V} \int_{t=0}^{t} dt \tag{2.14}$$

$$\frac{C_{CS} - C_C}{C_{CS} - C_{C0}} = e^{-\left(\frac{k_L A}{V}\right)t} \tag{2.15}$$

If pure solvent is used initially, $C_{C0}=0$, and then

$$1 - \frac{C_C}{C_{CS}} = e^{-\left(\frac{k_L A}{V}\right)t} \tag{2.16}$$

$$C_C = C_{CS}\left(1 - e^{-\left(\frac{k_L A}{V}\right)t}\right) \tag{2.17}$$

### 2.2.2 Heat and Mass Balance Equations for SFMAE

In order to formulate the heat and mass balance, material will be considered, as suggested by Navarrete et al. [24], as a capillary-porous media that includes the insoluble solids, bound and free water, and air. Heat is generated and conducted in

the capillary-porous medium. The vapor phase forms an homogeneous system, and heat convection can be neglected. Steam is removed from the system instantaneously, that is, no diffusion or convection was considered. The evaporation of water consumed all heat generated in the system. MAE is considered to be performed in a fixed bed formed by the plant material packed inside the extraction vessel. During the extraction, system temperature will be equal to or less than the boiling temperature. So long as the temperature in a given location of the bed did not reach the boiling temperature, the general heat transfer equation or the thermal conduction equation can be used to estimate the heat transfer flux and describes the space and time behavior of the temperature field [24]:

$$\rho_S C_P \frac{\partial T}{\partial t} - \nabla \bullet \left(K_t \nabla \mathrm{T}\right) = \mathrm{P} \tag{2.18}$$

where $\rho_S$ represents the solid material apparent density (kg m$^{-3}$), $C_P$ is the specific heat capacity (J kg$^{-1}$ K$^{-1}$), and $K_t$ is the thermal conductivity (A V$^{-1}$ m$^{-1}$). $T = T$ ($x$, $y$, $z$, $t$) is the absolute temperature and P = P ($x$, $y$, $z$, $t$) is the microwave energy power dissipated per volume unit; this corresponds to the heat generated by the interaction between microwaves in the plant material. Note that the parameters $\rho_S$, $C_P$ and $K_t$ should be estimated for the lumped capillary-porous media as already described. The moisture content varies during the extraction process, and these parameters vary with the moisture of the system: for MAE these parameters are not constant. Nonetheless, for other systems in which only heating is the important phenomenon, these parameters are usually taken as constants that are independent of position, time, and temperature, which simplifies the solution of the heat transfer equation. According to Navarrete et al. [24], the time-average power dissipated in a plant material per unit volume can be calculated from

$$P = \frac{1}{2}(K_t + \omega \varepsilon_o \varepsilon'')\left|E\right|^2 \tag{2.19}$$

where $\omega$ is the angular frequency of the electromagnetic wave, $\varepsilon_o$ is the vacuum permittivity ($8.8542 \times 10^{-12}$ F m$^{-1}$), $\varepsilon$ is the dielectric loss factor, and $E$ is the electric field (V m$^{-1}$).

After the system temperature has reached the boiling temperature, the energy generated will be used for the evaporation of water. Therefore, the evaporation rate will be given by Navarrete et al. [24]:

$$\frac{\partial C_w}{\partial t} = R_w \tag{2.20}$$

where $C_w$ (kg m$^{-3}$) is the water concentration per unit volume of extractor vessel and $R_w$ is the water evaporation rate (kg s$^{-1}$ m$^{-3}$)

So long as water is evaporating, the rate of evaporation can be estimated from Navarrete et al. [24]:

$$R_w = \frac{P}{\lambda_w} \tag{2.21}$$

Equations 2.6 and 2.7 were proposed by Navarrete et al. [24] to describe the SFME (solvent-free microwave extraction) of Lavandin essential oil. To solve Eqs. (2.5), (2.6), and (2.7), the authors estimated the system properties using the equations of Datta [21, 22], Navarrete et al. [24], and Sihvola [25]. The specific heat of the lumped system as a function of system moisture was estimated using [21]

$$C_P = \rho_S C_{PS}\left(1-\varphi\right) + \rho_w C_{Pw} \varphi S_w + \rho_g C_{Pg} \varphi (1 - S_w) \tag{2.22}$$

where $C_{Pg}, C_{PS}$ and $C_{Pw}$ are the air, insoluble solid, and water specific heat, and $\rho_g, \rho_S$ and $\rho_w$ are the air, insoluble solid, and water densities. $S_w$ is the amount of water in pores and is generally referred to as the water saturation; it is calculated from [21]

$$S_w = \frac{M_w (1-\varphi)\rho_S}{(1-M_w)\varphi\rho_S} \tag{2.23}$$

where $M_w$ is the plant material moisture, which is calculated from

$$M_w = \frac{C_w V}{C_w V + \left(1 - M_{wo}\right) m_o} \tag{2.24}$$

where $M_{wo}$ and $m_o$ are the initial moisture content of the plant material and the mass of feed, respectively. $\varphi$ is the bed porosity and is calculated using [21]

$$\varphi = 1 - \frac{\rho_B (1 - M_w)}{\rho_S} \tag{2.25}$$

where $\rho_B$ is the bed apparent density.

## 2.3 Important Parameters in Microwave-Assisted Extraction and Mechanism of Action

The optimization of MAE conditions has been studied in several applications. The efficiency of the process is directly related to the operation conditions selected. Special attention should be given to usually studied parameters that may influence the performance of MAE such as solvent composition, solvent-to-feed ratio, extraction temperature and time, microwave power, and the characteristics of the matrix including its water content. Comprehension of the effects and interactions of these factors on the MAE process is significant. Thus, this topic emphasizes some of the

parameters that affect MAE, presenting guidelines regarding the selection of proper operation conditions, and also discusses the interaction between these parameters.

### *2.3.1 Effect of Solvent System and Solvent-to-Feed Ratio (S/F)*

The most important factor that affects MAE process is solvent selection. A proper solvent choice will provide a more efficient extraction process. Solvent selection depends on the solubility of the compounds of interest, solvent penetration and its interaction with the sample matrix and its dielectric constant [26], and the mass transfer kinetics of the process [27]. The solvent should preferably have a high selectivity toward the solutes of interest excluding undesired matrix components. Another important aspect is that the optimal extraction solvents cannot be selected directly from those used in conventional extractions: it depends on the capacity of the solvent to absorb the microwave energy and consequently heat up [7, 8, 13, 28].

In general, the capacity of the solvent to absorb microwave energy is high when the solvent presents high dielectric constant and dielectric loss [27]. Solvents that are transparent to microwaves do not heat when submitted to them. Hexane is an example of microwave-transparent solvent whereas ethanol is an excellent microwave-absorbing solvent [13, 29]. Both polar and nonpolar solvents can be used in MAE, and solvents such as ethanol, methanol, and water are sufficiently polar to be heated by microwave energy [30]. In this context, the properties of the solvent can be modified when combining different solvents, which allow varying the solvent selectivity for different compounds [30]. The addition of salts to the mixture can also increase the heating rate, because besides dipole orientation the ion conductivity is the main origin of polarization and corresponds to losses to heat in dielectric heating [27]. Studies have shown that small amounts of water in the extracting solvent make possible the diffusion of water into the cells of the matrix, leading to better heating and thus facilitating the transport of compounds into the solvent at higher mass transfer rates.

In the case of volatile compounds, the addition of a solvent with relatively low dielectric properties can be used to ensure that the solvent temperature is kept lower to cool off the solutes once they are liberated into the solvent [7]. Generally, hexane is used for the extraction of volatile oils [13]. In addition, the solvent-free MAE (SFMAE) process has been designed for aromatic herbs rich in volatile oils; in this case, the moisture content within the plant matrix itself serves for extraction and no solvent is used [29, 31].

Studies have reported that ethanol or water can be added into poor microwave absorbers, such as hexane, to improve the extraction efficiency. One of the most used solvent mixtures is hexane-acetone [8], and only a small amount of water (about 10%) must be added in nonpolar solvents such as hexane, xylene, or toluene to improve the heating rate [8]. Zhou and Lui [32] evaluated different mixtures of ethanol and hexane in the extraction of solanesol from tobacco leaves; the 1:3 ratio gave the best yield. Comparing isopropanol and hexane for rice bran oil extraction,

hexane at 40°C extracted approximately 40% more oil than isopropanol. Although by increasing the temperature hexane did not extract significantly more amount of oil, isopropanol extracted about 25% more rice bran oil at 120°C [33].

Some authors studied the use of combined solvents in MAE according to the polarity of the target compounds. A methanol–water (85:15) combination proved to be a good solvent for MAE of gymnemagenin from *Gymnema sylvestre* R. Br. Higher water concentration reduced the extraction yield because high water content increases the mixture polarity to a degree where it is no longer is favorable for extraction. The same was observed by Talebi et al. [34] when extracting paclitaxel from *Taxus baccata*: a methanol–water (90:10) mixture was the best combination. Song et al. [35], extracting sweet potato leaves, found that 60–80% (v/v) ethanol concentration in water was optimal within proportions of 40% and 80% (v/v).

The solvent-to-solid (feed) ratio (S/F) is an important parameter to be optimized. The solvent volume must be sufficient to guarantee that the entire sample is immersed in the solvent throughout the entire irradiation process, especially when using a matrix that will swell during the extraction [8, 13, 29].

In conventional extractions, the use of large volumes of solvent increases the extraction recovery. Studies reported that the extraction solution must not exceed 30–34% (w/v) [8]. In many applications a ratio 10:1 (ml/mg) to 20:1 (ml/mg) was found to be optimal [34, 36]. In addition, the solvent volume is an important factor to be considered because too much of the extracting solvent means more energy and time is required to condense the extraction solution in the later step and purification process. On the other hand, MAE may give lower recoveries because of nonuniform distribution and exposure to microwaves [37].

In some cases, small amounts of solvent are sufficient to extract the compounds of interest. The phenol and methylphenol extracted from oils had optimal conditions when S/F reached 2 [38]. A different behavior was observed in the MAE of artemisinin from *Aretimisia annua* L.: a higher extraction rate was achieved by a greater amount of solvent [39]. In *Ganoderma atrum*, the yield of triterpenoid saponins increased with the increase of amount of solvent until the S/F reached 25, and then it decreased rapidly [40].

### *2.3.2 Effect of Extraction Time and Cycle*

In MAE the period of heating is another important factor to be considered. Extraction times in MAE are very short compared to conventional techniques and usually vary from a few minutes to a half-hour, avoiding possible thermal degradation and oxidation [20, 28], which is especially important for target compounds sensitive to overheating of the solute–solvent system. Overheating occurs because of the high dielectric properties of the solvent, especially ethanol and methanol, and further dilution with water that increases the heat capacity of the solvent combination [7]. Higher extraction time usually tends to increase the extraction yield. However, this increase was found to be very small with longer time [41]. Irradiation time is also

influenced by the dielectric properties of the solvent. Solvents such as water, ethanol, and methanol may heat up tremendously on longer exposure, thus risking the future of thermolabile constituents [13].

Occasionally, when longer extraction time is required, the samples are extracted in multiple steps using consecutive extraction cycles, which are also an example of the use of a larger amount of solvent and higher microwave application time [28, 42]. In this case, the fresh solvent is fed to the residue and the process is repeated to guarantee the exhaustion of the matrix. With this procedure, the extraction yield is enhanced, avoiding long heating [7, 28]. The number of process cycles will depend on the type of matrix and the solute. According to Li et al. [43], three cycles of 7 min were appropriate for MAE of triterpene saponins from yellow horn, whereas in optimization of triterpenoid saponins MAE from *Ganoderma atrum*, cycles of 5 min each were recommended [26]. Yan et al. [44] found that three extraction cycles of 5 min each are optimal for extracting astragalosides from *Radix astragali.* They also found that increasing the irradiation time from 1 to 5 min increases the extraction yield rapidly; extraction reaches its maximum at 5 min, and then the yields decreased with the extension of the irradiation time. In the case of flavonoids extraction from *R. astragali*, there was an increase in yield with time up to an exposure of 25 min and then the extraction yield started to decrease [42]. In the work of Chen et al. [26] it was observed that triterpenoid saponins yield from *Ganoderma atrum* reached its maximum at 20 min; after this time, the target compounds easily decomposed because of long exposure to high temperature. The same behavior was found by Song et al. [35].

### *2.3.3 Effect of Microwave Power and Extraction Temperature*

Microwave power and temperature are interrelated because high microwave power can bring up the temperature of the system and result in the increase of the extraction yield until it becomes insignificant or declines [4, 42, 45]. It is known that the temperature is controlled by incident microwave power that controls the amount of energy provided to the matrix, which is converted to heat energy in the dielectric material.

At high temperatures the solvent power increases because of a drop in viscosity and surface tension, facilitating the solvent to solubilize solutes, and improving matrix wetting and penetration [13, 43, 46]. In addition, when MAE is performed in closed vessels, the temperature may reach far above the boiling point of the solvent, leading to better extraction efficiency by the desorption of solutes from actives sites in the matrix [8]. However, Routray and Orsat [7] state that the efficiency increases with the increase in temperature until an optimum temperature is reached and then starts decreasing with the further increase in temperature: this happens because the selection of ideal extraction temperature is directly linked with the stability and, therefore, with the yield of the target compound.

Microwave power is directly related to the quantity of sample and the extraction time required. However, the power provides localized heating in the sample, which

acts as a driving force for MAE to destroy the plant matrix so that the solute can diffuse out and dissolve in the solvent. Therefore, increasing the power will generally improve the extraction yield and result in shorter extraction time [4, 28]. On the other hand, high microwave power can cause poor extraction yield because of the degradation of thermally sensitive compounds. Also, rapid rupture of the cell wall takes place at a higher temperature when using higher power, and as a result impurities can also be leached out into the solvent together with the desired solute [13]. Therefore, it is important to properly select the MAE power to minimize the time needed to reach the set temperature and avoid a "bumping" phenomenon in temperature during the extraction [8]. Moreover, the overexposure to microwave radiation, even at low temperature or low operating power, was found to decrease the extraction yield because of the loss of chemical structure of the active compounds.

Knowing that power level alone does not gives sufficient information about the microwave energy absorbed into the extraction system, Alfaro et al. [47] created a term to study the effect of microwave power on MAE: energy density, defined as the microwave irradiation energy per unit of solvent volume for a given unit of time (W/ml). According to Li et al. [43], the energy density should be considered as a parameter as power level alone. In this study, the anthocyanin extraction rates from grape peel were different under the same microwave power level, extraction time, and S/F because the energy density levels were different.

Raner et al. [48] reported that variation of power from 500 to 1,000 W had no significant effect on the yield of flavonoids. The decrease in extraction yield was found at temperatures higher than 110°C because of instability of flavonoids and consequent thermal degradation [42]. In another case, higher microwave power led to thermal degradation of phenols when it was higher than 350 W (between 150 and 550 W) [35]. The temperature behavior was the same in other studies. In extracting astragalosides from *Radix astragali*, Yan et al. [44] also found that yield increased remarkably with temperature increase from 50°C to 70°C; above 70°C, the yields of astragalosides increased slowly and even decreased.

### *2.3.4 Effect of Contact Surface Area and Water Content*

Not only the parameters already discussed but the characteristics of the sample also affect the MAE process. It is known that in a higher contact surface area the extraction efficiency increases. Also, finer particles allow improved or much deeper penetration of the microwave [49]. On the other hand, very fine particles may pose some technical problems; consequently, centrifugation or filtration is applied to prepare the matrix [13, 29]. In the preparation step the sample is grinded and homogenized to increase the contact area between the matrix and the solvent. The particle sizes are usually in the range of 100 μm to 2 mm [8]. In some cases soaking of the dried plant material in the extracting solvent before MAE has resulted in improved yield. This procedure is called pre-leaching extraction [13].

In many cases the extraction recovery is improved by the matrix moisture, which acts as a solvent. The moisture in the matrix is heated, evaporated, and generates

internal pressure in the cell, which ruptures the cell to release the solutes, hence improving the extraction yield [31]. When increasing the polarity of the solvent, water addition has a positive effect on the microwave-absorbing ability and, hence, facilitates the heating process [8, 28]. Moreover, the additional water promotes hydrolyzation, thus reducing the risk of oxidation of the compounds [41].

In extraction of astragalosides from *Radix astragali*, extraction efficiency was improved by the addition of water. The possible reason for the increased efficiency is the increase in swelling of plant material by water, which enhances the contact surface area between the plant matrix and the solvent [44].

### *2.3.5 Effect of Stirring*

The effect of stirring is directly related to the mass transfer process in the solvent phase, which induces convection in the headspace. Therefore, equilibrium between the aqueous and vapor phases can be achieved more rapidly. The use of agitation in MAE accelerates the extraction by enhancing desorption and dissolution of active compounds bound to the sample matrix [50]. Through stirring, the drawbacks of the use of low solvent-to-solid ratio (S/F) can be minimized, together with the minimization of the mass transfer barrier created by the concentrated solute in a localized region resulting from insufficient solvent [28]. In the work by Kovács et al. [51] it is possible to observe the difference between suspensions with and without stirring. The authors found that when the suspensions were agitated with magnetic stirrers the temperature reached its maximum value within a shorter time, and the temperature differences inside individual vessels were not significant.

## 2.4 Comparison of Microwave-Assisted Extraction (MAE) with Other Solid–Liquid Extraction Techniques

To introduce bioactive plant extracts in pharmaceutical and cosmetic formulations, industries are looking for green and efficient extraction processes free of toxic solvents. Methodologies using biodegradable and nontoxic solvents such as water and ethanol are being developed [52].

The traditional techniques of solvent extraction of plant materials are based on the correct choice of solvents and the use of heat or/and agitation to increase the solubility of the desired compounds and improve the mass transfer. Soxhlet extraction is the most common and is still used as a standard in all cases [53]. As a result of several secondary metabolites, the development of high performance and rapid extraction methods is an absolute necessity [54]. The new extraction techniques with shortened extraction time, reduced solvent consumption, increased pollution prevention, and with special care for thermolabile constituents have gained attention. In the many published papers comparing MAE with other advanced and conventional extraction methods, MAE has been accepted as a potential and powerful alternative for the extraction of organic compounds from plant materials [55].

The ideal extraction technology depends on the type of compound to be extracted, whereas the extraction method efficiency is based on the highest recovery, especially of the effective constituents, the shortest processing time, the lowest production cost, and use of minimum organic solvent [56]. There have been numerous reviews and research on the advances of different extraction techniques, comparing their results. In the extraction of bioactive compounds from plants, MAE was reported to be more efficient compared to conventional techniques such as Soxhlet and advanced methods of extraction including ultrasound-assisted extraction (UAE), pressurized liquid extractions (PLE), and supercritical fluid extraction (SFE), which have emerged as energy-saving technologies. Over the years the procedures based on MAE have replaced some conventional extraction methods and have been adopted over decades in laboratories and industry.

In addition, the progress in microwave extraction gave rise to other categories of techniques to improve its performance: (1) microwave-assisted distillation (MAD) for the isolation of essential oils from herbs and spices [57]; (2) microwave hydrodiffusion and gravity (MHG), a combination of microwave heating and distillation at atmospheric pressure that requires less energy and no solvent and simply combines microwaves and earth gravity at atmospheric pressure [58]; (3) vacuum microwave hydrodistillation (VMHD), which uses pressures between 100 and 200 mbar to evaporate the azeotropic mixture of water–oil from the biological matrix [59]; (4) microwave-integrated Soxhlet extraction (MIS), a combination of microwave heating and Soxhlet [60]; and (5) solvent-free microwave extraction (SFME), based on the combination of microwave heating and distillation, which is performed at atmospheric pressure [61]. If these techniques are explored scientifically, they can be proven to be efficient extraction technologies for ensuring the quality of herbal medicines worldwide [13].

As already mentioned, MAE is increasingly employed in the extraction of natural products as an alternative to traditional techniques of extraction for several reasons: reduced extraction time, reduced solvent consumption, and less environmental pollution as a result of increased efficiency and clean transfer of energy to the matrix; improved extraction yield and product quality, because materials can be rapidly heated, and often processed at lower temperatures; up to 70% energy saving compared to conventional energy forms from the high energy densities and the direct absorption of energy by the materials; compact systems, as small as 20% of the size of conventional systems; and selective energy absorption resulting from the dielectric properties of the material and applicator design [52, 55, 62].

On the other hand, some disadvantages can also be mentioned: additional filtration or centrifugation is necessary to remove the solid residue after the process; the efficiency of microwaves can be poor when the target compounds or solvents are nonpolar, or when they are volatile; and the use of high temperatures that can lead to degradation of heat-sensitive bioactive compounds [63].

Considering these advantages and drawbacks of MAE compared to other techniques, a discussion on MAE performance compared to conventional and advanced techniques as Soxhlet, SFE, UAE, and PLE is appropriate. Table 2.2 presents their advantages and drawbacks; and Table 2.3 shows studies comparing the extraction technologies and their respective optimization.

**Table 2.2** Comparison of traditional and advanced extraction techniques for analytical-scale extraction (adapted from Ref. 8 and 64)

| | Extraction technique | | | | |
|---|---|---|---|---|---|
| | Soxhlet | Microwave-assisted extraction (MAE) | Ultrasound-assisted extraction (UAE) | Supercritical fluid extraction (SFE) | Pressurized solvent extraction (PLE) |
| Brief description | Sample is placed in a glass fiber thimble and by using a Soxhlet extractor, the sample is repeatedly percolated with recondensed vapors of the solvent | Sample is immersed in a microwave-absorbing solvent in a closed vessel and irradiated with microwave energy | Sample is immersed in solvent in a vessel and submitted to ultrasonic using US probe or US bath | Sample is loaded in a high-pressure vessel and extracted with supercritical fluid (most commonly carbon dioxide at pressures of 150–450 bar and temperatures of 40°–150°C). The analytes are collected in a small volume of solvent, in a separator or onto a solid-phase trap, which is rinsed with solvent in a subsequent step | Sample and solvent are heated and pressurized in an extraction vessel; when the extraction is finished, the extract is automatically transferred into a vial |
| Extraction time | 3–48 h | 3–30 min | 10–60 min | 10–60 min | 5–30 min |
| Sample size | 1–30 g | 1–10 g | 1–30 g | 1–5 g | 1–30 g |
| Solvent use | 100–500 ml | 10–40 ml | 30–200 ml | 2–5 ml (solid trap)<br>5–20 (liquid trap) | 10–100 ml |
| Investment | Low | Moderate | Low | High | High |
| Advantages | Easy to handle, no filtration necessary, high matrix capacity | Fast and multiple extraction, easy to handle, moderate solvent consumption, elevated temperatures | Easy to use, multiple extractions | Fast extraction, low solvent consumption, concentration of the extract, no filtration necessary, possible high selectivity, low temperatures, no use of toxic solvents, automated systems | Fast extraction, no filtration necessary, low solvent consumption, elevated temperature, automated systems |
| Drawbacks | Long extraction time, large solvent volume, cleanup step is needed | Extraction solvent must absorb microwave energy, filtration step required, waiting time for the vessels to cool down | Large solvent volume, filtration step required, repeated extractions may be required | Many parameters to optimize, especially analyte collection | Possible degradation of thermolabile analytes, cleanup step is needed |

**Table 2.3** Comparison on the extraction yield between MAE and other techniques

| Plant material | Operational conditions: type of solvent(s), solvent to feed ratio (S/F), temperature (T), pressure (P), time (t), raw material moisture content (h), rotation (r), frequency (f), power (Pw), flow rate (v), power to feed ratio (P/F), humidity (h) | Bioactive compound extracted and extraction yield (dry basis, db; wet basis, wb) | References |
|---|---|---|---|
| Sweetgrass leaves (*Hierochloe odorata* L.) | MAE:<br>Pw = 200 W; s = acetone; S/F = 10; T = 80 C;<br>P = $P_{atm}$; t = 15 min; one-step extraction | 5,8-Dihydroxycoumarin (0.42% db[a])<br>5-Hydroxy-8-*O*-β D-glucopyranosyl-benzopyranone (0.11% db[a]) | [65] |
| | SFE:<br>Two-step:<br>(1) = 35 MPa; T = 40 C<br>(2) = P = 25Mpa; T = 40 C<br>S = ethanol (20%); t = 2 h; v = 0.5 l/min | 5,8-Dihydroxycoumarin (0.49% db[a])<br>5-hydroxy- 8-*O*-β - D-glucopyranosyl-benzopyranone (0.06% db[a]) | |
| | Soxhlet:<br>S/F = 50; s = acetone; t = 6 h | 5,8-Dihydroxycoumarin (0.46% db[a])<br>5-Hydroxy-8-*O*-β -D-glucopyranosyl-benzopyranone (0.08% db[a]) | |
| *Artemisia annua* L. | MAE:<br>Pw = 650 W; s = solvent oil; S/F = 15; T = ambient; t = 12 min | Artemisinin (92.1% db[a]) | [66] |
| | SFE:<br>P = 30 MPa; s = $CO_2$; S/F = 6; T = 35 C; t = 2.5 h | Artemisinin (33.2% db[a]) | |
| | Soxhlet:<br>s = Solvent oil; S/F = 11.67; T = 35 C; t = 6 h | Artemisinin (60.4% db[a]) | |
| Licorice roots (*Glycyrrhizia glaubra*) | MAE:<br>Pw = 700 W; s = ethanol; S/F = 10; T = 85 –90 C; t = 4 min | Glycyrrhizic acid –GA (2.26%[a]) | [67] |
| | US:<br>s = ethanol; S/F = 10; t = 20.5 h | Glycyrrhizic acid –GA (2.26%[a]) | |
| | Soxhlet:<br>s = ethanol; S/F = 10; t = 10 h | Glycyrrhizic acid –GA (2.5%[a]) | |

| | | | |
|---|---|---|---|
| Green tea leaves | MAE:<br>Pw = 700 W; s = ethanol: water (1:1 v/v); S/F = 20; T = 20 C; t = 4 min | Tea polyphenols (30%[a])<br>Tea caffeine (4%[a]) | [36] |
| | UAE:<br>s = ethanol: water (1:1 v/v); S/F =; T = 20 C–40 C; t = 90 min | Tea polyphenols (28%[a])<br>Tea caffeine (3.6%[a]) | |
| | Heat reflux extraction:<br>s = ethanol: water (1:1 v/v); S/F =; T = 85 C; t = 45 min | Tea polyphenols (28%[a])<br>Tea caffeine (3.6%[a]) | |
| Grapefruit | MAE:<br>Pw = 0.9 kW; s = water; S/F = 30; T = 20 C; t = 6 min | Pectin (27.81% db[a]) | [68] |
| | UAE:<br>s = water; S/F = 30; T = 70 C; t = 25 min | Pectin (17.92% db[a]) | |
| | UAE + MAE:<br>Pw = 0.45 kW; S/F = 30;<br>t = 30 min (UAE) and 10 min (MAE) | Pectin (31.88% db[a]) | |
| | Heat batch:<br>s = water; S/F = 30; T = 90 C; t = 90 min | Pectin (19.16% db[a]) | |
| *Ganoderma atrum* | MAE:<br>s = ethanol: water (9.5: 0.5 v/v); S/F = 25; T = 90 C; t = 5 min | Global yield (5.11% db[a]) | [69] |
| | UAE:<br>s = ethanol: water (9.5: 0.5 v/v); S/F = 25; t = 30 min; f = 33 kHz | Global yield (1.72% db[a]) | |
| | SFE:<br>P = 25 MPa; T = 55 C; s = $CO_2$ + ethanol; t = 3 h | Global yield (1.52% db[a]) | |
| | Shaking:<br>s = ethanol: water (9.5: 0.5 v/v); t = 3 h | Global yield (2.58% db[a]) | |
| | HRE:<br>s = ethanol (9.5: 0.5 v/v); S/F = 25; T = 95 C; t = 1 h | Global yield (2.22% db[a]) | |

(continued)

**Table 2.3** (continued)

| Plant material | Operational conditions: type of solvent(s), solvent to feed ratio (S/F), temperature (T), pressure (P), time (t), raw material moisture content (h), rotation (r), frequency (f), power (Pw), flow rate (v), power to feed ratio (P/F), humidity (h) | Bioactive compound extracted and extraction yield (dry basis, db; wet basis, wb) | References |
|---|---|---|---|
| Yellow horn (*Xanthoceras sorbifolia* Bunge.) | MAE:<br>Pw = 900 W; s = ethanol:water (40: 60 v/v); S/F = 30;<br>T = 50 C; t = 7 min × 3 cycles | Global yield (11.62% db[a]) | [43] |
| | UAE:<br>Pw = 250 W; s = ethanol:water (40: 60 v/v); S/F = 30;<br>T = 50 C; t = 60 min × 3 cycles | Global yield (6.78% db[a]) | |
| | HRE:<br>Pw = 800 W; s = ethanol: water (40: 60 v/v); S/F = 30;<br>T = 50 C; t = 90 min × 3 cycles | Global yield (10.82% db[a]) | |
| Turmeric plant (*Curcuma longa* L.). | MAE:<br>Pw = 60 W; s = acetone; S/F = 3; T = 50 C; t = 5 min | Curcumin (90.47% db[a]) | [70] |
| | UAE:<br>Pw = 150 W; s = acetone; S/F = 3; T = 21 C; t = 5 min | Curcumin (71.42% db[a]) | |
| | Soxhlet:<br>s = acetone; S/F = 5; t = 8 h | Curcumin (2.10% db[a]) | |
| | SFE:<br>P = 30 MPa; s = $CO_2$ + ethanol (10%); T = 50 C;<br>t = 240 min; v = 5 ml/min | Curcumin (69.36% db[a]) | |
| *Silybum marianum* (L.) (milk thistle) | MAE:<br>Pw = 600 W; s = ethanol: water (80:20 v/v); S/F = 25;<br>t = 2 min × 6 cycles | Silybinin (1.37% db[a]) | [56] |
| | Soxhlet:<br>s = ethanol: water (80:20 v/v); S/F = 100; t = 12 h | Silybinin (1.09% db[a]) | |
| | Stirring:<br>s = ethanol: water (80:20 v/v); S/F = 100; t = 24 h | Silybinin (0.48% db[a]) | |
| | Maceration:<br>s = ethanol: water (80:20 v/v); S/F = 100; t = 24 h | Silybinin (0.36% db[a]) | |

| | | | |
|---|---|---|---|
| *Coriandrum sativum* | MAE:<br>Pw = 200 W; s = ethanol: water (50:50 v/v); S/F = 20;<br>T = 50 C, t = 18 min | Phenolics content (0.082% db[b]) | [71] |
| | UAE:<br>s = ethanol: water (50:50 v/v), S/F = 10; t = 30 min | Phenolics content (0.041% db[b]) | |
| *Cinnamomum zeylanicum* | MAE:<br>Pw = 200 W; s = ethanol: water (50:50 v/v); S/F = 20;<br>T = 50 C, t = 18 min | Phenolics content (1.679% db[b]) | [71] |
| | UAE:<br>s = ethanol: water (50:50 v/v), S/F = 10; t = 30 min | Phenolics content (0.506% db[b]) | |
| *Cuminum cyminum* | MAE:<br>Pw = 200 W; T = 50 C, s = ethanol: water (50:50 v/v);<br>S/F = 20; t = 18 min | Phenolics content (1.159% db[b]) | [71] |
| | UAE:<br>s = ethanol: water (50:50 v/v), S/F = 10; t = 30 min | Phenolics content (0.290% db[b]) | |
| *Crocus sativus* | MAE:<br>Pw = 200 W; T = 50 C, s = ethanol: water (50:50 v/v); S/F = 20; t = 18 min | Phenolics content (2.939% db[b]) | [71] |
| | UAE:<br>s = ethanol: water (50:50 v/v), S/F = 10; t = 30 min | Phenolics content (0.500% db[b]) | |

(continued)

**Table 2.3** (continued)

| Plant material | Operational conditions: type of solvent(s), solvent to feed ratio (S/F), temperature (T), pressure (P), time (t), raw material moisture content (h), rotation (r), frequency (f), power (Pw), flow rate (v), power to feed ratio (P/F), humidity (h) | Bioactive compound extracted and extraction yield (dry basis, db; wet basis, wb) | References |
|---|---|---|---|
| Sea buckthorn | MHG: | Isorhamnetin 3-*O*-rutinoside (0.123% db[a]) | [2] |
| (*Hippophae rhamnoides)* | Pw = 400 W; t = 15 min; h = 57% | Isorhamnetin 3-*O*-glucoside (0.097% db[a]) | |
| | | Quercetin 3-*O*-Glucoside (0.025% db[a]) | |
| | | Isorhamnetin (0.00084% db[a]) | |
| | Agitated: | Isorhamnetin 3-*O*-rutinoside (0.187% db[a]) | |
| | s = methanol: water (80:20 v/v); S/F = 10; t = 8 min | Isorhamnetin 3-*O*-glucoside (0.162% db[a]) | |
| | | Quercetin 3-*O*-Glucoside (0.016% db[a]) | |
| | | Isorhamnetin (0.00064% db[a]) | |
| Cranberry press cake | MAE:<br>s = ethanol; S/F = 5.7; T = 125 C; t = 10 min | Quercetin (0.1272% db[a]) | [72] |
| | Stirring:<br>s = ethanol; S/F = 5; t = 2 h | Quercetin (0.1537% db[a]) | |
| *Morinda citriflora* (roots) | MAE:<br>Pw = 720 W; s = ethanol: water (80:20 v/v); S/F = 100; T = 60 C; t = 15 min | Global yield (95.91% db[a]) | [73] |
| | UAE:<br>s = ethanol; S/F = 100; T = 60 C; t = 60 min | Global yield (62.23% db[a]) | |
| | Maceration:<br>s = ethanol; S/F = 100; t = 3 days | Global yield (63.33% db[a]) | |
| | Soxhlet:<br>s = ethanol; S/F = 100; T = 100 C; t = 4 h | Global yield (97.74% db[a]) | |

| | | | |
|---|---|---|---|
| Soybean germ | MAE:<br>S/F = 17.5; T = 120 C; t = 0.5 h | Global yield (16.5% wb[a]) | [74] |
| | MAE + UAE:<br>Pw = 60 W (UAE) and 100 W (MAE); S/F = 5;<br>T = 45 C; t = 1 h | Global yield (14.1% wb[a]) | |
| | Soxhlet:<br>s = hexane; S/F = 6.67; t = 4 h | Global yield (8.65% wb) | |
| *Lavandula angustifolia* Mill., Lamiaceae (lavender flowers) | MASD:<br>Pw = 500 W; s = water; S/F = 4; t = 10 min | Monoterpenes (3.54% db[a])<br>Oxygenated monoterpenes (78.29% db[a])<br>Sesquiterpenes (2.77% db[a])<br>Global yield (8.86% db[a]) | [75] |
| | SD:<br>s = water; S/F = 4; t = 90 min | Monoterpenes (4.92% db[a])<br>Oxygenated monoterpenes (75.14% db[a])<br>Sesquiterpenes (2.87% db[a])<br>Global yield (8.75% db[a]) | |
| Caraway (*Carum carvi* L.) | MDG:<br>Pw = 100 W; t = 45 min | Global yield (2.59% db[a])<br>Carvone (67.59% db[a])<br>Limonene (30.10% db[a]) | [76] |
| | Hydrodistillation:<br>S/F = 5; t = 300 min | Global yield (2.54% db[a])<br>Carvone (66.89% db[a])<br>Limonene (30.30% db[a]) | |
| Tomato | MAE:<br>Pw = 100 W; s = methanol; S/F = 50; t = 45 min | Total phenolic contents (0.646% db[b]) | [77] |
| | Shaker:<br>s = ethanol: water (60: 40 v/v); S/F = 50; T = 45 C;<br>r = 400 rpm; t = 15 h | Total phenolic contents (0.603% db[b]) | |

(continued)

**Table 2.3** (continued)

| Plant material | Operational conditions: type of solvent(s), solvent to feed ratio (S/F), temperature (T), pressure (P), time (t), raw material moisture content (h), rotation (r), frequency (f), power (Pw), flow rate (v), power to feed ratio (P/F), humidity (h) | Bioactive compound extracted and extraction yield (dry basis, db; wet basis, wb) | References |
|---|---|---|---|
| *Foeniculum vulgare* Miller (seeds) | MWHD:<br>Pw = 300 W; s = water; S/F = 2; T = 100 C; t = 200 s | Global yield (1.14% db[a]) | [78] |
| | HD:<br>Pw = 300 W; s = water; S/F = 8; t = 319 s; T = 100 C; r = 50 rpm | Global yield (0.265% db[a]) | |
| *Iochroma gesnerioides* (leaves) | MAE:<br>Pw = 25 W; s = methanol; S/F = 50; t = 40 s | Withaferin A (0.48% db[a])<br>Iochromolide (0.85% db[a])<br>Withacnistin (0.39% db[a]) | [79] |
| | Soxhlet:<br>(1) s = water; S/F = 6; t = 15 min<br>(2) s = methanol; S/F = 100; t = 6 h | Withaferin A (0.41% db[a])<br>Iochromolide (0.81% db[a])<br>Withacnistin (0.38% db[a]) | |
| *Xanthoceras sorbifolia* Bunge. (yellow horn) | MAE:<br>Pw = 900 W; s = ethanol: water (40: 60 v/v); S/F = 30; T = 50 C; t = 7 min × 3 cycles | Triterpene saponins (11.62% wb[a]) | [43] |
| | UAE:<br>Pw = 250 W; s = ethanol: water (40: 60 v/v); S/F = 30; T = 50 C; t = 60 min × 3 cycles | Triterpene saponins (6.78%[a]) | |
| | Reflux:<br>Pw = 800 W; s = ethanol: water (40: 60 v/v); S/F = 30; T = 50 C; t = 90 min × 3 cycles | Triterpene saponins (10.82%[a]) | |
| *Ocimum Basilicum* L. (basil) | SFME:<br>Pw = 500 W; T = 100 C; t = 30 min | Eugenol (43.2% wb[a])<br>Linalool (25.3% wb[a])<br>Global yield (0.029% wb[a]) | [80] |
| | HD:<br>s = water; S/F = 12; T = 100 C; t = 4.5 h | Eugenol (11.0% wb[a])<br>Linalool (39.1% wb[a])<br>Global yield (0.028% wb[a]) | |

| | | | |
|---|---|---|---|
| *Mentha crispa* L. (garden mint) | SFME:<br>Pw = 500 W; T = 100 C; t = 30 min<br><br>HD:<br>s = water; S/F = 12; T = 100 C; t = 4.5 h | Limonene (9.7% wb[a])<br>Carvone (64.9% wb[a])<br>Global yield (0.095% wb[a])<br>Limonene (20.2% wb[a])<br>Carvone (52.3% wb[a])<br>Global yield (0.095% wb[a]) | [80] |
| *Thymus vulgaris* L. (thyme) | SFME:<br>Pw = 500 W; T = 100 C; t = 30 min<br><br>HD:<br>s = water; S/F = 12; T = 100 C; t = 4.5 h | γ-Terpinene (17.1% wb[a])<br>Eugenol (51.0% wb[a])<br>Global yield (0.160% wb[a])<br>γ-Terpinene (22.8% wb[a])<br>Eugenol (40.5% wb[a])<br>Global yield (0.161% wb[a]) | [80] |
| *Elletaria cardamomum* L. (cardamom) | SFME:<br>Pw = 390 W; T = 100 C; h = 67%; t = 75 min<br>HD:<br>s = water; S/F = 10; T = 100 C; t = 6 h | Global yield (2.70% db[a])<br><br>26.23 1,8-Cineole<br>5.29 Linalool<br>2.60 Terpin-4-ol<br>3.88 α-Terpineol<br>3.63 Linalyl acetate<br>45.45 α -Terpinyl acetate | [81] |
| *Gymnema sylvestre* R. Br. | MAE:<br>Pw = 280 W; s = methanol: water (85: 15 v/v); S/F = 25; t = 6 min<br>Reflux:<br>s = methanol: water (85: 15 v/v); S/F = 100; T = 95 C; t = 6 h<br>Maceration:<br>s = methanol: water (85: 15 v/v); S/F = 100; t = 24 h<br>Stirring:<br>s = methanol: water (85: 15 v/v); S/F = 100; t = 24 h | Gymnemagenin (4.3% db[a])<br><br>Gymnemagenin (3.3% db[a])<br><br>Gymnemagenin (1.7% db[a])<br><br>Gymnemagenin (2.2% db[a]) | [82] |

(continued)

**Table 2.3** (continued)

| Plant material | Operational conditions: type of solvent(s), solvent to feed ratio (S/F), temperature (T), pressure (P), time (t), raw material moisture content (h), rotation (r), frequency (f), power (Pw), flow rate (v), power to feed ratio (P/F), humidity (h) | Bioactive compound extracted and extraction yield (dry basis, db; wet basis, wb) | References |
|---|---|---|---|
| (*Melilotus officinalis* (L.) Pallas) (yellow sweet clover) | MAE:<br>Pw = 100 W; s = water: ethanol (50: 50 v/v); S/F = 20; T = 50 C; t = 5 min × 2 cycles<br>USAE:<br>s = water: ethanol (50: 50 v/v); S/F = 20; t = 60 min<br>Soxhlet:<br>s = ethanol: water (95: 5 v/v); S/F = 16.67; t = 8 h | Coumarin (0.3978% db)<br>*O*-Coumaric acid (0.1257% db[a])<br>Melilotic acid (0.9052% db[a])<br>Coumarin (0.3569% db[a])<br>*O*-Coumaric acid (0.1269% db[a])<br>Melilotic acid (0.8092% db[a])<br>Coumarin (0.2156% db[a])<br>*O*-Coumaric acid (0.0708% db[a])<br>Melilotic acid (0.6314% db[a]) | [83] |
| *Salvia miltiorrhiza* Bunge. (dried root) | MAE:<br>s = ethanol: water (95: 5 v/v); S/F = 10; T = 80 C; t = 2 min<br>Reflux:<br>s = ethanol: water (95: 5 v/v); S/F = 10; t = 45 min<br>UAE:<br>s = ethanol: water (95: 5 v/v); S/F = 10; t = 75 min<br>Soxhlet:<br>s = ethanol: water (95: 5 v/v); S/F = 10; t = 95 min | Tanshinone IIA (0.29% db[a])<br>Cryptotanshinone (0.23% db[a])<br>Tanshinone I (0.11% db[a])<br>Tanshinone IIA (0.25% db[a])<br>Cryptotanshinone (0.24% db[a])<br>Tanshinone I (0.11% db[a])<br>Tanshinone IIA (0.28% db[a])<br>Cryptotanshinone (0.25% db[a])<br>Tanshinone I (0.10% db[a])<br>Tanshinone IIA (0.33% db[a])<br>Cryptotanshinone (0.25% db[a])<br>Tanshinone I (0.12% db[a]) | [84] |

| | | | |
|---|---|---|---|
| *Radix astragali* (dried root) | MAE:<br>Pw = 700 W; s = ethanol: water (80:20 v/v); S/F = 25; T = 70 C; t = 5 min × 3 cycles<br><br>Soxhlet:<br>s = ethanol: water (80:20 v/v); S/F = 20; T = 90 C; t = 4 h<br><br>Reflux:<br>s = ethanol: water (80:20 v/v); S/F = 20; T = 90 C; t = 1 h<br><br>UAE:<br>Pw = 100 W; s = ethanol: water (80:20 v/v); S/F = 20; t = 40 min<br><br>Maceration:<br>s = ethanol: water (80:20 v/v); S/F = 20; t = 12 h | Astragalosides I (0.0788% db[a])<br>Astragaloside II (0.0351% db[a])<br>Astragaloside III (0.0206% db[a])<br>Astragaloside IV (0.0278% db[a])<br>Astragalosides I (0.770% db[a])<br>Astragaloside II (0.347% db[a])<br>Astragaloside III (0.193% db[a])<br>Astragaloside IV (0.242% db[a])<br>Astragalosides I (0.761% db[a])<br>Astragaloside II (0.352% db[a])<br>Astragaloside III (0.203% db[a])<br>Astragaloside IV (0.257% db[a])<br>Astragalosides I (0.519% db[a])<br>Astragaloside II (0.302% db[a])<br>Astragaloside III (0.190% db[a])<br>Astragaloside IV (0.225% db[a])<br>Astragalosides I (0.411% db[a])<br>Astragaloside II (0.299% db[a])<br>Astragaloside III (0.166% db[a])<br>Astragaloside IV (0.206% db[a]) | [44] |
| Sweet potato [*Ipomoea batatas* (L.) Lam.] | MAE:<br>Pw = 123 W; s = ethanol: water (53: 47 v/v); S/F = 25; t = 2 min<br>CSE:<br>s = ethanol: water (60: 40 v/v); S/F = 30; t = 120 min | Total phenolics (6.115% db[b])<br><br>Total phenolics (5.969% db[b]) | [35] |

(continued)

**Table 2.3** (continued)

| Plant material | Operational conditions: type of solvent(s), solvent to feed ratio (S/F), temperature (T), pressure (P), time (t), raw material moisture content (h), rotation (r), frequency (f), power (Pw), flow rate (v), power to feed ratio (P/F), humidity (h) | Bioactive compound extracted and extraction yield (dry basis, db; wet basis, wb) | References |
|---|---|---|---|
| Tobacco leaves | MAE:<br>Pw = 700 W; s = hexane: ethanol (1: 3 v/v) + NaOH (0.05 mol/l); S/F = 10; t = 40 min | Solanesol (0.91% db[a]) | [32] |
| | HRE:<br>s = hexane: ethanol (1: 3 v/v) + NaOH (0.02 mol/l); S/F = 10; T = 60 C; t = 180 min | Solanesol (0.87% db[a]) | |
| *Lavandula angustifolia* Mill., Lamiaceae (lavender flowers) | MSD:<br>Pw = 200 W; v = 8 g/min; t = 6 min | 1,8-Cineole (14.40% db[a])<br>Linalool (42.52% db[a])<br>Global yield (2.7% db[a]) | [85] |
| | SD:<br>t = 30 min | 1,8-Cineole (13.71% db[a])<br>Linalool (40.43% db[a])<br>Global yield (2.7% db[a]) | |
| *Radix astragali* (root of *Astragalus*; Huangqi) | MAE:<br>s = ethanol: water (95: 5 v/v); S/F = 25; T = 110 C; t = 25 min × 2 cycles | Flavonoids (0.1190%[a]) | [42] |
| | Soxhlet:<br>s = methanol; S/F = 25; T = 85 C; t = 4 h | Flavonoids (0.1292%[a]) | |
| | UAE:<br>s = methanol; S/F = 20;T = 60 C; t = 30 min × 2 cycles | Flavonoids (0.0736%[a]) | |
| | HRE:<br>s = ethanol: water (90: 10 v/v); S/F = 25; T = 75 C; t = 2 h × 2 cycles | Flavonoids (0.0934%[a]) | |

| | | | |
|---|---|---|---|
| Yellow onion | VMHG:<br>Pw = 500 W; P = 700 mbar; P/F = 1 W/g; T = 81 C;<br>t = 26 min; h = 84.5% | Quercetin (0.662%db[a])<br>Global yield (3.18% db[a]) | [86] |
| | MHG:<br>P = 1 bar; T = 100 C; t = 23 min; h = 84.5% | Quercetin (0.283%db[a]) | |
| | CSE:<br>s = methanol: water (80: 20 v/v); S/F = 10;<br>r = 8,000 rpm; t = 5 min | Quercetin (0.890% db[a]) | |

*HRE* heat reflux extraction, *MHG* microwave hydrodiffusion and gravity, *MASD* microwave-accelerated steam distillation, *SD* steam distillation
*SFME* solvent-free microwave extraction, *MDG* microwave dry-diffusion and gravity, *MWHD* microwave-assisted hydrodistillation
*HD* hydrodistillation, *USAE* Soxhlet extraction, ultrasound-assisted extraction, *MSD* microwave steam distillation
*VMHG* vacuum microwave hydrodiffusion and gravity, *CSE* conventional solvent extraction
[a]Yield (%) = g compound per 100 g sample
[b]Yield (%) = g gallic acid equivalent (GAE) per 100 g mass of sample
Adapted from Eskilsson and Björklund [8]; Chemat et al. [64]

### 2.4.1 MAE Versus Soxhlet

Soxhlet is the typical technique and the main reference for evaluating the performance of other solid–liquid extraction methods as it has long been one of the most used solid–liquid extraction techniques. In Soxhlet extraction the solid material containing the solutes is placed inside a thimble holder, which is connected to a flask containing the extraction solvent, and submitted to reflux. After this process, the extract is concentrated by evaporation of the solvent [87]. This method has a large dependence on plant characteristics and particle size, as the internal diffusion may be the limiting step during extraction, and extraction and evaporation temperatures affect the quality of the final products [31].

It is a general and well-established technique, which surpasses in performance other conventional extraction techniques except, in a limited field of applications, the extraction of thermolabile compounds. Furthermore, it presents other disadvantages such as poor extraction of lipids, long operation time, high solvent consumption, and operation at the solvent's boiling point [88]. The advantages of this method include no requirement of a filtration step after leaching and the displacement of transfer equilibrium by repeatedly bringing fresh solvent into contact with the solid matrix [31, 88].

Studies show that MAE allows the reduction of time and solvent consumption, as well as improvement in global yield. Kaufmann et al. [89], extracting whitanolides from *Lochroma gesneroides*, showed a drastic reduction in solvent usage (5 vs. 100 ml) and in extraction time (40 s vs. 6 h). Another study concluded that the same quantity and quality of tanshiones from *Salvia miltiorrhiza* Bunge was obtained with 2 min of MAE and 90 min of Soxhlet [90]. Higher yield was obtained when extracting artemisinin from *Artemisia annua* L. by MAE; in 12 min, 92.1% of artemisinin was recuperated by MAE whereas several hours were needed by Soxhlet to reach only about 60% extraction efficiency [66].

### 2.4.2 MAE Versus Supercritical Fluid Extraction (SFE)

For green extraction, the use of SFE is very attractive because the solute is easily recovered and the solvent can be recycled by the simple manipulation of parameters such temperature and/or pressure. Supercritical fluids present liquid-like densities, whereas their viscosity is near that of normal gases and their diffusivity is about two orders of magnitude higher than in typical liquids [91]. Carbon dioxide ($CO_2$) is the most used solvent in SFE because it is safe, nontoxic, and generally available at a reasonable cost. However, even at high densities, $CO_2$ has a limited ability to dissolve highly polar compounds. The addition of modifiers to $CO_2$ can improve the extraction efficiency by increasing the solubility of the solute in the solvent.

The ease of tuning the operating conditions to increase the solvation power makes this technology a good option for the selective recovery of several types of

substances. This combination of properties makes SFE an important process in the food, pharmaceutical, and cosmetic industries because it is possible to fabricate products without toxic residues, with no degradation of active principles, and with high purity. Thus, SFE can be a fast, efficient, and clean method for the extraction of natural products from vegetable matrices [92].

Compared to SFE, MAE has a disadvantage, because cleanup is usually needed for this relatively selective technique [8, 63]. However, method development is often more complex in SFE and additionally sample throughput is not as high as in MAE [8]. Furthermore, the efficiency of MAE can be poor when either the target compounds or solvents are nonpolar, or when they are volatile. According to Stalikas [93], drying of the samples can be avoided for sample preparation with MAE, whereas samples are usually dried before SFE.

From the economic point of view, MAE is feasible as it requires moderate cost for equipment setup [63] and is much cheaper as compared to SFE. Moreover, MAE has low risks and no major safety issues as most extractions are generally carried out under atmospheric condition [28].

Several studies compared SFE and MAE. Hao et al. [66] extracted artemisinin from *Artemisia annua* L. by MAE, Soxhlet, and SFE. They found that MAE saves much time (12 min) and gives a high extraction rate (92.1%); SFE gives the lightest extract color but the lowest extraction yield while several hours were needed for Soxhlet. The same results were found by Grigonis et al. [64] comparing MAE with SFE and Soxhlet. The MAE gave the most concentrated extract with 8.15% of 5,8-dihydroxycoumarin (extract yield, 0.42%) from sweet grass. In addition, only 5 min gave the highest yield of triterpenoid saponins (0.968%), whereas SFE and UAE required several hours or even more than 10 h and gave a lower yield [40].

### *2.4.3 MAE Versus Ultrasound-Assisted Extraction (UAE)*

Ultrasound-assisted extraction (UAE) in the food industry has been the subject of research and development; its emergence as a green novel technology has also attracted attention to its role in environmental sustainability [94]. Ultrasound has been used in various processes of the chemical and food industries; it is a rapid technique, consumes small amounts of fossil energy, and allows reducing solvent consumption, thus resulting in a more pure product and higher yields.

The principle of high-power ultrasound has been attributed to the acoustic cavitation phenomenon that appears when high-intensity acoustic waves are generated in a fluid [95]. The extraction mechanism involves two types of physical phenomena: diffusion through the cell walls and washing out the cell content once the walls are broken [96]. Ultrasound waves modify their physical and chemical properties after their interaction with subjected plant material, and their cavitational effects facilitate the release of extractable compounds and enhance mass transport by disrupting the plant cell walls [94, 97, 98].

Developments in ultrasound technology and its potential benefits have triggered interest in the application of power ultrasound on a wider range of chemistry processing [99].

The combination of sonication and microwaves was studied for extraction of lipids from vegetables and microalgae sources. Ultrasonication alone, microwave irradiation alone, or a combination of both techniques gave excellent extraction efficiencies in term of yield and time, with a tenfold reduction in the time needed with conventional methods, and increase of yields from 50% to 500% [74]. MAE possessed higher efficiency (11.62%) for the extraction of triterpene saponins from yellow horn (*Xanthoceras sorbifolia* Bunge.) compared with UAE (6.78%) and reflux extraction (10.82%) [43].

### *2.4.4 MAE Versus Pressurized Liquid Extraction (PLE)*

Pressurized liquid extraction (PLE), also referred to as pressurized solvent extraction (PSE) and accelerated solvent extraction (ASE), is now well accepted as an alternative to Soxhlet extraction [100] and has been successfully used to isolate antioxidants from plants [101], such as thermolabile anthocyanins from jabuticaba (*Myrciaria cauliflora*) [102].

The use of the PLE technique is an attractive alternative because it allows fast extraction and reduced solvent consumption [102]. This technique allows the use of solvents or solvent mixtures with different polarities under high pressures (up to 20 MPa), keeping the extraction solvent in the liquid state [103], and temperatures ranging from room temperature up to 200°C [104].

The pressurized solvent at a determined temperature is pumped into an extraction vessel containing the sample matrix. Using high temperature accelerates the extraction process by increasing the solubility of the analytes in the solvent and thus increasing the kinetic rate of desorption of the solute from the sample matrix; this occurs because the pressurized solvent remains in the liquid state well above its boiling point, allowing high-temperature extraction [103]. Considerable increase in the mass transfer rates results from the decrease of viscosity and superficial tension of the solvent.

Moreover, the use of high temperatures, which on the one hand increases extraction rates, on the other hand may lead to degradation of thermolabile compounds [105]. PLE uses liquid solvents; therefore, its basic principle is considered similar to those of classic extraction. Partly because these newer technologies are automated and the solvents are under "superheated" conditions (the effect of microwaves in MAE or elevated temperature or pressure in PLE), they are more user friendly, much quicker, and require significantly less organic solvent [49].

Although in PLE the filtration step is "included" in the process, in MAE a cleanup step is often needed. MAE is considered an easy technique, and compared to SFE and PLE, it is less expensive [8].

Although good recovery rates were obtained with both extraction methods, MAE provided advantages with regard to sample handling, cost, analysis time, and solvent consumption.

## 2.5 Conclusion

There has been much research and many advances in development in the microwave-assisted extraction of a number of plant compounds. This chapter showed the phenomena of mass and heat transfer of the MAE process as well the parameters that influence MAE extraction of bioactive compounds. Therefore, optimized operating parameters can improve MAE performance. Also, MAE is better or comparable with other techniques. As a concluding remark, the MAE system is considered a promising technique for plant extraction because of its use of different physical and chemical phenomena compared to those in conventional extractions.

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# Chapter 3
# Microwave-Assisted Extraction of Essential Oils and Aromas

**Farid Chemat, Maryline Abert-Vian, and Xavier Fernandez**

## 3.1 Essential Oils and Aromas: Chemistry, Extraction, and Applications

Essential oils, which must be isolated by physical means only, are defined as products obtained from raw plant materials. The physical methods used are distillation (steam, steam/water, and water), squeezing (also known as cold pressing for citrus peel oils), or dry distillation (also known as pyrolysis) of natural materials. The essential oil is physically separated from the water phase by distillation. The volatile compounds have the property to solubilize in fatty oils and fats so that they have been called "essential oils." The term oil is used to denote the hydrophobic and viscous characteristics (not water soluble) whereas the term essential is used to denote the native essence and typical fragrance of the plant (Fig. 3.1).

The traditional way of isolating volatile compounds as essential oils from plant material is distillation. During distillation, fragrant plants are exposed to boiling water or steam, releasing their essential oils through evaporation. Recovery of the essential oil is facilitated by distillation of two immiscible liquids (water and essential oil) based on the principle that the combined vapor pressure equals the ambient pressure at the boiling temperature. Thus, the ingredients of essential oil, for which boiling points normally range from 200°C to 300°C, are evaporated at a temperature close to

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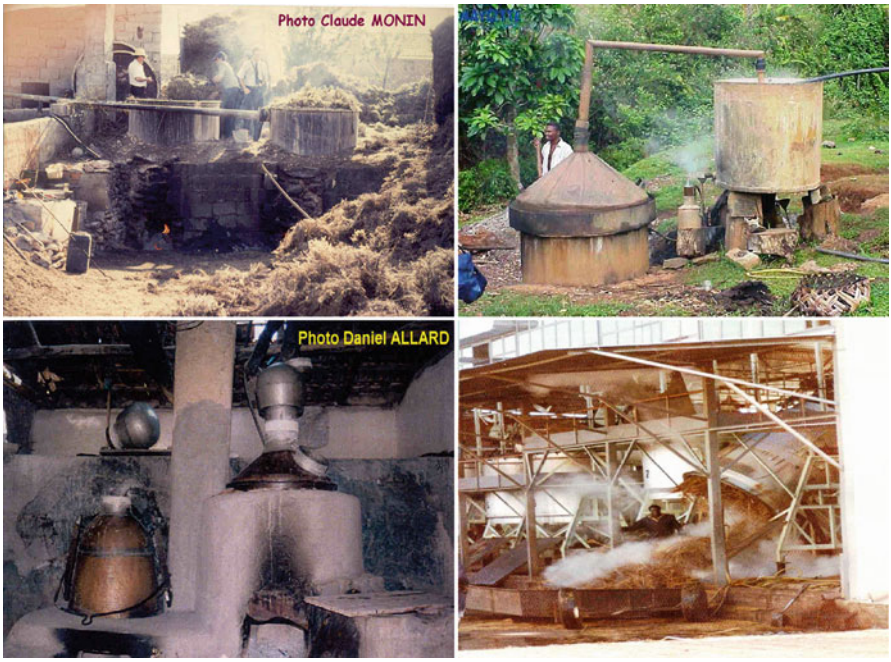


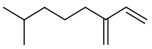
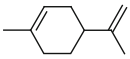
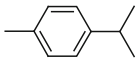
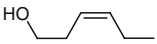
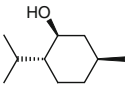
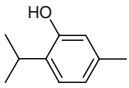
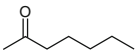
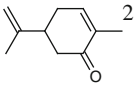
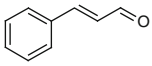
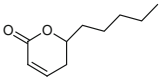
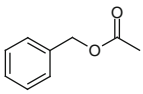
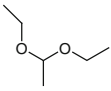
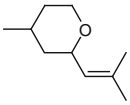
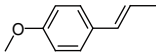
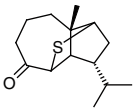
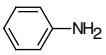

**Fig. 3.1** Conventional steam distillation or hydrodistillation of essential oils around the world

that of water. The laden steam of the essential oil rises and enters narrow tubing that is cooled by an outside source. As steam and essential oil vapors are condensed, both are collected and separated in a vessel traditionally called the Florentine flask. The essential oil, which is lighter than water, floats at the top while water goes to the bottom and can easily be separated. The amount of essential oil produced depends on four main criteria: the length of distillation time, the temperature, the operating pressure, and, most importantly, the type and quality of the plant material. Typically, the yield of essential oils from plants is between 0.005% and 10%.

Essential oils are highly concentrated aromatic oily liquids obtained from a variety of spices and aromatic plant materials. Numerous publications have presented data on the composition of the various essential oils, which comprise more than 100 individual components. Essential oils can be classified into two main groups: hydrocarbons, which consist of terpenes, such as monoterpenes, sesquiterpenes, and diterpenes; and oxygenated compounds, such as esters, aldehydes, ketones, alcohols, phenols, oxides, acids, and lactones. Nitrogen and sulfur compounds also occasionally exist.

Terpene is the generic name of a group of natural products, structurally based on isoprene (2-methyl butadiene) units that have the molecular formula $(C_5H_8)_n$. This term may also refer to oxygen derivatives of these compounds that are known as the terpenoids. They are normally classified into groups based on the number of isoprene units from which they are biogenetically derived. Monoterpenes contain two isoprene units. They are widely distributed in nature, particularly in essential oils, and are important in the perfumery and flavor industries. Sesquiterpenes contain three isoprene units. They can be found in many living systems but particularly in higher plants. Diterpenes,

**Table 3.1** Molecular diversity of essential oil components

| | Chain | | | |
|---|---|---|---|---|
| | | Linear | Cyclic | Aromatic |
| Function | Hydrocarbon |  |  |  |
| | | Myrcene | Limonene | *p*-Cymene |
| | | Mastic, celery | Citrus | Oregano, thyme |
| | Alcohol |  |  |  |
| | | (*Z*)-Hexen-3-ol | Menthol | Thymol |
| | | Mustard, ylang-ylang | Mint | Oregano, thyme |
| | Carbonyl |  |  |  |
| | | Heptan-2-one | Carvone | Cinnamaldehyde |
| | | Ginger | Caraway seed, mint | Cinnamon |
| | Ester/lactone |  |  |  |
| | | Ethyl isobutyrate | Massoïa lactone | Benzyl acetate |
| | | Myrte | Massoïa wood | Ylang-ylang |
| | Ether |  |  |  |
| | | 1,1-Diethoxyethane | Rose oxide | Anethol |
| | | Rose | Rose | Badiane |
| | Sulfur |  |  |  |
| | | Dimethyl trisulfide | Mint sulfide | Benzothiazole |
| | | Garlic, onion, mustard | Rose, mint, sage | Ginger, Chinese rose |
| | Nitrogenous |  | |  |
| | | Aniline | | 2,4-Diméthyl pyrazine |
| | | Fenugreek | | Iris |

which are made from four isoprene units, contain 20 carbon atoms in their basic skeletons. They occur in almost all plant families and belong to more than 20 major structural types. Table 3.1 presents the molecular diversity of essential oils.

Essential oils are obtained from a variety of aromatic plant materials including flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots. These aromatic compounds are formed by plants as by-products or indeed as final metabolic products, and they are stored in certain organs of the plant (Table 3.2).

**Table 3.2** Plant organs yielding essential oils (EO)

| Plant organs | Example |
|---|---|
| Aril | Mace of nutmeg |
| Barks | Cinnamon, tea tree, gaïac |
| Berries | Black pepper, juniperus |
| Buds | Clove |
| Bulbs | Onion, garlic, leek |
| Flower | Rose, neroli |
| Pistil | Saffron |
| Kernel | Nutmeg |
| Leaf | Basil, geranium, mint, sage |
| Rhizome | Ginger, iris |
| Latex from rhizome | Asafoetida |
| Roots | Angelica |
| Seeds | Caraway, coriander, dill, fennugrek |

**Table 3.3** Important flavor compounds in essential oils

| Essential oil | Important flavor compounds |
|---|---|
| Allspice | Eugenol, β-caryophyllene |
| Anise | (E)-Anethole, methyl chavicol |
| Black pepper | Piperine, δ3-carene, β-caryophyllene |
| Caraway | Carvone, carvone derivatives |
| Cinnamon | Cinnamaldehyde |
| Coriander | Linalool |
| Mustard | Allyl isothiocyanate |
| Parsley | Apiol |
| Peppermint | Menthol, menthone, menthfuran |
| Spearmint | (L)-Carvone |

About 3,000 essential oils are known, of which about 300 are commercially important and widely used in the perfume, cosmetic, pharmaceutical, agricultural, and food industries. It has long been recognized that some of them have shown antimicrobial, antioxidant, antiviral, anti-mycotic, anti-toxigenic, anti-parasitic, and insecticidal properties. These characteristics are possibly related to the function of these compounds in plants. For example, monoterpenes and sesquiterpenes serve as anti-herbivore agents that have significant insect toxicity while having negligible toxicity to mammals. Essential oil can also be used as a compound source, in particular in flavors and fragrances. Table 3.3 presents important flavor compounds present in essential oils of herbs and spices.

## 3.2 Compressed Air Microwave Distillation (CAMD)

This process was proposed in 1989 by Craveiro et al. [1] and recognized as the first microwave technique for extraction of essential oils from aromatic plants and spices. This system is composed of three parts: a compressor that injects air into the container with the

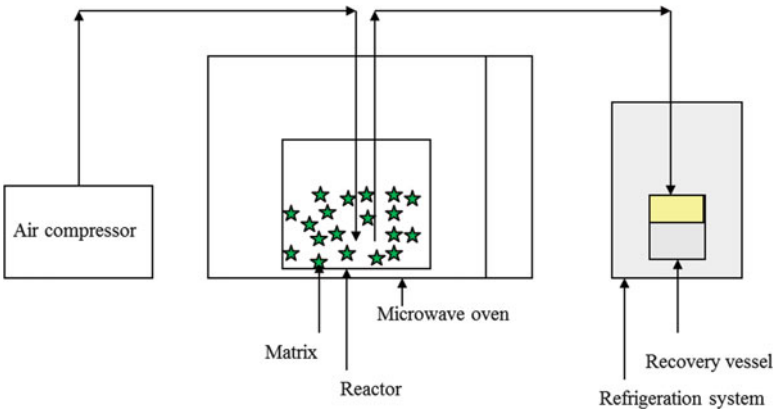



**Fig. 3.2** Compressed air microwave distillation (CAMD)

matrix, a microwave oven, and a refrigeration system (Fig. 3.2). This technique relies on the steam entrainment principle and uses compressed air instead of steam to extract the essential oil. Compressed air is continuously injected in the reactor where the matrix is immersed in water and heated by microwaves. The steam becomes saturated in volatile molecules and then is driven to a recovery vessel immersed in a refrigeration system situated outside the microwave oven. After only a few minutes, water and aromatic molecules are condensed and recovered in similar proportions to that of a conventional process.

In 2010, Lee [2] patented a similar system: "Microwave apparatus and method of extracting essential oils, essence and pigments with gas flow." A condenser is used at temperatures between −20°C and −15°C to cool the gas that flows out of the extraction cartridges. As no organic solvents or artificial chemical compounds are added, this extraction method is environmentally friendly.

## 3.3 Microwave Hydrodistillation (MWHD)

The process microwave hydrodistillation (MWHD), which was developed by Stashenko et al. in 2004 [3], is based completely on the classical hydrodistillation principle; a part of the hydrodistillation assembly line is placed in the microwave oven (Fig. 3.3). The matrix is installed with water into a reactor that has already been placed inside the microwave oven. The refrigeration system and the part estimated to recover essences are situated outside the oven.

A hydrodistillation apparatus is placed inside a domestic microwave oven with a side orifice, through which an external glass condenser joins the round flask with the matrix (*Xylopia aromatica*) (100 g) and water (2 l) inside the oven. The oven is operated for 30 min at 800 W, which causes vigorous reflux boiling of the water. Essential oil is decanted from the condensate and dried with anhydrous sodium sulfate. The major component of this essential oil is the β-phellandrene (65%) [3].

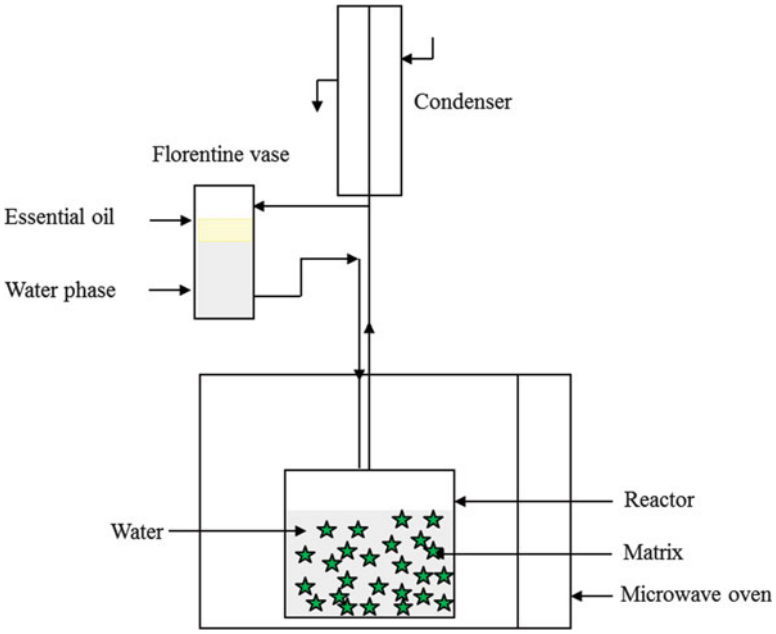



**Fig. 3.3** Microwave hydrodistillation (MWHD)

This technique was applied for essential oil extraction from many aromatic plants and spices [3–10]. Plants that have proceeded to an essential oils extraction by microwave hydrodistillation are inventoried in Table 3.4. For example, operating conditions for *Thymus vulgaris* L. [5] were 60 g of matrix with 2,000 ml water, microwave power was 990 W during 120 min at atmospheric pressure, and 2.52% essential oil was obtained. As another example, *Zataria multiflora* Boiss. [6] was extracted at 990 W during 120 min at atmospheric pressure; 60 g of this matrix with 1,200 ml water provided a yield of 3.7%. In *Satureja montana* [7], 0.7% of essential oil was obtained with 60 g matrix, 1,200 ml water, and microwave heating at 660 W during 90 min at atmospheric pressure.

An improvement of MWHD was developed by Flamini et al. in 2006 [11] by introducing an insulated microwave coaxial antenna inside a glass flask containing matrix and water. This in situ microwave heating appears to be safe and versatile. It shows advantages of time and energy savings and can be useful for industrial applications.

Another innovation, microwave steam distillation (MSD), was developed in 2008 by Chemat et al. [12]. The process is based on the conventional steam distillation principle in which microwave radiation is only applied on the extraction reactor (Fig. 3.4). The refrigeration system and the part estimated to recover essences are situated outside the oven. This method was applied for essential oil extraction of lavender flowers (*L. angustifolia* Mill.).

**Table 3.4** Essential oils extracted by microwave hydrodistillation (MWHD)

| Botanical species | Extraction operating conditions | References |
|---|---|---|
| *Xylopia aromatica* (Lamarck) | 100 g, 2,000 ml water, P(atm), 800 W, T = 30 min | [3] |
| *Lippia alba* (Mill.) | 100 g, 1,000 ml water, P(atm), 800 W, T = 30 min | [4] |
| *Thymus vulgaris* L. | 60 g, 2,000 ml water, P(atm), 990 W, T = 120 min, R = 2.52% | [5] |
| *Zataria multiflora boiss* | 60 g, 1,200 ml water, P(atm), 990 W, T = 120 min, R = 3.7% | [6] |
| *Satureja hortensis* | 30 g, 600 m water, P(atm), 660 W, T = 180 min, R = 3.1% | [7] |
| *Satureja montana* | 60 g, 1,200 ml water, P(atm), 660 W, T = 90 min, R = 0.7% | [7] |
| *Eryngium foetidum* L. | 300 g, 1,000 ml water, P(atm), 900 W, T = 27 min, R = 0.061% | [8] |
| *Lavandula angustifolia* Mill. | 80 g, 1,500 ml water, P(atm), 500 W, T = 20 min, R = 7.40% | [9] |
| *Anethum graveolens* L. | 100 g, 1,000 ml water, P(atm), 500 W, T = 60 min, R = 2% | [10] |
| *Coriandrum sativum* L. | 100 g, 1,000 ml water, P(atm), 500 W, T = 60 min, R = 0.4% | [10] |

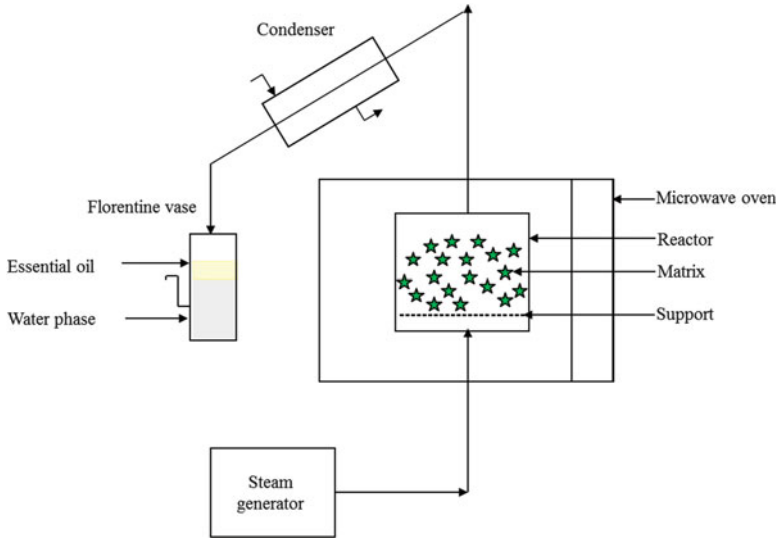



**Fig. 3.4** Microwave steam distillation (MSD)

## 3.4 Solvent-Free Microwave Extraction (SFME)

Solvent-free microwave-assisted extraction (SFME) was developed and patented in 2004 by Chemat et al. [13, 14]. SFME is one of the newest techniques for essential oil extraction assisted by microwaves, without solvent and water at atmospheric pressure. The process SFME principally consists of four parts: one reactor where only matrix to be processed is placed, one microwave oven, one refrigeration system, and an essence container where essential oil is recovered (Fig. 3.5).

Based on a relatively simple principle, this process describes a dry distillation assisted by microwaves that places the fresh matrix in a microwave reactor without adding water or organic solvent. Water heating of the raw material breaks the glands containing essential oil. This phase releases the essential oil, which is then driven by steam produced from matrix water. A cooling system placed outside the microwave oven allows the continuous condensation of the distillate, which is composed of water and the essential oil, and the excessive water returns inside the balloon, which allows maintenance of the proper humidity rate of the matrix.

A Milestone "DryDist" microwave laboratory oven (1,000 W maximum) is used to perform solvent-free microwave extraction (SFME). This is a multimode microwave reactor of 2,450 MHz. The experiment was conducted at atmospheric pressure with 250 g matrix during 30 min at 500 W without solvent or water. This extraction was continued at 100°C until no more essential oil was obtained. At the end, essential oil is collected, dried with anhydrous sodium sulfate, and stored at 4°C. Then, essential oils are analyzed by gas chromatography coupled with mass spectrometry (GC–MS). Yields of essential oils obtained by SFME are 0.029% for basil, 0.095% for crispate mint, and 0.160% for thyme [15].

This process was applied to several kinds of fresh and dry plants, such as spices, aromatic herbs, and citrus fruits [15–30]. Table 3.5 inventories plants that have been used in essential oils extraction by the SFME process. The first example is *Ocimum*

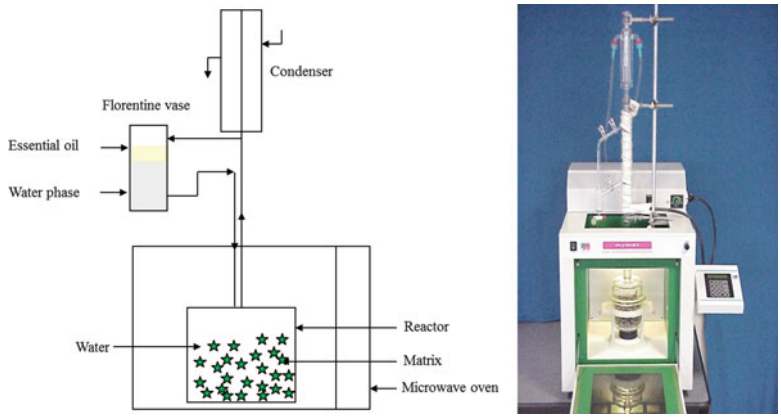



**Fig. 3.5** Solvent-free microwave extraction (SFME)

**Table 3.5** List of plants exposed to an essential oils extraction by solvent-free microwave-assisted extraction (SFME)

| Botanical species | Extraction operating conditions | References |
|---|---|---|
| *Ocimum basilicum* L. | 250 g, P(atm), 500 W, T = 30 min, R = 0.029% | [15] |
| *Mentha crispa* L. | 250 g, P(atm), 500 W, T = 30 min, R = 0.095% | [15] |
| *Thymus vulgaris* L. | 250 g, P(atm), 500 W, T = 30 min, R = 0.160% | [15] |
| *Carum ajowan* L. | 250 g soaked in water during 1 h, P(atm), 500 W, T = 60 min, R = 1.41% | [16] |
| *Cuminum cyminum* L. | 250 g soaked in water during 1 h, P(atm), 500 W, T = 60 min, R = 0.63% | [16] |
| *Illicium verum* | 250 g soaked in water during 1 h, P(atm), 500 W, T = 60 min, R = 1.38% | [16] |
| *Elletaria cardamomum* L. | 100 g soaked in water (67% of humidity), P(atm), 390 W, T = 75 min, R = 2.70% | [17] |
| *Citrus sinensis* L. | 200 g, P(atm), 200 W, T = 30 min, R = 0.42% | [18] |
| *Citrus sinensis* L. | 200 g, P(atm), 200 W, T = 10 min, R = 0.4% | [19] |
| *Citrus limon* L. | 200 g, P(atm), 200 W, T = 30 min, R = 0.24% | [20] |
| *Rosmarinus officinalis* L. | 200 g, P(atm), 200 W, T = 30 min, R = 0.57% | [21] |
| *Rosmarinus officinalis* L. | 250 g, P(atm), 500 W, T = 40 min, R = 0.39% | [22] |
| *Nigella sativa* L. | 150 g, P(atm), 850 W, T = 10 min, R = 0.20% | [23] |
| *Origanum vulgare* L. | 150 g soaked in water during 1 h, P(atm), 622 W, T = 35 min, R = 0.054 ml/g | [24] |
| *Origanum glandulosum* Desf. | 25 g soaked in water during 1 h, P(atm), 850 W, T = 20 min, R = 3.3% | [25] |
| *Laurus nobilis* L. | 150 g soaked in water during 1 h, P(atm), 622 W, T = 85 min, R = 0.0235 ml/g | [26] |
| *Saccocalyx satureioides* Coss. and Dur. | 25 g soaked in water during 1 h, P(atm), 622 W, T = 20 min, R = 1.9% | [27] |
| *Melissa officinialis* L. | 280 g soaked in water, P(atm), 85 W, T = 50 min, R = 0.15% | [28] |
| *Laurus nobilis* L. | 140 g soaked in water, P(atm), 85 W, T = 50 min, R = 0.42% | [28] |
| *Bupleurum fruticosum* L. | P(atm), 500 W, T = 30 min, R = 0.21% | [29] |
| *Calamintha nepeta* L. Savi | 60 g, P(atm), 250 W, T = 40 min, R = 0.38% | [30] |

*basilicum* L. [15]; 0.029% of essential oil was obtained by extracting 250 g of the plant at 500 W during 30 min at atmospheric pressure. The second example, *Mentha crispa* L. [15], was heated under 500 W during 30 min at atmospheric pressure with 250 g matrix, providing 0.095% essential oil. *Thymus vulgaris* L. [15], the third example, supplied as the final result 0.16% essential oil for 250 g matrix heated at 500 W during 30 min at atmospheric pressure.

Improved SFME was proposed by Wang et al. in 2006 [31]. The method lies in the use of a solvent-free extraction assembly assisted by microwaves in which a carbonyl iron powder (CIP) is added and mixed with dry matrix at the bottom of the reactor (Fig. 3.6). The spherical particles of carbonyl iron (CIP) are able to absorb a part of the emitted microwave energy and restore it in the middle as heat form. Thus, the matrix can be heated by simple conduction without any auxiliary energy. Different

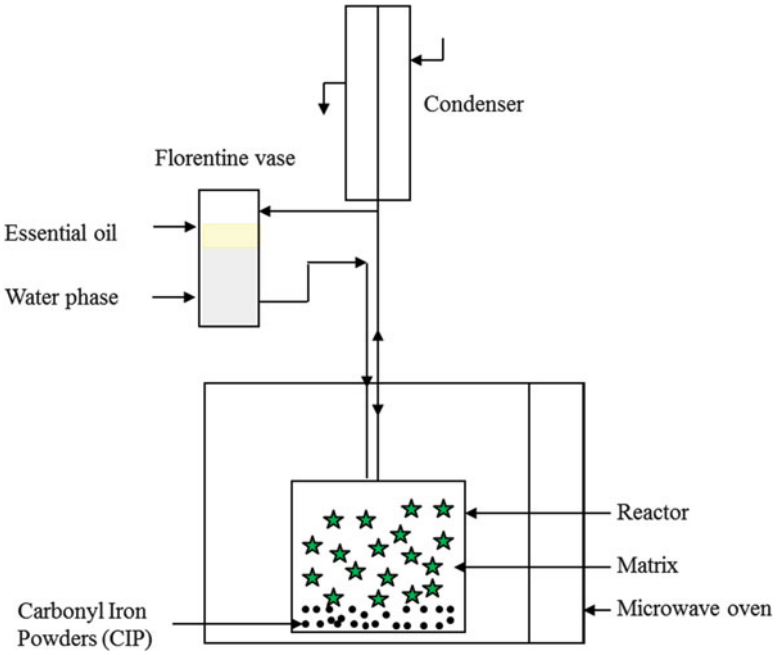



**Fig. 3.6** Improved solvent-free microwave extraction (improved SFME)

**Table 3.6** List of plants exposed to an essential oils extraction by improved SFME

| Botanical species | Extraction operating conditions | References |
|---|---|---|
| *Cuminum cyminum* L. | 100 g + 20 g carbonyl iron powder (CIP), P(atm), 85 W, T = 30 min | [31] |
| *Zanthoxylum bungeanum* Maxim. | 100 g + 20 g CIP, P(atm), 85 W, T = 30 min | [31] |
| *Citrus reticulata* Blanco | 100 g + 20 g CIP, P(atm), 85 W, T = 30 min | [34] |
| *Mentha haplocalyx* Briq. | 100 g + 20 g CIP, P(atm), 85 W, T = 30 min | [34] |
| *Illicium verum* Hook. f. | 100 g + 20 g CIP (or + 20 g GP or + 20 g ACP), P(atm), 85 W, T = 30 min | [32] |
| *Zingiber officinale Rosc.* | 100 g + 20 g CIP (or + 20 g GP or + 20 g ACP), P(atm), 85 W, T = 30 min | [32] |
| *Illicium verum* Hook. f. | 20 g + 15 ml ionic liquid (IL), P(atm), 440 W, T = 15 min | [33] |
| *Cuminum cyminum* L. | 20 g + 15 ml IL, P(atm), 440 W, T = 15 min | [33] |

types of materials such as graphite (graphite powders), active carbon (active carbon powders), and ionic liquid (1-hexyl-3-methylimidazolium hexafluorophosphate) can absorb microwave radiation [32, 33].

The improved SFME process was applied to different kinds of dry and fresh plants such as spices, citrus, and aromatic herbs [31–34] (Table 3.6). For example, 100 g *Cuminum cyminum* L. [31] and 20 g CIP were heated at 85 W during 30 min at atmospheric pressure. Another plant, *Citrus reticulata* Blanco [34], was also extracted at 85 W for 30 min at atmospheric pressure by taking 100 g matrix with

20 g CIP. In *Mentha haplocalyx* Briq. [34], the same extraction operating conditions were employed, that is, 100 g plant with 20 g CIP heated at 85 W during 30 min at atmospheric pressure.

## 3.5 Microwave Hydrodiffusion and Gravity (MHG)

Microwave hydrodiffusion and gravity (MHG) extraction was patented by Chemat et al. in 2008 [35]. This process was designed for essential oil extraction of different matrices by hydrodiffusion through microwave radiation at atmospheric pressure. The MHG system is principally composed of four parts: one reactor where only processed matrix is placed, a microwave oven, a refrigeration system, and an essence container where the essential oil is recovered (Fig. 3.7).

This technique consists of placing matrix in a reactor inside the microwave oven without adding water or organic solvent. Microwaves induce warming of the water contained in the matrix, which allows the destruction of cells containing essential oil. Essential oils, as well as the internal water of the matrix, are

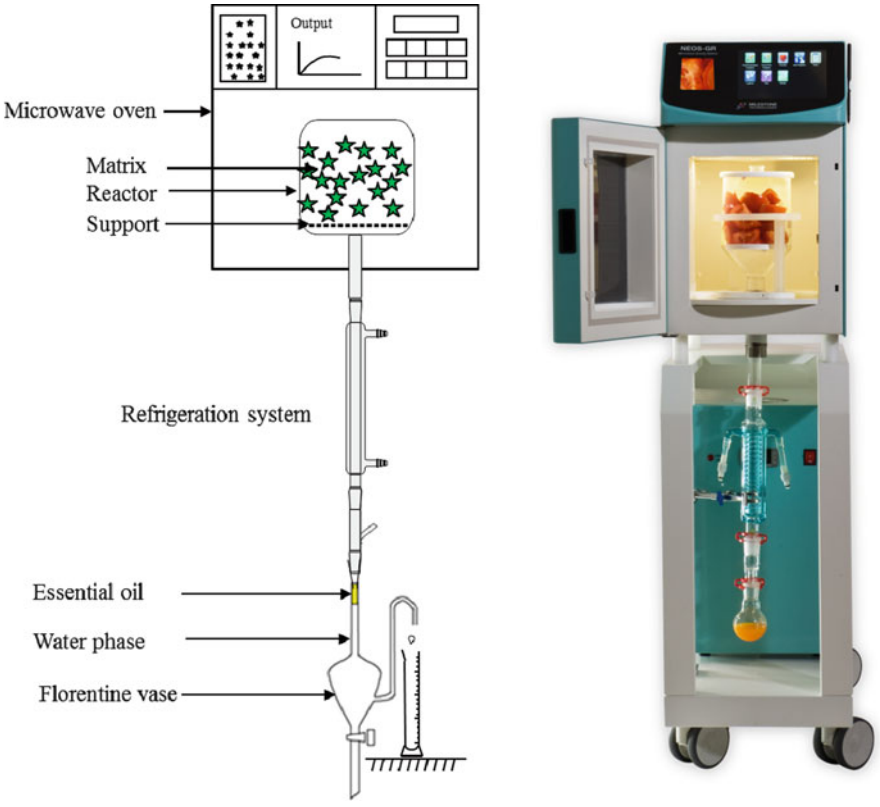



**Fig. 3.7** Hydrodiffusion microwave and gravity (MHG)

**Table 3.7** List of plants exposed to an essential oils extraction by hydrodiffusion microwave and gravity (MHG)

| Botanical specie | Extraction operating conditions | References |
|---|---|---|
| *Menthe pulegium* L. | 500 g, P(atm), 500 W, T = 20 min, R = 0.95% | [36] |
| *Menthe spicata* L. | 500 g, P(atm), 500 W, T = 20 min, R = 0.6% | [36] |
| *Citrus limon* L. | 500 g, P(atm), 500 W, T = 15 min, R = 0.7% | [37] |
| *Citrus limon* L. | 500 g, P(atm), 500 W, T = 15 min R = 1.6% | [37] |
| *Citrus aurantifolia* (Chrism.) Swing | 500 g, P(atm), 500 W, T = 15 min, R = 0.8% | [37] |
| *Citrus paradise* L. | 500 g, P(atm), 500 W, T = 15 min, R = 1% | [37] |
| *Citrus sinensis* L. | 500 g, P(atm), 500 W, T = 15 min, R = 1.2% | [37] |
| *Citrus sinensis* L. | 500 g, P(atm), 500 W, T = 15 min, R = 1% | [37] |
| *Citrus sinensis* L. | 500 g, P(atm), 500 W, T = 15 min, R = 0.9% | [37] |
| *Citrus paradisi* Macf. | 500 g, P(atm), 500 W, T = 15 min, R = 1.2% | [37] |
| *Rosmarinus officinalis* L. | 500 g, P(atm), 500 W, T = 15 min, R = 0.33% | [38] |

released and transferred from inside to the outside of the plant: this is the hydrodiffusion phenomenon. A cooling system placed outside the microwave oven allows the condensation of the distillate.

A Milestone NEOS-GR microwave laboratory oven (900 W maximum) is used to perform the microwave hydrodiffusion and gravity (MHG) extraction: this is a multimode microwave reactor of 2.45 GHz. Temperature is monitored by an external infrared (IR) sensor. The experiment is conducted at atmospheric pressure with 500 g matrix during 20 min at 500 W without solvent or water. At the end, essential oil is collected, dried with anhydrous sodium sulfate, and stored at 4°C. Yields of essential oils obtained by MHG are 0.6% for spearmint and 0.95% for pennyroyal. This extraction method offers the benefits of cost reduction and savings in time and energy [36].

This process has been applied to many kinds of plants such as aromatic plants and citrus [36–38]. Table 3.7 inventories plants that have been used for essential oil extraction by the MHG process. The first example is the *Menthe pulegium* L. extraction [36], where 0.95% essential oil was obtained by the heating of 500 g matrix at 500 W during 20 min at atmospheric pressure. For *Citrus limon* L. [37], 500 g matrix was also treated at 500 W for 15 min, with two yields of 0.7% and 1.6%, respectively, of essential oil obtained at atmospheric pressure. In another example, *Rosmarinus officinalis* L. [36] was tested by taking 500 g plant at 500 W during 15 min, providing 0.33% essential oil.

## 3.6 Cost, Energy, and Environmental Issues

The reduced cost of essential oils extraction is an undoubted advantage for the proposed microwave methods in terms of energy and time. Power consumption has been determined with a wattmeter at the entrance of a microwave generator

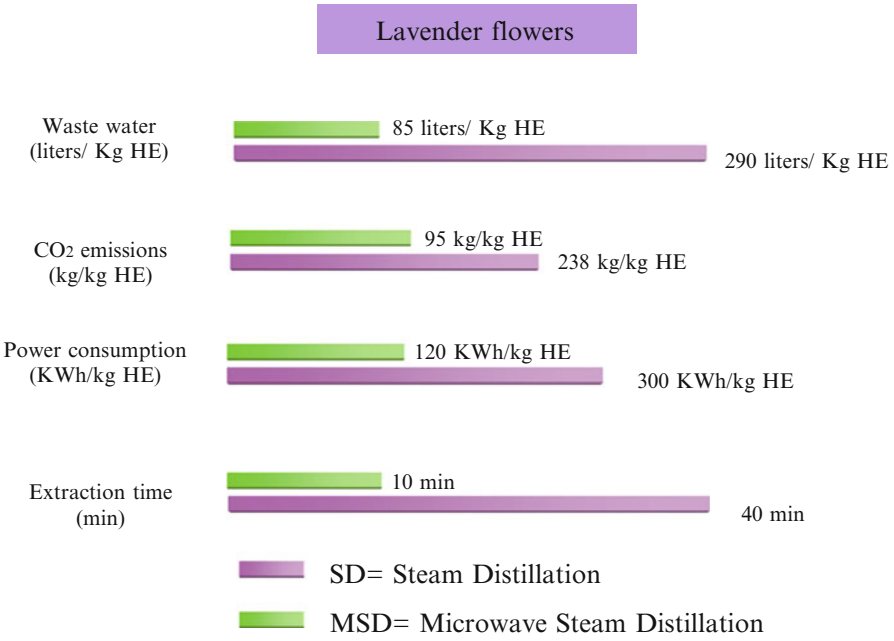



**Fig. 3.8** Comparison of microwave steam distillation (MSD) and steam distillation (SD) for lavender flowers extraction with an identical essential oil yield

and the power supply of an electrical heater. Regarding environmental impact (Fig. 3.8), the calculated quantity of carbon dioxide injected to the atmosphere is higher in the case of conventional steam distillation (SD) (238 kg $CO_2$ $kg^{-1}$ oil) than that in microwave steam distillation (MSD) (95 kg $CO_2$ $kg^{-1}$ oil). These calculations have been made according to the literature [39]: to obtain 1 kWh from coal or fuel, 800 g $CO_2$ will be injected into the atmosphere during combustion of fossil fuel. Wastewater was also reduced from 290 l/kg essential oil with SD to 85 l/kg essential oil with MSD.

## 3.7 Industrial Applications

Vacuum microwave hydrodistillation (VMHD) was elaborated and patented by Archimex in the 1990s [40]. This technique is based on selective heating by microwaves combined with application of sequential vacuum. The plant material is placed in a microwave cavity with water to refresh the dry material. The plant material is afterward exposed to microwave radiation for release of the natural extracts. Reducing the pressure to between 100 and 200 mbar allowed the evaporation of the azeotropic water–volatile oil mixture from the biological matrix. The procedure is repeated in a stepwise fashion, which can

**Fig. 3.9** Vacuum microwave hydrodistillation (VMHD) (100 l)

help to extract all the volatile oil from the plant. Up to 100 kg of materials can be treated per hour (Fig. 3.9). The Gattefossé company in France (www.gattefosse.com) commercialize a variety of products named "original extracts" obtained by microwave distillation of in situ water of herbs, spices, and fruits without adding any water or solvent. These original extracts are used for cosmetic, perfumes, and nutraceuticals.

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# Chapter 4
# The Role of Microwaves in the Extraction of Fats and Oils

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## 4.1 Introduction

### *4.1.1 Properties of Fats and Oils*

Fats is the general name given to a category of lipids usually referred to as acylglycerides, which are esters in which two or three fatty acids are bonded to a glycerol molecule forming monoglycerides, diglycerides, or triglycerides, respectively. The most common fats are triglycerides; these are triesters of glycerol and fatty acids, and can be solid or liquid at room temperature depending on their particular structure and composition. Although the names "oils," "fats," and "lipids" are widely used to refer to fats, the words oils and fats usually apply to lipids that are liquid and solid, respectively, at ambient temperature.

The types of fatty acids constituting a given ester (particularly, their degree of unsaturation) dictate its physical state at ambient temperature. Thus, fats are formed mainly from fatty acids with a saturated long chain (more than eight carbon atoms) including lauric, myristic, and palmitic acids. Fatty acids are present in bacon, cocoa, and various other foods and are believed to raise the levels of low-density

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lipoproteins (LDL) in plasma. However, the most appreciated edible oils are formed mainly from unsaturated fatty acids such as oleic, palmitoleic, and linoleic acids. Typical examples of edible oils include those obtained from olives, sunflower seeds, corn, rapeseed, or soya, which have beneficial effects on human health via their action on plasma lipids.

Polyunsaturated acids, to which category belong the controversial omega-3 and omega-6 series, are mainly found in blue fish such as tuna or salmon, and also in nuts such as walnuts, hazelnuts, and almonds.

A distinction must be made between essential fatty acids (EFAs), which are not synthesized by humans and must thus be acquired through the diet, and nonessential fatty acids (NEFAs), which are synthesized by humans. Fats, which are present in animals and plants, have both structural and metabolic functions. Also, they are major ingredients of the animal diet (human diet included).

### *4.1.2 Raw Materials for Extraction of Fats and Oils*

The primary raw materials containing extractable fats and oils come from plants or animal sources.

Ambient-temperature solid fats from plant materials (e.g., cocoa butter, coconut fat, palm butter) are obtained by using a specific method for each raw material. These fats are widely used in the food industry, usually as fat filling (e.g., cocoa butter in the chocolate industry).

Vegetable oils come from fruits (e.g., olives) or from oily seeds (e.g., sunflower, soybean, corn, safflower, cotton). Oils from fruits or seeds are used as such or for frying at both household and industrial levels. Hydrogenating seed oils increases their melting point and allows their use as margarines. Edible oils are also, but less often, obtained from dried fruits such as nuts, hazelnuts, or almonds, as well as from grape seeds (a by-product of wine production).

Some vegetable fats and oils are also used in the cosmetics industry. Also, linseed oil, which has a high content in polyunsaturated fatty acids and possesses semi-drying properties, is used in varnishes and paints.

Animal fat producers include terrestrial and marine animals. The most common animal fat is lard, which comes exclusively from porcine adipose tissue and is widely used in cakes and pastries. Tallow is obtained from sheep, goats, or, for the greatest quality, cattle. Meat from birds such as chickens and turkeys has a low fat content.

Food products from terrestrial animals, which have variable contents of fats and oils, are used mainly to obtain butter, cheese, and milk.

Fatty fish such as sardine, anchovy, mackerel, tuna, bonito, and salmon are fat rich and contain essential omega-3 fatty acids (C20:5 and C22:6). By contrast, whitefish and lean fish contain little fat (2.5% at most). Finally, semi-fatty fish (bream, red mullet, sea bass) contain 2.5–6% fat.

### 4.1.3 Traditional and Modern Methods for Extracting Fats and Oils

Solvent extraction of solid samples, which is commonly known as "solid–liquid extraction" but should rather be named "leaching" or "lixiviation" to more strictly adhere with its physicochemical foundation, is one of the oldest techniques for solid sample preparation. Traditionally, leaching and liquid–liquid extraction have been the techniques of choice for extracting fats from solid and liquid matrices, respectively.

Laboratory-scale conventional methods for lipid extraction are based either on hydrolysis (whether acid, alkaline, or enzymatic) before solvent extraction (Weibull–Berntrop, Röse–Gottlieb, Mojonnier, Folch, Werner–Schmid, Bligh–Dyer) or on direct extraction (Soxhlet: reference AOAC method [1], Lickens–Nickerson). Despite the modifications in solvent mixtures and laboratory practices [2–5], these methods have improved little during the past decades; also, they involve long preparation times and, frequently, a second extraction step to ensure complete removal of lipids [6]. These methods provide a lipid extract that is usually quantified by gravimetry or, less commonly, by titration.

Modern methods for lipid isolation are based on supercritical fluid extraction [7], accelerated solvent extraction [8], ultrasound-assisted extraction [9], or, especially, microwave-assisted extraction [10]. Also, the methods for the subsequent quantification of the analytes, with or without individual separation, identification, and *cis/trans* discrimination [11, 12], have shifted to the use of infrared spectrometry [13, 14], gas chromatography (GC) with flame-ionization detection (FID) [15], GC with mass spectrometry (MS) detection [16, 17], and liquid chromatography (LC) with MS [18] or nuclear magnetic resonance (NMR) detection [19].

For nutrition labeling purposes, fat has been designated triglycerides, which are extracted with ether, or total lipids [20–22]. To unify criteria, the U.S. Food and Drug Administration, through the Nutritional Labeling and Education Act of 1990, defined total fat as the combination of all fatty acids obtained in the lipid extract, expressed as triglycerides [23]. Complete extraction of lipids from the sample is therefore required.

### 4.1.4 Types of MAE Extractants: The Influence of Sample–Microwave Interaction on the Choice of Extractant (Economic Aspects)

The extractant used for MAE has a strong impact on performance. The choice should be dictated by its microwave-absorbing properties, its interactions with the sample matrix, and the solubility of the target compound(s). In solid–liquid extraction, the solvent or extractant should have a high selectivity toward the target compound(s) and exclude unwanted matrix components so far as possible. The

extractant should be compatible with the analytical method to be used for determination and have no toxic effects (or be easily removed, if toxic) when operating for nutritional purposes at an industrial scale. The optimal extractants for MAE need not coincide with those used in conventional procedures. Thus, a solvent absorbing no microwave radiation can hardly be an effective extractant unless used in combination with special bars of a chemically inert fluoropolymer to absorb microwave energy and facilitate transfer of the heat to the surrounding medium [23].

Extraction under heating may occur via a number of mechanisms depending on the particular extractant. Thus, the sample can be immersed in a single solvent or in a mixture capable of strongly absorbing microwave energy (mechanism I). Alternatively, the sample can be extracted into a combination of solvents with both high and low dielectric losses mixed in variable ratios (mechanism II). Also, a sample with a high dielectric loss can be extracted with a microwave-transparent solvent (mechanism III). Finally, if both the sample and extractant are transparent to microwaves, heating can be accomplished by using special bars of a chemically inert fluoropolymer (mechanism IV). Usually, solute extraction and partitioning occur via one of these mechanisms or a combination thereof. The temperature reached by each solvent on application of a given amount of energy differs widely, and so does its effect on MAE efficiency.

The analytical extraction of lipids from tissues requires using a sufficiently polar extractant or extractant mixture to ensure quantitative extraction of both neutral lipids and membrane-associated polar lipids. Water in the sample matrix (e.g., fish tissue) additionally requires that the solvent be sufficiently water soluble to facilitate thorough penetration in the sample. Mixtures of *n*-hexane and acetone are the most frequently used solvents for MAE of lipids [24]. However, ethyl acetate/cyclohexane mixtures have also proved effective for extraction from tissues [25]. The ethyl acetate/cyclohexane azeotrope has an almost equivolume composition (54:46) [26] and a boiling point of 72.8°C, which is 23°C higher than that of the *n*-hexane/acetone mixture [26]. Also, it has a high enough dielectric constant and can be directly heated without the need for a microwave transformer [27, 28]. In addition, it plays a dual role because it acts as a nonpolar system but does accept some water. Evaporation of water in the sample decomposes the cell structure and supports removal of lipids from their association with cell membranes and lipoproteins. From this aspect, the presence of water in the sample is advantageous for accelerating extraction (particularly under microwave irradiation). However, water increases the polarity of the extracting solvent, which decreases its efficiency and also, possibly, lipid yields. For this reason, aqueous tissue samples are usually dried before extraction [29]. Water in the sample forms a ternary azeotrope with the solvent (an ethyl acetate–cyclohexane mixture), the boiling point of which is lower than that of the binary azeotrope.

Liquid–liquid partitioning with chloroform–methanol according to Bligh and Dyer [30] is widely considered the most effective method for polar lipid extraction and commonly used to determine lipid contents in environmental samples [31, 32]. However, this method is time consuming; also, it uses chlorinated solvents, which are environmentally unfriendly.

The present trend toward green processes has also reached extraction, where it has revived the battle for and against replacing toxic solvents such as *n*-hexane with others of lesser or no toxicity; such is the case with limonene (a major component among the by-products of the citrus fruit industry), which has proved more efficient than *n*-hexane for the MAE of oil from olive drupes [33].

## 4.2 Microwave-Assisted Equipment for Extraction of Fats and Oils

A wide range of laboratory equipment from household ovens to commercial dedicated systems is currently available for MAE of lipids. A detailed description of their performance and uses can be found in the literature and in their respective users' manuals. MAE pilot plants have traditionally been sparse, usually under patent, and with inaccessible literature. At present, manufacturers of new and traditional MAE devices offer commercial or tailor-made pilot plants based on the use of MAE energy to extract a number of different compound families (particularly lipids) from vegetables.

### *4.2.1 Laboratory Equipment*

Commercially available equipment for MAE of lipids such as that marketed by CEM or Milestone (see Chap. 1) affords the simultaneous processing of several samples and temperature and pressure control, among other functions. Other systems, however, require user involvement (e.g., manual introduction of the sample, extractants, and reagents into the vessel) and dramatically increase the overall processing time as a result, for example, of the need to allow the vessel to cool down before it can be opened after extraction. This limitation has promoted the development of commercial and, occasionally, patented, laboratory-scale equipment to circumvent these shortcomings [34, 35].

The traditional equipment for Soxhlet extraction of lipids has led to the development of microwave-assisted Soxhlet extraction (MASE). MASE differs mainly in four aspects from other microwave-assisted techniques, namely (1) the extraction vessel is open, so it always works under normal pressure; (2) microwave irradiation is focused on the sample; (3) the extraction step is totally or partially performed as in the conventional technique (i.e., with permanent sample–fresh extractant contact); and (4) no subsequent filtration is required. Therefore, MASE retains the advantages of conventional Soxhlet extraction while overcoming its limitations concerning throughput, automatability, and the ability to quantitatively extract strongly retained analytes, for example. Microwave-assisted Soxhlet extraction can be implemented on the Soxwave-100 extractor, which was patented and made

commercially available by Prolabo (Paris, France), and on the focused microwave-assisted Soxhlet extractor (FMASE), which was designed by the authors' group and the first prototype of which was also constructed by Prolabo [36] (Fig. 4.1a). The main differences between the two extractors are as follows:

(a) The principle behind the Soxwave-100 is similar to Kumagawa extraction and its operation similar to that of the Soxtec$^{R}$ System HT [37], the process involving extraction in three steps. The FMASE works similarly to a conventional Soxhlet apparatus; thus, it performs a series of cycles whereby the extractant is completely renewed but the sample is irradiated with microwaves for a preset time each cycle.
(b) The Soxwave-100 uses a single heating source (focused microwaves) acting on both the sample and solvent, whereas the FMASE uses two energy sources (microwaves for sample irradiation and electrical heating for the extractant). This latter dissimilarity has led to a number of differences in performance, as follows:

    1. Because the heating source of the solvent is microwaves, the dielectric constant of the extractant is of paramount importance in the Soxwave-100; thus, polar extractants are more efficient here than are nonpolar and low-polar extractants, so this is not the more appropriate choice for lipid extraction. By contrast, extractant distillation in FMASE is accomplished by electrical heating and is thus unaffected by the solvent polarity.
    2. Because the amount of energy required by the extractant differs from that needed to remove the target analytes from the sample, a compromise must inevitably be made in this respect in Soxwave-100 extraction. This is not the case with FMASE, where the operating conditions can be optimized independently at each temperature.
    3. Operation with the Soxwave-100 involves a preliminary step in which the sample is immersed in the boiling extractant, followed by lifting the cartridge over the solvent and continuous dropping of the condensate on the cartridge. In this step, a matrix–extractant partitioning equilibrium of extractable species is established while microwave radiation acts on both the sample and extractant. In the second step, the partitioning equilibrium is displaced to extraction completion by effect of the sample coming into contact with fresh extractant in the absence of microwave irradiation. In FMASE, clean extractant and microwave irradiation are simultaneously supplied, which facilitates mass transfer and shortens extraction times as a result.

Although the Soxwave-100 has retained its original design, FMASE has been the subject of continual improvements based on alterations of the initial prototype or its subsequent incarnations.

The first, simplest prototype designed by the authors and constructed in 1998 by Prolabo (Paris, France) consisted of a modified Microdigest A301 focused-microwave digestor (200 W maximum power) where a hole is made at the bottom of the irradiation zone to connect the cartridge compartment with the distillation flask

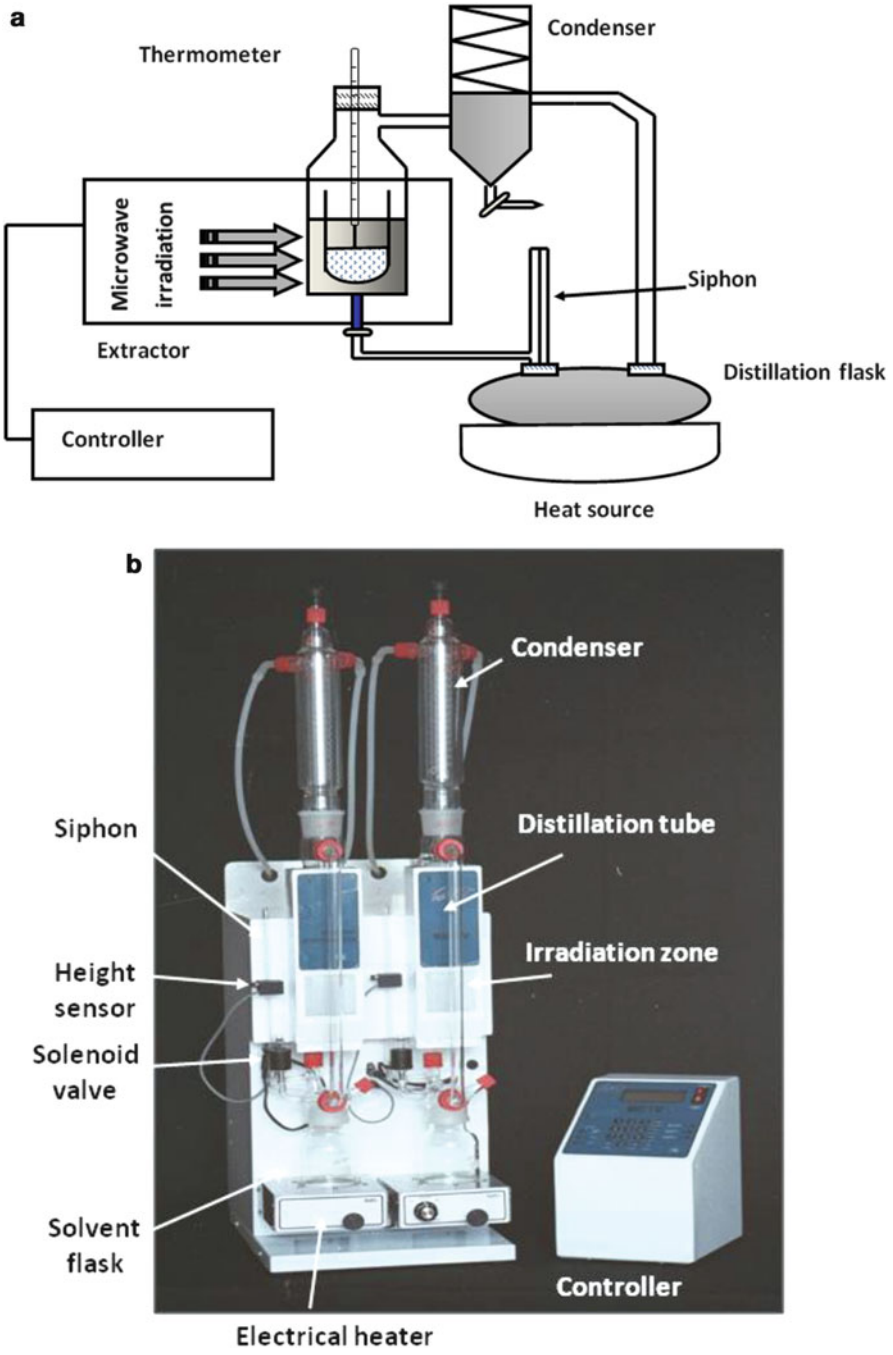


**Fig. 4.1** (**a**) The first focused microwave-assisted Soxhlet extractor (FMASE) prototype. (Reproduced with permission of Elsevier. From Luque de Castro and Priego-Capote [36]). (**b**) Automatic FMASE. (Reproduced with permission of Elsevier. From Luque de Castro and Priego-Capote [36])

through a siphon (Fig. 4.1a). This adaptation allows the cartridge compartment of a conventional Soxhlet unit to be accommodated in the irradiation zone of the microwave oven. Operationally, the extractor is identical with a conventional Soxhlet apparatus except that it affords irradiation with focused microwaves for a preset time during each extraction cycle while fresh extractant (condensed vapors from the distillation flask) is dropped on and percolated through the solid sample. In this way, breaking of the analyte–matrix bonds is facilitated by application of suitable energy. A Prolab Megal 500 thermometer is used to monitor the extraction temperature. Also, a controller is used for the microwave unit and thermometer, and an electrical isomantle furnished with a rheostat is used as a heating source for the distillation flask. The operational variables amenable to optimization in the FMASE prototype are the irradiation power, irradiation time, and number of cycles.

This device retains the advantages of conventional Soxhlet extraction while overcoming restrictions such as its long extraction times, nonquantitative extraction of strongly retained analytes, which is enabled by easier cleavage of analyte–matrix bonds by effect of interactions with focused microwave energy, difficulty of automation, which is relieved by replacing glassware with pumps, and the large volumes of organic solvent that are wasted. Differing from a conventional Soxhlet extractor, the microwave-assisted Soxhlet system allows up to 75–85% of the total extractant volume to be recycled by evaporation–collection of most of the extractant volume. Electrical heating of the extractant, the efficiency of which is independent of its polarity, is also crucial here. Moreover, the efficiency is unaffected by the moisture content of the sample.

This prototype, which is especially flexible, has been used in a number of modifications or combinations including a reverse configuration [38], its coupling for extraction monitoring [39], and an automatic configuration [40].

A second prototype was developed to facilitate the use of high-boiling extractants for "green" applications based on the use of water as extractant [41], the glassware being replaced with piston pumps and Teflon tubing. A third prototype afforded the simultaneous processing of two samples for replicate extraction and automation with the aid of an optical sensor, a solenoid valve, and control via microprocessor software [42] (Fig. 4.1b).

Recently, a new, more compact prototype called Accesox (Barcelona, Spain) was developed to reach a wider market. This device has the additional choice of the maximum temperature to be reached in the sample–extractant medium during microwave irradiation. In this way, the temperature of the leaching process can be effectively controlled, which is especially desirable with thermolabile compounds.

The Chemat team developed a commercial microwave-assisted extractor that was deemed similar to a Soxhlet extractor—in fact, they call it microwave integrated Soxhlet (MIS)—but which in fact differs markedly from the classic extractor in operational terms [43]. Thus, as can be seen in Fig. 4.2, the sample is never brought into contact with fresh extractant and the extract is not siphoned; also, the extractant is heated by microwaves (similarly to the Soxwave-100) and a filtration step is required because the sample is not held in a cartridge, but rather dispersed in the extractant. Low-polar and nonpolar extractants are heated to their boiling points

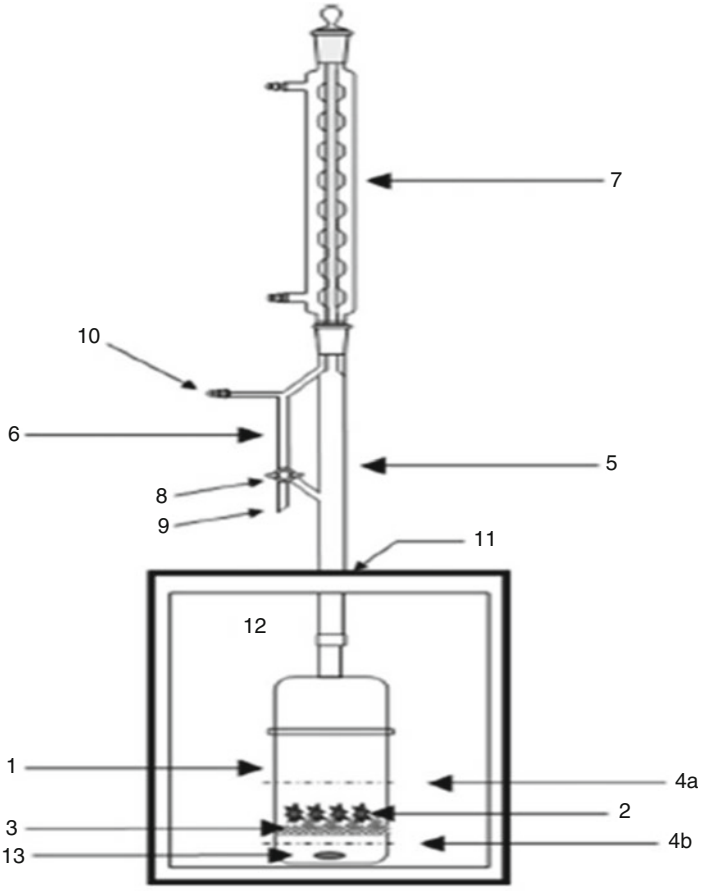


**Fig. 4.2** A microwave integrated Soxhlet (MIS) extractor. *1* flask, *2* solid sample, *3* inner support, *4a* solvent (in a quantity sufficient to immerse the solid material), *4b* the level of the solvent is lowered below the sample, *5* extraction tube, *6* side arm, *7* condenser, *8* valve, *9* and *10* outlets, *11* upper surface, *12* microwave oven, *13* magnetic stirrer. (Reproduced with permission of Elsevier. From Virot et al. [43])

by microwaves while stirring with a Weflon magnetic bar to absorb microwave radiation. In this way, solvent vapors penetrate through the sample and are liquefied on arrival at the condenser. Then, the condensate is dropped down onto the sample by switching a three-way valve. As can be seen, this operation does not rely on the Soxhlet principle, which exploits contact between the sample and fresh extractant in each leaching cycle; therefore, displacement of the partitioning equilibrium to complete extraction is impossible. Extraction must inevitably be followed by filtration to separate the remaining solid matrix from the extract. Despite its name, the device does not integrate Soxhlet extraction and microwaves.

Other characteristics of MIS are as follows: it uses a Milestone ETHOS multimode microwave oven with a twin magnetron (2×800 W, 2.45 GHz) delivering a

maximum power of 1,000 W in 10 W increments. Time, temperature, pressure, and power are controlled with the software "easy WAVE". The flask holding the solid material (see (1) in Fig. 4.2) is suitable for microwave radiation and contains a polytetrafluoroethylene/graphite stir bar capable of absorbing microwaves and diffusing the heat to the surroundings; this is essential with solvent-transparent microwave radiation. The vessel contains an inner support (3) for placing the solid material (2) to be extracted. The support is preferentially made of polytetrafluoroethylene (PTFE) and placed at a preset distance from the vessel bottom. After the method has been applied, the solid material (2) placed on the support is separated from residual solvent, which is collected at the bottom of the vessel. A condenser (7) is placed on top of the extraction tube (5), in which switching valves allow the solvent present in the base vessel to reflux upon microwave irradiation and either repeatedly percolate the sample to ensure thorough extraction or be removed from the extractor to concentrate the extract. The solid material is extracted by immersing the sample into the vessel containing the solvent under reflux and repeated percolation with the same organic solvent. The four stages of the process are preceded by placement of the sample onto a preset amount of raw material for lipid extraction and the addition of extractant (usually *n*-hexane), into which the sample is immersed, the condenser being placed on the extraction tube and extraction started. First, the solvent is heated up to its boiling point by microwave irradiation and stirred with a Weflon magnetic stirrer (13). The solvent vapors penetrate through the sample and condense on the condenser. Then, the condensate drips down onto the sample and extraction proceeds for a preset time. Second, the level of solvent is lowered below the sample (4b) by switching the three-way valve accordingly for a given time. Third, repeated leaching with only clean, fresh solvent follows for a preset time with the valve adjusted such that the condensate is driven back into the extraction tube. Finally, the solvent level is lowered to concentrate the extract.

The main difference between these laboratory-designed extractors, which are more or less similar to a conventional Soxhlet extractor, and the commercial devices from CEM or Milestone is that the former use open vessels. As a result, the maximum temperature reached in the extraction vessel in these open systems is strongly dependent on the boiling point of the extractant. Also, extraction takes more time than in closed vessels, which, however, require waiting for the vessel to cool before it can be opened. Finally, the open-vessel systems are better suited to thermolabile lipids.

Household MW ovens operating at fixed frequency of 2.45 GHz and delivering a power of 700–1,200 W have been used for leaching in the analytical laboratory from the start [44] and are still in use [45] even though they afford little or no control of their performance via extraction-related variables.

### *4.2.2 Pilot-Plant and Industrial MAE Equipment*

Developments in MAE equipment for industrial lipid extraction have and continue to follow a rather different path from those in MAE laboratory equipment for the

same purpose. Although most MAE pilot plants are documented to a greater or lesser extent, the design of industrial-scale plants usually remains secret.

MAE pilot plants usually require using a microwave frequency of 915 MHz instead of 2,450 MHz. Microwaves at 915 MHz, which are the choice for industrial work, have a much greater penetration ability than microwaves at 2,450 MHz, which are more commonplace at the laboratory scale.

MAE pilot plants can operate in a continuous or discrete mode. The latter uses a tank of appropriate dimensions and material that is irradiated with MWs at an appropriate power for a preset, previously optimized time. In continuous flow microwave-assisted extraction (CMAE), the solid raw material and the extractant are continuously pumped and heated in a microwave cavity. This process is made more complex by the addition of momentum transfer to the heat generation from microwave heat transfer in the extractant–solid matrix and mass transfer through the solid–extractant [46]. The increased penetration ability of 915 MHz MWs used in CMAE affords much larger tube diameters and processing flow rates, as well as the use of microwave generators with significantly increased power and efficiency. By contrast, a single-mode MW device, and also a multimode applicator such as those commonly used in household MW ovens, focuses microwaves on the center of the applicator, to which the material is driven via a processing tube. This resonance mode allows for very high electric field values, which increase the heating rate; also, focused MWs create an electrical field distribution where the highest values are in the centre of the applicator tube and decrease toward the tube walls. Therefore, provided the flow in the tube is laminar, the fluid with highest velocity, in the center, will receive the greatest amount of microwave energy. On the other hand, the fluid with the lowest velocity, near the wall, will receive smaller amounts of energy and exhibit a more uniform temperature distribution when exiting the microwave applicator [47]. Although this difference in electric field distribution may not play a prominent role with tubes of a small diameter, temperature uniformity is important when scaling-up to higher flow rates, which requires larger tube diameters.

Developments in pilot plants for lipid extraction have resulted from work at three different levels, namely (1) research in technological laboratories, whose designs are severely limited by budget availability; (2) patented designs, which are very scant; and (3) the development of dedicated pilot-plant or industrial setups designed according to the client's specific needs (a) or tailor-made methods for previously deployed pilot plants (b).

Investigations at the Technological Institute of Durango (Mexico) [48], and at the Agricultural and Mechanical College of Louisiana State University [49], illustrate the results of research in technological laboratories. Figure 4.3a shows a design for the extraction of oil from oregano. An Erlenmeyer flask holding a powdered oregano sample is placed on the rotary platform of a household MW oven and heated by effect of MW irradiation to remove the volatile oil, which is then cooled in a refrigerant and dropped into a collector vessel. No information is available as to the type, power, and specifications of the MW device used. Figure 4.3b shows a pilot-scale continuous MAE system consisting of a feed tank furnished with an industrial stirrer, a Seepex progressive cavity pump coupled with an 1-HP motor

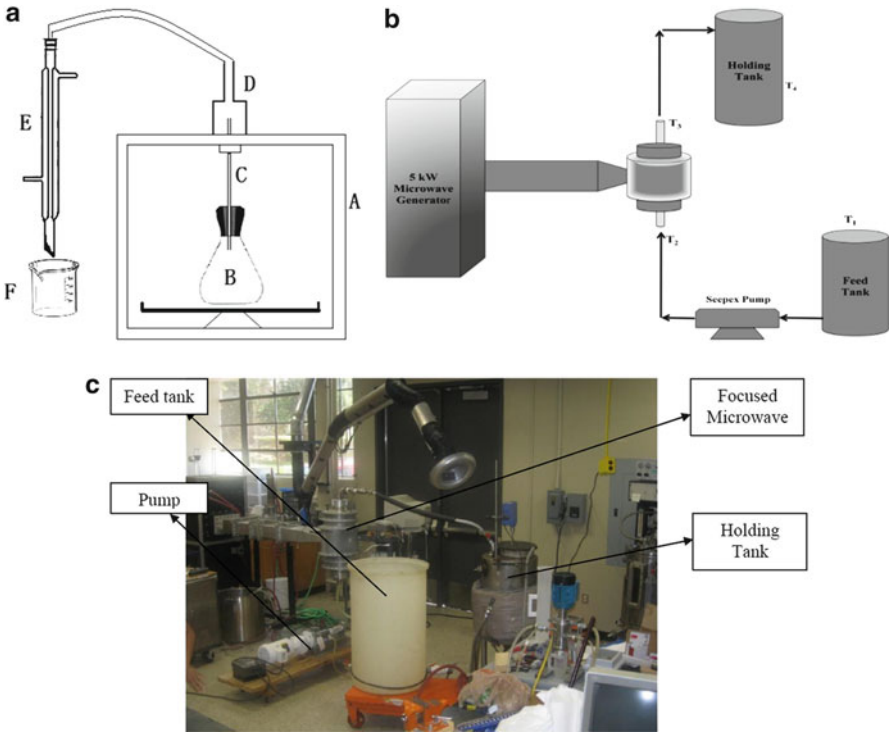


**Fig. 4.3** (**a**) Design for extraction of oil from oregano. (From Contreras Flores [48]). (**b**) *1* Schematic design of pilot-scale continuous microwave-assisted extraction, *2* pilot-scale continuous microwave-assisted extraction. (From Terigar [49])

and controlled by an AC Tech MC Series controller to provide flow rates of process mixture from 0.6 to 6.0 l/min, a 5 kW, 915 MHz continuous microwave system (Industrial Microwave Systems, Morrisville, NC, USA), and an insulated stainless steel holding tank with sampling ports. A scheme and a photograph of the overall design are shown in parts 1 and 2, respectively, of Fig. 4.3b. The literature on pilot plants focusing on other aspects of potential industrial development lacks information about the plants themselves or the types of MW devices used to facilitate extraction (e.g., Ching-Lung et al. [50] simply stated that "The maximum power of the industrial microwave oven employed in this study was 1,600 W").

Patents on pilot plants for lipid extraction are concerned mainly with the extraction of essential oils (the subject matter of Chap. 3), so only one example is discussed here. Figure 4.4 depicts the pilot plant developed by Paré for the continuous extraction of volatile oils from plant materials [51]. The overall system consists of two reservoirs (for extractant and clean solution), two inlets to the extraction chamber (one for the raw material and the other for the extractant), a valve to regulate extractant input, a stirrer to ensure proper contact between the extractant and raw

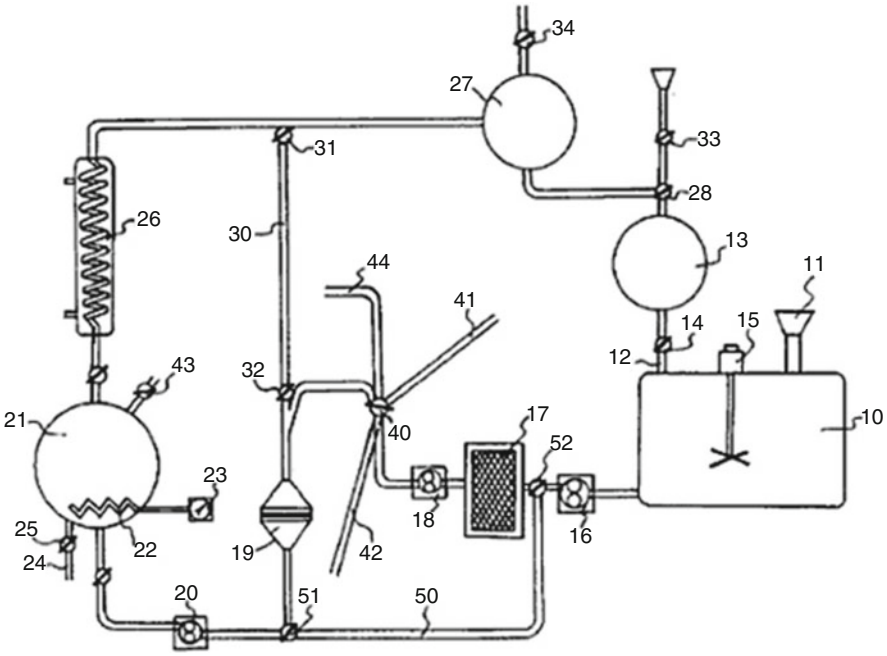


**Fig. 4.4** Scheme of the pilot plant reported by J. Paré for continuous extraction of volatile oils from vegetal materials. (From Paré [51])

material, an MW applicator (200–10,000 W, 2,000–30,000 MHz), a pump to feed the filter with the solid–liquid medium after MW irradiation, and a filtration system. Several filters can be arranged in parallel to facilitate uninterrupted removal of solids. An additional feeding pump, valves, and tubing are used to recycle the extract for increased enrichment; a condenser and a reservoir to recover the extractant; and an additional reservoir to collect the extract at the required preconcentration level.

The commercial world of pilot-scale and industrial plants revolves largely around the models from Radient Technologies and AIREM. The former is a prototype from a manufacturer meeting clients' demands at the pilot-plant or industrial scale. As heirs of the technology developed by Paré's team through MAP patents [52], Radient Technologies defines its products as "exclusive worldwide licenses to technology invented by Environment Canada, began in Ontario, moving into Alberta." This firm started business through contract manufacturing but later focused on MW-assisted extraction of added-value ingredients from natural biomass supplemented with downstream purification steps.

Radient Technologies offers manufacturing contracts on behalf of third-party clients by using a plant with a capacity of 0.8 tonnes input per day (or a plant of input 5 tonnes per day). In addition, Radient Technologies licenses or transfers MAP technology, but does not manufacture or sell microwave equipment. Apart from

this, the firm is open to clients' demands such as (a) working with reliable suppliers of intermediate support for preclinical studies and clinical trials; (b) maximizing yields in highly valuable compounds from expensive raw materials; (c) maximizing the purity of crude extracts; and (d) developing efficient purification/isolation procedures. The firm is also open to the development of efficient and scalable processes affording (a) efficient recovery of active compounds (65–95% greater than with conventional methods); (b) reduced extraction times (from 8 h to 30 min); (c) average crude extract concentrations of 8–13%; (d) the replacement of toxic extractants with environmentally benign, green substitutes; (e) solvent ratios from 10:1 to 4:1 (and reduced solvent and energy costs as a result); and (f) simplified purification and isolation affording greater protection to operators.

SAIREM (Lyon, France) is a typical example of a firm designing and constructing tailor-made pilot plants according to clients' specifications. In collaboration with the École Nationale de Chimie in Montpellier, SAIREM has installed a platform (unique in Europe, SAIREM claims) for experimentation at the pilot-plant or semi-industrial scale that can provide customized solutions for continuous flow or batch work using frequencies tailored to the thickness and other characteristics of the products to be processed. SAIREM also offers synergistic association of other energies such as infrared, forced air, or steam cooking to maximize treatment efficiency. This firm has developed a comprehensive range of standard machines to meet their clients' special needs, and claims that its offer does not end with the delivery of turnkey-ready equipment, but rather includes counseling its clients toward continually improving production quality, as well as providing system maintenance throughout the world.

SAIREM explains, albeit without detailed schemes because it uses tailor-made approaches, the main parameters of their pilot-scale installation in Montpellier: an installed maximum microwave power of 30 kW at 915 MHz; fast control of forward and reflected power; high attainable microwave power densities; integration between microwave generator and reactor to ensure internal compatibility and control of all system components; a continuous reactor operating at flow rates up to 5 l/min; a batch reactor of up to 60 l with variable speed mechanical stirring specially suited to plant-type extraction in water or various solvents; recirculation of the extracted product back into the reaction mixture; processing under an inert atmosphere ($N_2$, Ar, $CO_2$,); the possibility of online filtration or distillation of the products; cooling of the reactor via an external jacket with automated temperature control; in situ temperature measurements; and a range of interlocks for safe, reliable operation. Figure 4.3b (1 and 2) is the only scheme of SAIREM pilot plants available on their web page and is intended to represent their offerings.

SAIREM uses what it calls INTLI technology to develop its LABOTRON modular systems for the extraction of delicate biological or plant-specific molecules (lipids included). According to SAIREM, INTLI technology provides the following advantages: (a) a high density of radiated power (up to several kilowatts per liter) inside the biomass; (b) microwave energy can be selectively concentrated inside the biomass by using a relatively MW-transparent extractant; (c) extraction can also be carried out in aqueous media; (d) external cooling via a metal jacket can maintain

the biomass and extraction products at very low temperatures to avoid degradation of heat-sensitive products; (e) the ability to operate in the continuous flow or batch mode; (f) that to easily incorporate various types of reactors, whether standard or custom made, and with or without mechanical stirring, into the same microwave head; (g) a PLC-based controller and a touch-screen user interface; (h) all system functions and status, including recipe changes, alarms, and chemical levels are accessible from the touch-screen display; (i) online reagent addition and product removal and sampling; and (j) a mobile platform for quick installation and positioning. For higher production or testing capacities, SAIREM also offers the possibility of using their pilot-scale test facility with microwave power levels up to 100 kW, 915 MHz, and a throughput up to 700 l/h.

## 4.3 Aims of Laboratory MAE and Industrial MAE of Fats and Oils

Because laboratory-scale and industrial MAE differ markedly in their aims, the two are discussed separately here.

### *4.3.1 Aims of Laboratory-Scale MAE*

Laboratory-scale MAE methods are usually developed with the aim of (a) improving previous extraction methods, whether conventional or using auxiliary energies such as ultrasound or a high pressure–high temperature combination; (b) extracting lipids from new raw materials, or a specific class, category, or family of lipids; or (c) preliminary optimization of an extraction system to be implemented at the industrial scale. The first two aims are usually fulfilled by using similar criteria, but the third requires considering the specific target industrial application.

MAE for analytical purposes is used to ensure exhaustive extraction without degradation of the given lipids with a view to determining their total content in the sample. This intent entails using multivariate optimization designs and appropriate tests to compare the results of each new method with those of a standard or official method for the same analyte–matrix couple [53]. The extractant cost and volume, the amount of energy used, and the efficiency with which the extractant and energy can be recovered are usually regarded as secondary or unimportant when chemical “greenness” is the main goal. Using a nonpolar extractant is no longer mandatory as a result of the availability of fluoropolymers with a high microwave-absorption ability.

When the final aim is to obtain a “rough sketch” of the industrial utility of MAE, opting for focused or multimode MW irradiation is a crucial decision because an industrial plant based on a multimode MW system is usually less expensive to construct than one using a monomode system. The nature, cost,

toxicity, and polarity of the extractant, and the amount used, are also highly influential. Interestingly, *n*-hexane has traditionally been the extractant of choice for edible (refined) oils despite its toxic nature because its low boiling point makes their removal relatively easy and complete. The potential use of heat exchangers and ways to recover the energy used in the process so far as possible should always be considered in developing a laboratory-scale MAE method to be subsequently implemented at the industrial scale.

### *4.3.2 Aims of Industrial MAE*

The industrial production of oils from olives and various seeds (sunflower, soybean) has traditionally relied on extraction with organic solvents to obtain refined oils. As already stated, hexane, by virtue of its low boiling point, is the solvent of choice for this purpose. The solid performance of existing methods, however, is hindering changes in their implementation.

The industrial uses of MAE for oil production are limited to new applications such as the extraction of essential oils (see Chap. 3) or, in general, lipids with a high added value by the cosmetic and pharmaceutical industries. A number of firms present on the Internet provide services such as the development of MW-assisted pilot plants or optimization of pilot-plant conditions for improved industrial extraction processes.

Among other factors, optimizing one of these MW-assisted plants requires the following: (a) using the more economical MW mode (usually multimode MW); (b) making an acceptable compromise in selecting the extractant by choosing one polar enough for efficient exploitation of MW benefits but capable of extracting the usually low-polar compounds present in the raw material with the assistance of a high enough MW power (water continues to be the most frequent choice despite its high polarity and dielectric constant); (c) avoiding toxic extractants as far as economically feasible; (d) balancing the extraction efficiency (e.g., dispensing with thorough extraction if the cost of the last portions surpasses the benefits of their extraction); and (e) recovery of the energy expended, usually by using heat exchangers.

## 4.4 Microwave-Assisted Extraction of Fats and Oils Versus Other Types of Accelerated Extraction and Conventional Extraction

MAE users have frequently compared the performance of their methods with that of methods using other types of auxiliary energy and, obviously, with conventional extraction methods.

### 4.4.1 MAE Versus Ultrasound-Assisted Extraction (USAE)

The enthusiasm aroused by MAE in some users has led them to compare its performance with that of USAE under dissimilar conditions. In most cases, a commercial MAE system specially designed for this purpose and equipped with a variety of devices for enhanced performance was measured against a low-price ultrasonic bath designed for cleaning and degassing, which usually exhibits power decay with time and heterogeneous distribution of ultrasonic energy [54]. The results thus obtained are therefore unreliable toward comparing the potential of the two types of energy for a given application. Tests with both types in appropriate systems have shown them to provide similar results [55, 56] or even revealed that ultrasound energy may be more convenient (especially with thermolabile, hard-to-oxidize compounds) [57–59]. Therefore, USAE and MAE have specific areas of application that can coincide in some cases. MAE is invariably the better choice when an increase in temperature favors extraction of thermostable analytes.

Similar to ultrasound, microwaves can favor emulsification of two immiscible extractants for the simultaneous extraction of polar and nonpolar compounds [60]. However, ultrasound causes emulsification via cavitation [61], whereas microwaves emulsify by boiling one of the extractants, that with the lower boiling point, thus producing effective emulsification by which the combination of a high contact surface area and an also high temperature greatly facilitate mass transfer from the solid to each of the liquid phases as a function of analyte affinity [60].

The combined use of ultrasound and microwaves for increased acceleration of extraction is known as UMAE. Research in this direction with laboratory-made devices [62] led to the development of a patented commercial device developed in the framework of the EU's COST Program [63]. The extractor consists of a microwave generator, a sonic probe connected to an ultrasound generator, and a chemically inert, MW-transparent vessel for placement in the microwave cavity. The sonic probe contacts the sample directly and the microwave generator is spaced away from the sample container. This device has been used to develop fast, efficient extraction methods for a variety of active compounds such as lycopene from tomatoes [64] and vegetable oils [65] and polysaccharides [66] from various plants. The UMAE extraction time for lycopene from tomatoes was 6 min (97.4% yield) as compared to 29 min (89.4% yield) with ultrasonic-assisted extraction. Chen et al. [66] compared UMAE and the conventional methods for the extraction of polysaccharides in *Inonotus obliquus*. Under optimal conditions, UMAE increased the extraction yield from 2.12% to 3.25%, and the extract purity from 73.16% to 64.03%, relative to a previously reported method based on traditional hot-water extraction.

### 4.4.2 MAE Versus Superheated Liquid Extraction (SHLE)

Virtually during the past two decades, SHLE, which uses a solvent at a high pressure and/or temperature below its critical point, has emerged as an efficient means

for increasing automatability, shortening process times, and reducing the amounts of solvent required to digest or leach solid samples.

There has been much debate over the name this technique should be given ever since its inception. Dionex Corporation initially patented it under the designation accelerated solvent extraction (ASE) [67], which was also applied to its commercial devices. The virtually exclusive use of this term in the earliest years was largely because the sole commercially available extractor for this purpose was manufactured by Dionex. At about the same time, Hawthorne, who was using water at high pressure and temperature as extractant, named the process subcritical water extraction [68]. With time, however, alternative names such as pressurized fluid extraction (PFE), pressurized hot solvent extraction (PHSE), high-pressure solvent extraction (HPSE), subcritical solvent extraction (SSE), superheated solvent extraction (SHSE), and superheated liquid extraction (SHLE) have gradually replaced ASE, a commercial designation that bears no relationship to the actual foundation of the technique. In the authors' opinion, high pressure is not its most salient feature because most often the only purpose of raising the pressure is to keep the extractant liquid and only in a very few cases has an increase in pressure above this level had any effects. On the other hand, the term subcritical solvent encompasses any temperature and pressure below the critical point, even at ambient conditions, and is therefore inappropriate as well. For these reasons, the authors suggest the generic name "superheated liquid extraction," which can be accomplished with an aqueous or organic solvent under static conditions, under dynamic conditions, that is, by continuously circulating the solvent through the sample, or a combination thereof [69].

Comparisons have frequently revolved around commercial extractors based on different principles such as those behind SHLE and MAE, which have led to some general conclusions, namely (a) the more drastic working conditions used in SHLE lead to dirtier extracts that make chromatograms more difficult to interpret and result in higher limits of detection and quantitation for the target analytes; (b) SHL extracts can be made cleaner by purification for a longer time, but this can make analyses more expensive and time consuming; (c) one potential hindrance to MAE is the need for additional handling to separate the extract from the solid sample matrix after extraction; and (d) wet samples can be simultaneously extracted in up to 40 vessels with MAE, but require the addition of anhydrous sodium sulfate with SLHE.

Comparisons of SLHE with MAE have involved mainly toxic compounds that are difficult to extract from a variety of different matrices including food (e.g., the extraction of PCBs from various types of foods [70]). A comparison of five different extraction methods based on liquid–liquid extraction (LLE), microwave-assisted extraction (MAE), focused microwave-assisted extraction (FME), solid-phase extraction (SPE), and superheated liquid extraction (SHLE) revealed that FME provided the best results for the extraction of polychlorinated biphenyls (PCBs), polybrominated diethyl ethers (PBDEs), organochlorine pesticides, and lipids in serum [71]

### 4.4.3 MAE Versus Conventional Extraction

Each new MAE method is invariably compared in performance with its conventional extraction counterpart (usually a reference or standard method).

Fat, expressed as the combination of fatty acids and expressed as triglycerides or the content in each lipid, is one of the major components of food. Fat is frequently determined in food analysis laboratories and has traditionally been isolated by Soxhlet extraction, which is the basis for a number of official methods despite its slowness.

FMASE has been widely used to develop methods for lipid extraction with excellent performance in the removal of fat from various matrices such as olive drupes [72] and oily seeds (sunflower, rape, soybean) [73], cheese [74], milk [75], fried and pre-fried foods [76], and sausage products [10]. In all instances, FMASE was much more expeditious than the corresponding reference methods for fat extraction. For example, the extraction of fat from fried and pre-fried foods takes 55 min with FMASE and 8 h with the reference method. Also, FMASE reduces the time needed to extract fat from seeds from 8 h to 20–25 min, and the procedure is less labor intensive than the official method, which, for example, requires halting the process twice to grind the sample [73]. With cheese, FMASE reduces extraction times from 6 h to 40 min; with milk [75], from 10 h to 50 min. In addition, FMASE dramatically expedites the prehydrolysis step required for digestion of dairy products (from 1 h to 10 min) and avoids the need for subsequent neutralization. Moreover, FMASE provides cleaner extracts, possibly as a result of its shorter operational times. For example, milk fat extracted by FMASE exhibits less marked chemical transformation of triglycerides during extraction [75]. The increased extraction efficiency of FMASE has been confirmed by some kinetics studies. Figure 4.5a illustrates the kinetics of extraction of fat from two bakery products by using FMASE, with and without microwave assistance (solid and dashed lines, respectively) [77]. As can be seen, seven cycles were required for complete fat isolation from a snack sample by FMASE. In the absence of microwaves, fat was extracted by 8.21% from snacks versus 26.22% with FMASE (data obtained by gravimetry). Therefore, microwave irradiation under optimal conditions had a substantial influence on the outcome. Figure 4.5b further illustrates the kinetics of conventional Soxhlet extraction with the isolation of fat from the same type of sample. The kinetic study was conducted over periods of 4 and 20 h to determine the time needed for complete extraction. Clearly, the extraction time was much longer in the absence of microwaves than in their presence, even though the duration of each FMASE cycle was identical with that of a conventional Soxhlet cycle. The most salient result was that the time required for complete fat isolation from snack samples was only 35 min with FMASE and 8 h with Soxhlet extraction [77], thus confirming the boosting effect of microwaves the extraction efficiency.

The same method used with the bakery products was used to determine *trans* fatty acids as a contribution to the initiative of adding *trans* fat content to the label of processed foods, either by legislation or as a quality index to be implemented in the near

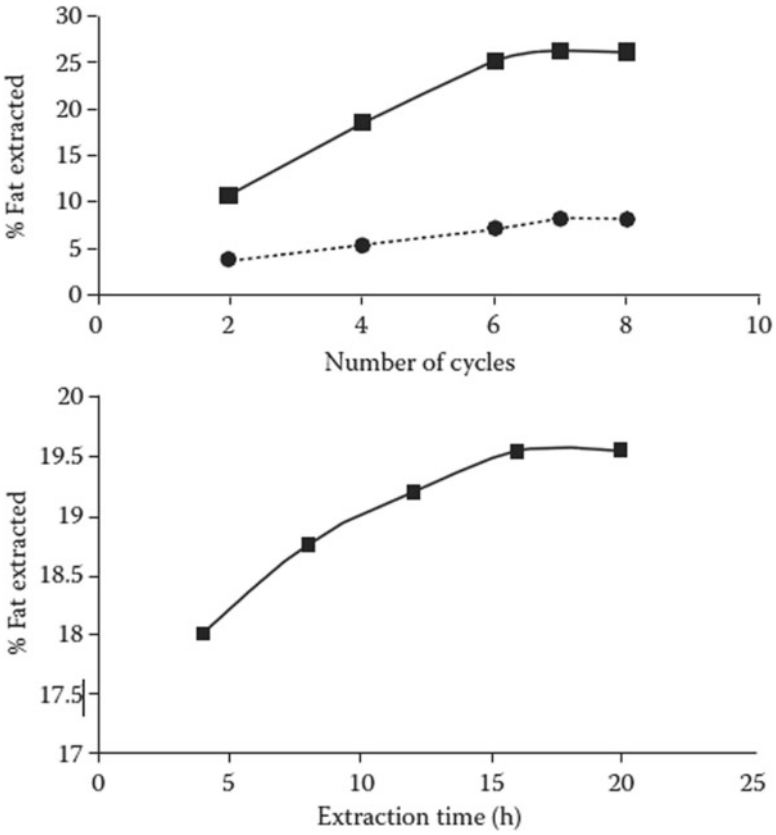


**Fig. 4.5** (**a**) Extraction kinetics of fat from bakery products as performed by FMASE (*solid line*) and without microwave assistance (*dashed line*). (Reproduced with permission of Elsevier. From Priego-Capote and Luque de Castro [77]). (**b**) Extraction kinetics of fat from bakery products as performed with the conventional Soxhlet technique. (Reproduced with permission of Elsevier. From Priego-Capote and Luque de Castro [77])

future. FMASE extracts can be used to quantify the content in *trans* fatty acids by middle infrared spectrometry (MIR) [13] and the profiles of individual compounds by GC–MS [16]. The FMASE results were compared with those provided by the Folch reference extraction method, which uses mild temperatures. Both extraction methods provided similar results in terms of total content in *trans* isomers and also of individual isomer concentrations. Therefore, a method based on FMASE sample preparation and subsequent MIR or GC–MS analysis has the potential to become an effective, expeditious alternative to the Folch method for routine analyses.

The advantages of MAE over Bligh and Dyer extraction of lipids from fish were demonstrated by Batista et al. [78] using a Prolabo Soxtec-100 system [78] (see Table 4.1). Both methods exhibited good reproducibility, but MAE used less material

**Table 4.1** Relative existence of fatty acids in comparison to palmitic acid (C16:0, peak height = 100) in cod liver and mackerel after microwave-assisted extraction (MAE) and Bligh and Dyer extraction 73

| | | | Cod liver | | Mackerel | |
|---|---|---|---|---|---|---|
| Fatty acid | Formula | $t_R$ (min)[a] | MAE ($n = 15$) | Bligh and Dyer ($n = 15$) | MAE (n = 15) | Bligh and Dyer (n = 9) |
| Lauric | C12:0 | 7.71 | 0.5±0.1 | 0.5±0.1 | 0.5±0.1 | 0.6±0.05 |
| U1 | ? | 7.83 | 12.6±1.6 | 9.8±1.4 | 9.9±2.3 | 9.1±1.1 |
| Tridecanoic | C13:0 | 9.12 | Qualitatively detected | | 0.3±0.06 | 0.3±0.2 |
| U2 | ? | 9.53 | 3.1±0.6 | 2.9±0.4 | Qualitatively detected | |
| Myristic | C14:0 | 10.86 | 23.0±0.8 | 22.5±0.7 | 42.9±2.4 | 42.0±2.7 |
| U3 | ? | 10.93 | 1.8±0.5 | 2.0±0.2 | 2.0±0.2 | 2.0±0.07 |
| U4 | ? | 11.36 | 0.7±0.1 | 0.8±0.1 | 0.3±0.2 | 0.4±0.04 |
| Myristoleic | C14:1n-5[b] | 11.64 | 0.6±0.1 | 0.06±0.1 | 0.3±0.06 | 0.4±0.1 |
| U5 | ? | 11.94 | 1.9±0.1 | 2.0±0.2 | 2.0±0.2 | 2.0±0.1 |
| Pentadecanoic | C15:0 | 13.21 | 4.4±0.1 | 4.3±0.1 | 3.4±0.1 | 3.3±0.1 |
| Palmitic | C16:0 | 16.38 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
| U6 | ? | 16.87 | 4.2±0.9 | 4.0±0.1 | 2.4±0.1 | 2.6±0.2 |
| Palmitoleic | C16:1n-7 | 17.21 | 85.8±0.4 | 87.1±2.1 | 26.1±0.2 | 25.0±0.2 |
| U7 | ? | 17.46 | 4.9±0.4 | 5.0±0.1 | 2.4±0.1 | 2.9±0.4 |
| U8 | ? | 17.82 | 3.8±0.3 | 3.9±0.1 | 1.5±0.2 | 1.2±0.2 |
| U9 | ? | 18.30 | 2.7±0.2 | 2.8±0.3 | 0.8±0.05 | 0.8±0.5 |
| | C16:2n-4 | 18.91 | 3.9±0.3 | 3.8±0.1 | 1.5±0.3 | 1.5±0.3 |
| U10 | ? | 19.02 | 3.2±0.3 | 3.3±0.1 | 4.3±0.5 | 3.4±0.3 |
| U11 | ? | 19.14 | 10.4±3.3 | 12.8±1.7 | Qualitatively detected | |
| Heptadecanoic | C17:0 | 19.38 | 4.8±0.2 | 4.9±0.1 | 3.2±1.6 | 2.7±0.2 |
| | C16:3n-4 | 19.97 | 4.3±0.3 | 4.2±0.2 | 1.6±0.9 | Qualitatively detected |
| U12 | ? | 20.05 | 3.1±0.3 | 3.5±0.2 | 2.1±0.7 | 2.8±0.1 |
| U13 | ? | 20.87 | 1.3±0.1 | 1.3±0.1 | 2.0±0.4 | 1.8±0.09 |
| U14 | ? | 21.74 | 2.0±0.2 | 1.9±0.1 | 1.3±0.3 | 1.1±0.3 |

(continued)

**Table 4.1** (continued)

| | | | Cod liver | | Mackerel | |
|---|---|---|---|---|---|---|
| Fatty acid | Formula | $t_R$ (min)[a] | MAE ($n=15$) | Bligh and Dyer ($n=15$) | MAE (n=15) | Bligh and Dyer (n=9) |
| Stearic | C18:0 | 22.55 | 26.6±0.7 | 27.3±0.9 | 16.5±0.8 | 16.3±2.0 |
| U15 | ? | 22.85 | 4.1±0.3 | 4.2±0.2 | Not detected | Not detected |
| Oleic | C18:1n-9[d] | 23.27 | 117.5±3.0 | 124.0±3.9 | 57.1±2.4 | 56.1±1.4 |
| Vaccenic | C18:1n-7 | 23.45 | 42.1±1.1 | 44.3±1.6 | 12.1±0.5 | 11.7±0.8 |
| U16 | ? | 23.72 | 5.0±0.4 | 5.0±0.2 | 2.9±0.4 | 3.0±0.2 |
| Linoleic | C18:2n-6 | 24.56 | 10.8±0.3 | 11.1±0.4 | 13.8±0.6 | 14.6±2.5 |
| U17 | ? | 25.13 | 2.2±0.1 | 2.2±0.1 | 0.6±0.1 | 0.5±0.07 |
| γ-Linolenic | C18:3n-6 | 25.45 | 1.4±0.1 | 1.3±0.1 | 1.0±0.4 | 1.5±0.7 |
| | C18:3n-4 | 26.04 | 1.9±0.2 | 1.9±0.1 | 0.3±0.2 | 0.3±0.08 |
| α-Linolenic | C18:3n-3 | 26.51 | 6.1±0.2 | 6.2±0.3 | 8.6±0.4 | 9.4±0.6 |
| Stearidonic | C18:4n-3 | 27.47 | 13.4±0.4 | 13.5±0.9 | 19.0±1.2 | 17.4±2.0 |
| Arachidic | C20:0 | 28.45 | 0.5±0.2 | 0.6±0.1 | 1.0±0.1 | 0.9±0.1 |
| U18 | ? | 28.92 | 14.1±0.6 | 15.4±1.1 | Not detected | Not detected |
| Eicosenoic | C20:1n-9 | 29.04 | 6.9±0.3 | 7.8±1.8 | 65.6±4.2 | 69.2±7.7 |
| U19 | ? | 29.31 | 19.1±0.8 | 20.6±1.3 | 1.5±0.2 | 1.2±0.2 |
| U20 | ? | 29.47 | 1.6±0.2 | 1.7±0.1 | 0.2±0.1 | 0.2±0.04 |
| U21 | ? | 29.62 | 1.4±0.2 | 1.5±0.1 | 0.5±0.2 | 0.5±0.1 |
| Eicosadienoic | C20:2n-6 | 30.41 | 4.1±0.2 | 4.4±0.3 | 1.8±0.2 | 1.8±0.1 |
| C-8, 11, 14-Eicosetrienoic | C20:3n-6 | 31.15 | 0.4±0.1 | 0.5±0.0 | 0.4±0.2 | 0.4±0.05 |
| Arachidonic | C20:4n-6 | 31.85 | 8.6±0.3 | 9.0±0.5 | 2.3±0.2 | 2.5±0.4 |
| Eicosatrienoic | C20:3n-3 | 32.27 | 1.8±0.1 | 1.8±0.2 | 0.9±0.1 | 1.0±0.1 |
| C-8, 11, 14, 17-Eicosetetrean | C20:4n-3 | 33.00 | 3.0±0.4 | 2.6±0.2 | 4.4±1.0 | 4.3±0.3 |
| Eicoasapentaenoic | C20:5n-3 | 33.76 | 67.5±3.1 | 69.8±3.1 | 21.7±2.1 | 21.4±4.1 |
| Behenic | C22:0 | 33.96 | 0.3±0.1 | 0.3±0.1 | 0.3±0.1 | 0.4±0.07 |
| C-9 docoseanoic | C22:1n-11 | 34.43 | 1.3±0.3 | 1.3±0.2 | 97.4±7.9 | 93.8±7.5 |
| Erucic | C22:1n-9 | 34.52 | 1.4±0.2 | 1.6±0.2 | Qualitatively detected[c] | |
| Docosadienoic | C22:2n-6 | 35.61 | 1.2±0.3 | 1.5±0.1 | 1.1±0.6 | 1.2±0.3 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Tricosanoic | C23:0 | 36.21 | 2.1±0.2 | 2.1±0.2 | 1.4±0.1 | 1.3±0.3 |
| U22 | ? | 36.87 | 4.1±1.4 | 4.0±0.6 | 1.3±0.6 | 1.6±0.5 |
| U23 | ? | 37.66 | 3.0±1.4 | 3.3±0.6 | 1.0±0.5 | 2.2±1.0 |
| Docosapentaenoic | C22:52-3 | 38.78 | 6.0±0.7 | 6.3±0.8 | 3.7±0.4 | 4.2±1.2 |
| U24 | ? | 39.36 | Qualitatively detected | | 1.4±0.3 | 1.1±0.5 |
| Docosehexaenoic | C22:6n-3/C24:1n-9 | 39.73 | 52.5±3.8 | 55.9±5.8 | 36.4±4.1 | 42.6±8.1 |

[a]Retention time, for gas chromatography (GC) parameters (Stabilwax column); see Materials and methods
[b]n designates the location of the first double bond from the methyl end of the molecule; all fatty acids were *cis*
[c]As a result of the high levels of C22: 1n-11, which eluted slightly earlier, exact determination of the area was not possible
[d]Oleic acid (C18: 1n-9*c*) co-elutes on Stabilwax with elaidic acid (C18: 1n-9*t*)
*Source:* Reproduced with permission of *European Food Research and Technology* (García-Ayuso et al. [73])

and solvent. Also, the MAE-based method used less toxic solvents and was thus "greener." One other advantage of the MAE method was the shorter time required (40 min vs. 160 min per sample), and its ability to process several samples simultaneously. A further disadvantage of the Bligh and Dyer method was that the water content had a strong influence on the accuracy of the results and that special care was needed to identify the optimal operating conditions; on the other hand, the water content of the samples had no influence on the MAE results.

## 4.5 Selected Applications of MAE for the Removal of Lipids

### *4.5.1 Laboratory-Scale Applications*

A very large number of applications of MAE for the removal of contaminants (particularly pesticides) from oil and fat matrices, testifying to the widespread use of this extraction technique, have been reported [27, 79–82] but are not the subject matter of this chapter. Rather, this section focuses on the distinction between applications using commercial extractors and those using prototypes or laboratory-designed devices for lipids extraction.

#### 4.5.1.1 Applications Based on Commercial Extractors

MAE is a suitable technique for the extraction of lipids from fish as a preliminary step in the determination of the fatty acid pattern [78]. The interest of this determination lies in the potential benefits and nutritional significance of long-chain polyunsaturated fatty acids (PUFAs), which are especially prevalent in fish and other marine lipids.

For this purpose, focused open-vessel MAE was performed in a Prolabo Soxwave 100 system. Microwaves of 2,450 MHz were delivered by a magnetron. The system, which operated at ambient pressure, was equipped with a reflux column (a condenser) to avoid solvent losses during extraction and afforded multistep programming of the MW power up to 300 W and of the irradiation time. Although the system allowed the use of a cartridge similar to that of a typical Soxhlet system, the samples were weighed directly into a 300-ml quartz glass vessel. The glass tube connecting the quartz vessel to the solvent condenser was fitted with a tap for solvent reduction. The volume above the tap (20 ml) was used as a water trap. Homogenized samples of mackerel fillet (approximately 10 g) and cod liver (approximately 5 g) were individually weighed, blended with 5 g of $Na_2SO_4$, and placed in the 300-ml quartz extraction vessel of the extractor. A volume of 50 ml 1:1 (v/v) cyclohexane–ethyl acetate mixture was then added and the vessel inserted in the extraction cavity, fitted with a condenser, and irradiated as follows: after 5 min at 30 W, the power was raised to 45 W, which was held for 5 min; then, the power was raised to 75 W and held for 30 min. After cooling, the extract was filtered through $Na_2SO_4$. The system was rinsed by passing approximately 20 ml solvent through the

condenser and the residue in the extraction vessel washed with a further 30 ml solvent that was subsequently added to the extract. The combined extracts were evaporated under vacuum at 35°C at 210 torr and dried with a stream of nitrogen.

The results of the extraction of the lipid content of frozen fish (horse mackerel, chub mackerel, and sardine) in a microwave accelerated reaction system (MARS-X), 1,500 W (CEM, Mathews, NC, USA) were compared with those provided by other extraction methods (Soxhlet, Bligh & Dyer, modified Bligh & Dyer, Folch, modified Folch, Hara & Radin, Roese-Gottlieb) [83]. The MAE, Bligh & Dyer, Folch, modified Folch, and Hara & Radin methods proved the most efficient. Although the results were not statistically different, there were some differences in terms of variability, with MAE showing the highest repeatability [coefficient of variation (CV) = 0.034). By contrast, the Roese–Gottlieb, Soxhlet, and modified Bligh & Dyer methods exhibited very poor efficiency and repeatability (CV, 0.13–0.18).

#### 4.5.1.2 Applications Based on FMASE

Meat products from Iberian pigs fed extensively with acorns are regarded as being of higher quality than those from pigs fed with mixed diets; analysis of the fat fraction of acorns is thus of paramount importance with a view to accurately assessing these products for quality. To ensure representative analyses, the extraction step preceding quantitation should in no way alter the original composition of the fat fraction (Fig. 4.6).

A method based on FMASE was used to extract oil from acorns [84] by using a prototype design in between those of Figs. 4.1 and 4.7. The method afforded total extraction of fatty acids in acorns within 30 min. The extraction program used for this purpose consisted of 15 cycles each involving the following four steps: (1) filling of the sample cartridge vessel to 54 ml with the vessel valve in the load position as a result of distillation of the solvent from the flask, condensation in the refrigerant, and dropping on the sample; (2) microwave irradiation of the cartridge at a fixed microwave power (300 W) for a pre-set time (60 s); (3) contact of the sample with the heated *n*-hexane for a pre-set delay time (40 s); and (4) unloading of the extraction vessel by switching the vessel valve to its unload position to deliver the vessel contents to the distillation flask; only the first step was repeated after the last cycle to reduce the volume of the extract contained in the distillation flask to about 50 ml. After the last cycle, the solvent was removed by rotary evaporation and the extract dissolved in 2 ml *n*-hexane.

Another salient application of FMASE in this context is the extraction of *trans* fatty acids in bakery products [16] by using the extractor of Fig. 4.1a. The extraction method was validated by comparison with the Folch reference method, which uses very mild conditions; the former caused no change in the original fat composition by effect of using microwave irradiation. The reduced extraction time (35 or 60 min vs. 3.5 h with the Folch method) and the similarity of the extract make this method an excellent choice for the treatment of solid samples before analysis for *trans* fatty acids. The results were consistent with those provided by the reference method, which testifies to the effectiveness of this approach for fat isolation in routine analyses.

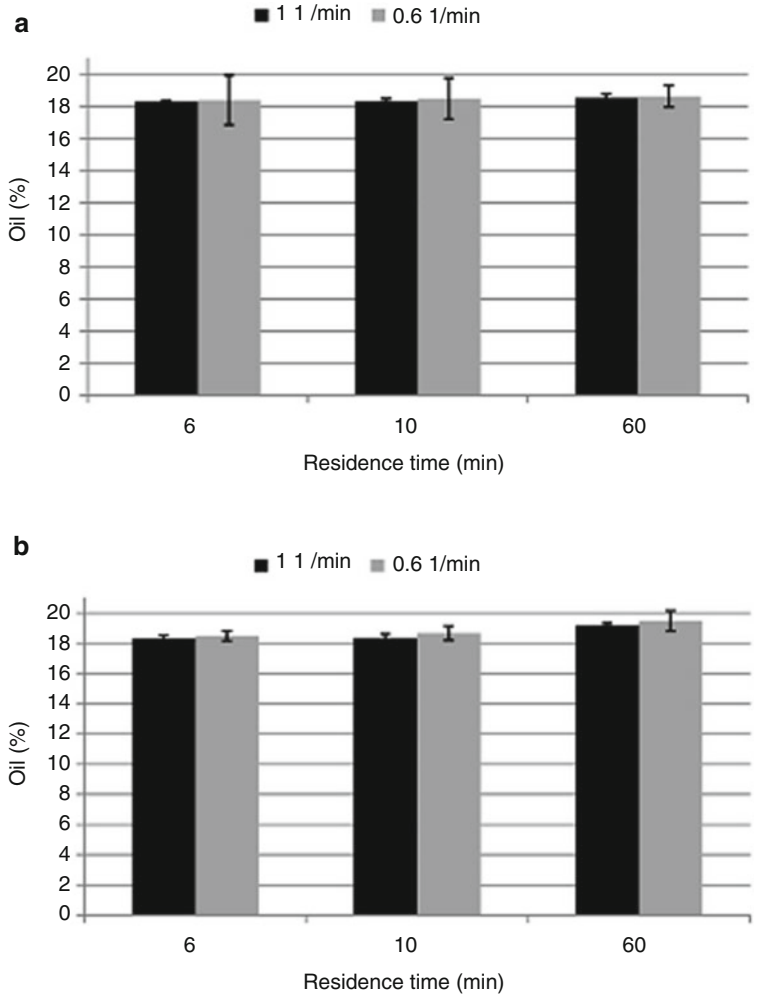


**Fig. 4.6** Oil extracted at different flow rates and extraction times from soybean (**a**) and rice bran (**b**). (From Terigar [49])

#### 4.5.1.3 Applications of MIS

One green, original alternative procedure for the extraction of fats and oils in oleaginous seeds was implemented with the extractor of Fig. 4.2 whereby a by-product of the citrus industry (D-limonene) was used as extractant instead of a hazardous solvent such as *n*-hexane [33]. Microwave radiation was applied in two steps; thus, the extraction was initially performed by MIS and followed by removal of the solvent from the medium by using a microwave Clevenger distillation system.

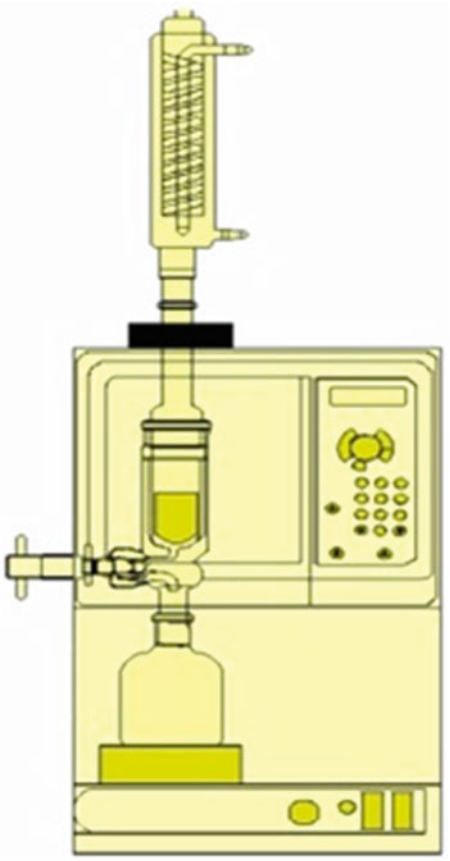

**Fig. 4.7** MIC II prototype of the focused microwave-assisted Soxhlet extractor. (Reproduced with permission of the American Chemical Society [41])

This method is an alternative for the determination of fats and oils in olive seeds with the added advantage of using a green solvent.

MIS extraction was compared with the conventional Soxhlet technique for the extraction of oil from olives [43]. The oils extracted by MIS for 32 min were quantitatively (yield) and qualitatively (fatty acid composition) similar to those obtained with conventional Soxhlet extraction for 8 h. Therefore, MIS seems to be an effective alternative to the extraction of fat and oils from food products.

### *4.5.2 Pilot-Plant Scale and Industrial-Scale Applications*

Similar to the equipment used, MAE applications to the extraction of lipids at the pilot-plant scale can be classified according to whether they have been developed in the academic or industrial realm.

Academic research in this field has provided rather dissimilar results. For example, that conducted at the University of Durango [48] has been the subject of no report, possibly because the results were poor, a logical outcome judging by the rudimentary MW equipment and additional units used. However, this type of research may be of great interest in a country such as Mexico, which is very rich in plants.

An M.Sc. student at the University of Louisiana [49] conducted research into the continuous MAE of oils from soybean and rice bran with a view to producing biodiesel. The procedure used MW energy to accelerate transesterification and ethanol as extractant. The pilot plant used is depicted in Fig. 4.3b.

The processing parameters (microwave exposure time, temperature, and holding times) previously established for small-scale continuous microwave extraction with the commercially available, fully instrumented, batch-type ETHOS E Microwave Extraction System from Milestone, Inc. CT, modified for continuous operation, were used as the basis for establishing the preliminary processing parameters at the pilot scale. The large amounts of products required to operate this system made it too expensive to test the scaled-up process on every parameter established with the smaller system. Rather, the oil extraction efficiency of the pilot plant was tested by using a feedstock/solvent ratio of 1:3 and a flow rate of 1 or 0.6 l/min. The microwave power input was adjusted so that the process temperature of the mixture leaving the microwave chamber would be 73°C (approximately 4.2 kW for 1 l/min, and 3 kW for 0.6 l/min). Figure 4.7 shows the extraction yields obtained under these working conditions.

The system of Fig. 4.3b can only be used at temperatures below the solvent boiling point. The increased temperatures required to boost extraction can be obtained by applying higher pressure to maintain the solvent in its liquid state beyond its atmospheric boiling point. Increasing the extraction time and temperature increases oil extraction yields, but not the quality of the extracted oil.

The University of Louisiana has also conducted research into the extraction of isoflavones. These compounds, which are present in soybeans, have been identified as alternative natural antioxidants to prevent rancidity in food fats. Researchers at this university investigated the microwave-assisted extraction of soy isoflavones from soy flour and assessed the ability of isoflavone extracts to prevent rancidity in fish oil. Experiments were performed with a continuous microwave extraction system and separation system. Soy flour and the extractant were continuously pumped together through the microwave cavity, where the solid–liquid system was heated to the temperature required for the oil to be extracted. Following heating and extraction of soybean oil from the flour, spent flour was separated from the extractant–oil mixture. Then, the extractant was removed and the oil collected. Oil was extracted with different solvents for variable times and the three most prevalent isoflavones were measured. Ethanol proved the most effective solvent for extracting total isoflavones. Increasing the extraction time with this solvent at 67°C increased yields by 330% relative to conventional solvent extraction (about 98% of total extractable oil in soy flour was recovered within 6 min).

The contents in total antioxidant phytochemicals and isoflavones of the defatted soy flour extract and the soy oil were compared. The defatted soy flour extract had much higher levels of phenolic compounds and isoflavones than the oil extracted

with hexane by a conventional method. The defatted soy flour extracts proved effective toward preventing fish oil oxidation. These results can be useful to the soybean industry, which has thus been provided with an alternative, more efficient method for extracting isoflavones from soy products and by-products. The new method has a high potential for revolutionizing the way bioactive components are industrially extracted from soybeans [85].

Firms developing pilot plants or using them to developed methods tailored to their clients' specific needs obviously do not publicize how such methods are devised, but only the materials or products in which they specialize. For example, Radient Technologies states the raw materials they address, which include plants (fresh and dried leaves, seeds, bark), single cells (algae, yeast, fungi), and other biomass (insects); the extractants they use (alcohols, alkanes, ketones, esters, mixed solvents, liquefied gases); and the products they extract, which include lipids (fatty acids, oils, essential oils), sterols, carotenoids, vanillin, and polyphenols.

### *4.5.3 Drawbacks of MAE*

Comparing MAE operational settings is a difficult task owing to the dissimilar design of microwave devices; thus, some afford temperature and pressure control, others only either and still others neither. Therefore, adapting existing MAE procedures when first addressing a given extraction can be difficult unless the same microwave device is used in all. In addition, large cooling or venting times after completion of the MAE step are usually needed to avoid losses of volatile lipids, which considerably lengthens the overall extraction process.

The temperature inside the extraction vessel can rise rapidly and cause the loss of volatile lipids to the headspace in closed-vessel microwave systems.

Poor selectivity is another drawback commonly reported in MAE applications. The high extractive capacity of MAE often requires subsequent extensive cleanup. Depending on the nature and polarity of the interferents present, co-extracted compounds can be removed by conventional solid-phase extraction (SPE) with disposable cartridges (C18, silica, alumina, ion exchangers), liquid–liquid extraction, or solid-phase microextraction (SPME).

An additional step usually required in MAE is filtration or centrifugation after extraction to separate the extract containing the lipids from the remaining solid, which obviously delays completion of the overall process.

## 4.6 Trends in MAE for Fats and Oils

The following are some of the foreseeable trends in the microwave-assisted extraction of lipids:

(a) The use of greener extractants to comply with the increasingly restrictive rules imposed by competent organisms and also with internal corporate policies. One

case in point is limonene [33], a clean solvent and one of the best extractants for MAE by virtue of its high polarity and dielectric constant.

(b) The conduct of research into the characteristics of monomode MW with a view to enabling its application to more specific targets.

(c) The development of more efficient magnetrons to reduce industrial costs and increase extraction yields.

These trends may lead to a wider industrial use of MAE, not only for extracting high added-value lipids, but also to obtain common edible oils (e.g., by extraction with water). The cost of MW energy would thus be offset by the savings in extractant (currently hexane) and in the energy needed for its complete removal from the extracted oil.

Although the utility of MAE for lipid extraction has been demonstrated beyond doubt at the laboratory scale, the growing trend to using new vegetables to extract edible oils and oils for biofuel production will require preliminary optimization at this scale to avoid the use of the greater amounts of raw material needed at the pilot-plant scale.

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## Abbreviations

| | |
|---|---|
| **ASE** | Accelerated solvent extraction |
| **CMAE** | Continuous flow microwave-assisted extraction |
| **EFAs** | Essential fatty acids |
| **FID** | Flame-ionization detection |
| **FMASE** | Focused microwave-assisted Soxhlet extractor |
| **FME** | Focused microwave-assisted extraction |
| **GC** | Gas chromatography |
| **HPSE** | High-pressure solvent extraction |
| **LC** | Liquid chromatography |
| **LDL** | Low-density lipoproteins |
| **LLE** | Liquid–liquid extraction |
| **MAE** | Microwave-assisted extraction |
| **MASE** | Microwave-assisted Soxhlet extraction |
| **MIR** | Middle infrared spectrometry |
| **MIS** | Microwave integrated Soxhlet |
| **MS** | Mass spectrometry |
| **NEFAs** | Nonessential fatty acids |
| **NMR** | Nuclear magnetic resonance |
| **PFE** | Pressurized fluid extraction |
| **PHSE** | Pressurized hot solvent extraction |

**PTFE** Polytetrafluoroethylene
**PUFAs** Polyunsaturated fatty acids
**SHLE** Superheated liquid extraction
**SHSE** Superheated solvent extraction
**SPE** Solid-phase extraction
**SPME** Solid-phase microextraction
**SSE** Subcritical solvent extraction
**UMAE** Ultrasound and microwaves for acceleration of extraction
**USAE** Ultrasound-assisted extraction

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# Chapter 5
# Microwave-Assisted Extraction of Antioxidants and Food Colors

**Ying Li, Anne-Sylvie Fabiano-Tixier, Maryline Abert-Vian, and Farid Chemat**

## 5.1 Brief Introduction

### *5.1.1 Antioxidants*

Natural antioxidants have a very strong appeal to consumers and are present in high content in plants. Antioxidants are commercial assets frequently highlighted by the cosmetic and dietary supplement industries in the means of promoting their production. The most abundant natural antioxidants in plant materials are the phenolic or polyphenolic compounds that can interfere with the formation of free radicals (i.e., the initiation reactions) and also detain the propagation of oxidation or free radical chain reactions, thus preventing formation of hydroperoxides.

The presence of antioxidants could inhibit lipid oxidation and scavenge free radicals in the form of molecules in living organisms, such as phenolic compounds, vitamins, and flavonoids. Numerous studies have demonstrated that some plants could slow or prevent the oxidation of the aforementioned molecules, which are essential for human health and food safety and quality. Over the course of the past decade, great interest has been shown in extracting natural antioxidants because of proven negative health effects caused by synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). However, the conventional techniques are not adequate because of the appearance of various undesirable by-products during the extraction process such as oils, waxes, and pigments. Therefore, chemists, chemical engineers, and biotechnologists are being asked to come

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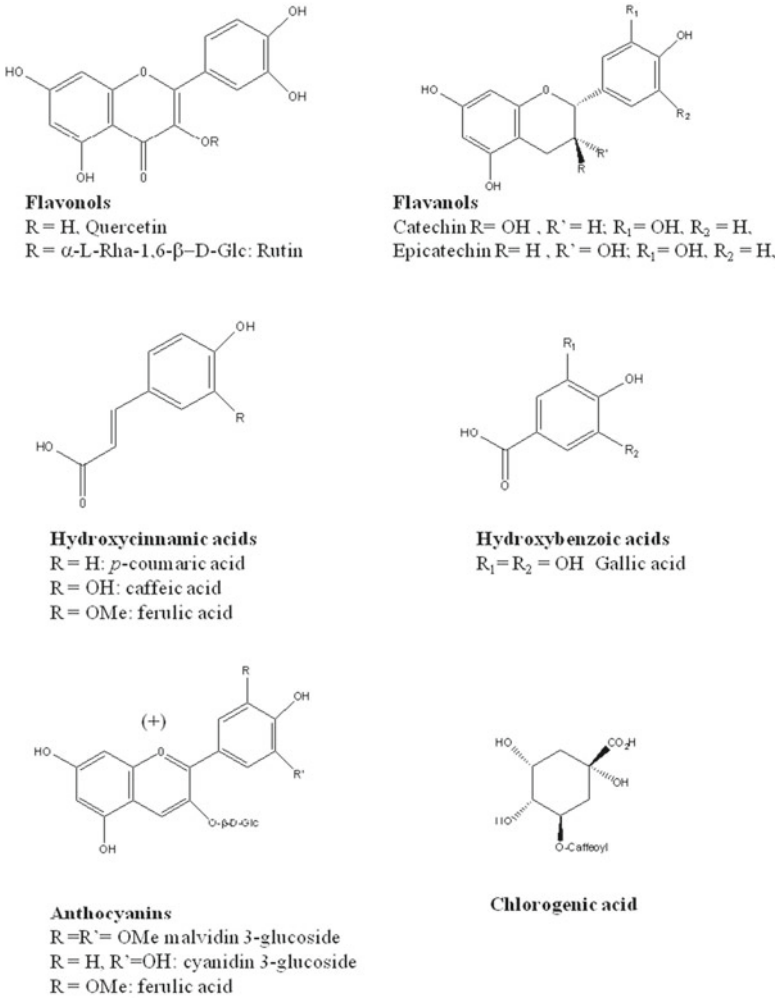



**Fig. 5.1** Examples of common polyphenols

up with innovative and effective ways to minimize the shortfalls that may exist in the intensified and multiplied solvent extraction processes which result in higher cost, energy consumption, and use of nonrenewable resources, and more individual operations, waste, and hazardous factors that can be harmful to humans as well as to the environment.

The majority of naturally occurring antioxidants are found as secondary metabolites in plants, named phenolic compounds or polyphenols [1, 2]. The main class of polyphenols occurring in abundance in foods are hydroxybenzoic acids and hydroxycinnamic acids, often presenting an acid function involved in an ester bond with a variety of alcohols (quinic acid, tartaric acid, sugar alcohols, OH

group of the pyran ring of flavanols), flavanols (condensed tannins), and the flavonols and anthocyanins (Fig. 5.1)

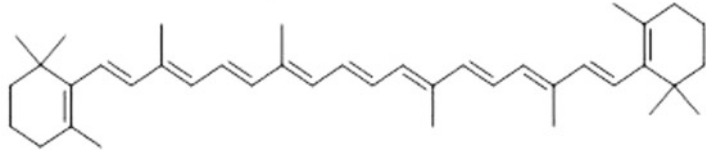
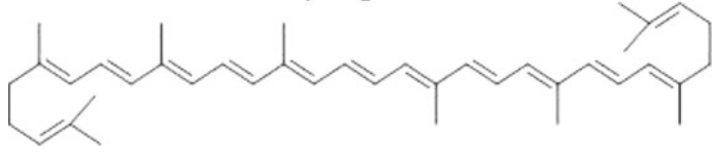
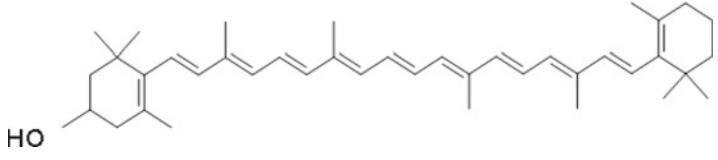
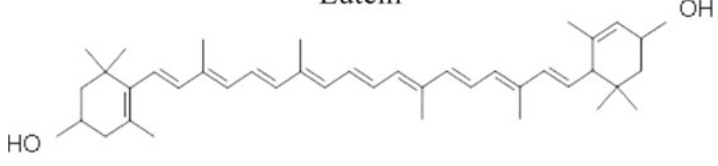
### *5.1.2 Natural Food Colors*

Throughout history, mankind has always been keen on exploring and learning more about the usage of color, which can make our life vivid. In our early history, people liked to color themselves or their animals for some special and meaningful occasions such as religious ceremonies, as well as during wars. They believed that the color would give them invincible power to conquer any enemies. As time went on, society became more complex and so did the use of colors. It is difficult to imagine a modern world without colors. Natural colorants and dyes can be produced from almost all parts of the plants such as leaves, flowers, fruits, seeds, and roots. Green is considered the mostly frequently seen color in plant leaves because of chlorophyll, which can convert sunlight into chemical energy through photosynthesis. Other different colors in plants not only can prevent the plants from attacks by their natural enemies but can also help to attract insects or other animals that can be intermediates in plant pollination and finally reproduction. It is worth mentioning that all these natural colors could be used to impart color to an infinite variety of materials (textiles, food, varnishes, cosmetics, etc.) so we can make our living world full of vigor and color.

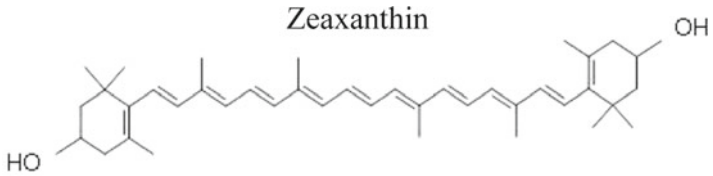
The natural colors comprise mainly one or more of the groups of color compounds, such as carotenoids, anthocyanins, betanin, chlorophyll, curcumin, and flavonoids [3]. In addition, chemical studies of pigment have concluded that a pigment molecule has two principal chemical groups, namely, chromophores and auxochromes. The chromophore, which is related to its coloring property, is normally compared of an aromatic ring having unsaturated bonds, and the number of unsaturated bonds decides the intensity of color; the auxochrome can help to combine the pigment molecule with the substrate, thus imparting color [4]. Colorants have extensively been used in a wide range of food products with the aim of enhancing the aesthetic value of foods. Because of increasing market demands, more and more artificial colorants and dyes synthesized from petrochemical origins have flown into our lives, which has resulted in a rapid decline of the use of natural colorant and dyes. However, people are inclined to use natural colors because of their health, safety, and environmentally friendly properties. Nevertheless, natural colors have not yet succeeded commercially for reasons of some technical hitches (lack of knowledge of extraction, difficulty in plant collection, etc.).

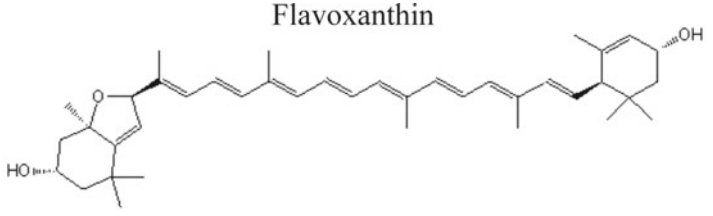
Carotenoids are the largest group of natural colorant. Indeed, many of the natural food colors are constituted of carotenoids, which are orange-red pigments found in many plant species such as tomato, orange, and carrot, but also in some animals. Carotenoids are composed of a chain of isoprene units. More than 500 different molecules of carotenoids have been identified so far. Carotenoids are hydrophobic molecules of long chains, possessing at least 40 carbon atoms and a chain of carbon–carbon conjugated double bonds. In the carotenoids class, it is possible to distinguish caro-

**Table 5.1** Example of carotenoids

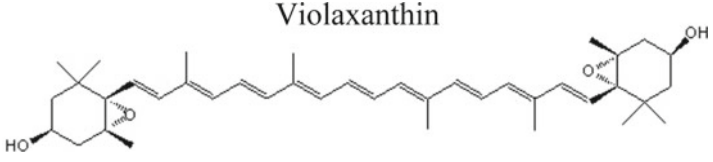
| Classification of carotenoids | Structure example |
|---|---|
| Hydrocarbon<br>α, β, γ-Carotene ($C_{40}H_{56}$)<br>Lycopene ($C_{40}H_{56}$)<br>Rhodopurpurine ($C_{40}H_{56}$ or $C_{40}H_{58}$)<br>Leprotene ($C_{40}H_{54}$) | β–carotene<br>  <br>Lycopene<br>  |
| Xanthophylls<br>Containing 1-OH group<br>Lycoxanthin ($C_{40}H_{56}O$)<br>Kryptoxanthin ($C_{40}H_{56}O$)<br>Gazaniaxanthin ($C^{40}H_{56-58}$)<br>Rubixanthin($C_{40}H_{56}O$)<br>Rhodopin ($C_{40}H_{56-58}$)<br>Containing 2-OH groups | Kryptoxanthin<br>  <br>Lutein<br>  |

Lutein ($C_{40}H_{56}O_2$)
Lycophyll($C_{40}H_{56}O_2$)
Zeaxanthin($C_{40}H_{60}O_2$)
Rhodoviolascin($C_{40}H_{60}O_2$)
Eschischolzanthin($C_{40}H_{54+2}O_2$)
Containing 3-OH groups
Flavoxanthin ($C_{40}H_{56}O_3$)
Antheroxanthin ($C_{40}H_{56}O_3$)

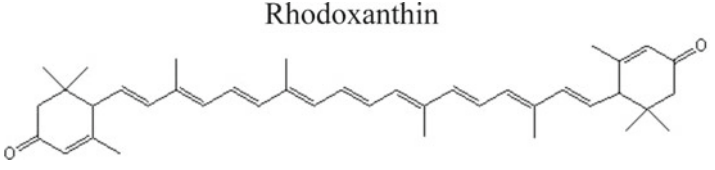



Petaloxanthin ($C_{40}H_{58(-2)}O_3$)
Eloxanthin ($C_{40}H_{56}O_3$)
Containing 4-OH groups
Violaxanthin ($C_{40}H_{56}O_4$)
Taraxanthin ($C_{40}H_{56}O_4$)

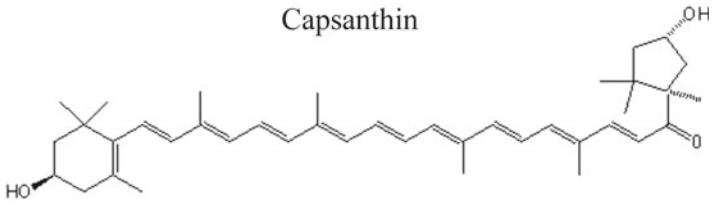
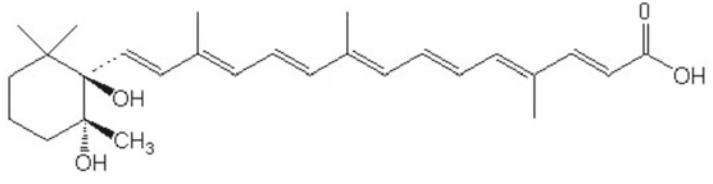
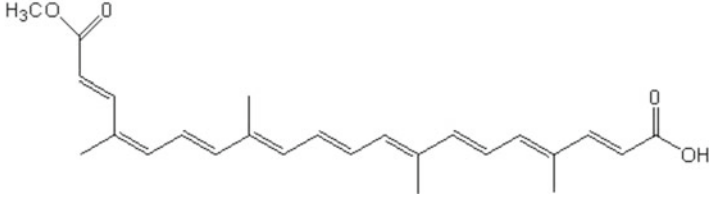



Ketones
Myxoxanthin($C_{40}H_{54}O$)
Alphanin ($C_{40}H_{54}O$)
Rhodoxanthin ($C_{40}H_{50}O_2$)




(continued)

**Table 5.1** (continued)

| Classification of carotenoids | Structure example |
|---|---|
| Hydroxyl carbonyl compounds<br>Capsanthin ($C_{40}H_{58}O_3$)<br>β-Citraurin($C_{30}H_{40}O_2$)<br>Capsorubin ($C_{40}H_{60}O_4$)<br>Astaxanthin ($C_{40}H_{52}O_4$)<br>Fucoxanthin ($C_{40}H_{60}O_4$) | Capsanthin   |
| Carboxylic compounds<br>Azafrin ($C_{27}H_{38}O_4$)<br>Bixin ($C_{25}H_{30}O_4$)<br>Crocetin ($C_{20}H_{24}O_4$) | Azafrin  <br>Bixin   |

tenes from xanthophylls because carotenes consist solely of carbon and hydrogen, and xanthophylls additionally contain oxygen atoms. There are also acids and esters (carotenoid acids and esters, xanthophylls esters, etc.), which are described in Table 5.1.

## 5.2 Relevant Microwave Extraction Techniques

Use of microwave energy was described for the first time in 1986 simultaneously by Gedye et al. [5] and Giguere et al. [6] in organic synthesis and by Ganzler et al. [7] and Lane and Jenkins [8] for extraction of biological samples for analysis of organic compounds. Since then, numerous laboratories have studied the synthetic and analytical possibilities of microwaves as a nonclassical source of energy. More than 2,000 articles have been published on the subject of microwave synthesis and more than 500 on the subject of extraction [9, 10].

In the past decade there has been an increasing demand for new extraction techniques, amenable to automation, with shortened extraction times and reduced organic solvent consumption, to prevent pollution and reduce the cost of sample preparation. Driven by these goals, advances in microwave extraction have given rise to two classes of techniques: microwave-assisted solvent extraction (MASE) and microwave solvent-free extraction (MSFE). Over the years, procedures based on microwave extraction have replaced some of the conventional processes and other thermal extraction techniques that have been used for decades in chemical laboratories.

### *5.2.1 Microwave-Assisted Solvent Extraction (MASE)*

MASE consists of treating an organic solvent (extractant) in contact with the sample, dry or wet, with microwave energy. The partitioning of the analytes from the sample matrix to the extractant depends on the temperature and the nature (polarity) of the solvent.

According to the dielectric characteristics of the solvent and the sample matrix, two cases should be considered:

- The solvent absorbs all the microwave energy: the polar solvent (ethanol, methanol, water, etc.) heats up until it reaches the boiling point, diffuses into the sample matrix, and solubilizes the analytes. Heat transfer in the solid matrix is caused by conduction from the solvent. In this case, the mechanism of extraction assisted by microwaves is not fundamentally different from that of the classical solid–liquid extraction. However, microwaves present an instantly controllable energy source that is precisely adjustable.
- The second scenario consists of direct heating of a wet matrix that directly absorbs microwaves: target compounds migrate from the matrix through the transparent nonpolar solvent (hexane, toluene…). This process was introduced by Paré, who has developed and patented a family of technologies called microwave-assisted process (MAP) [11–14] for extraction of various chemical

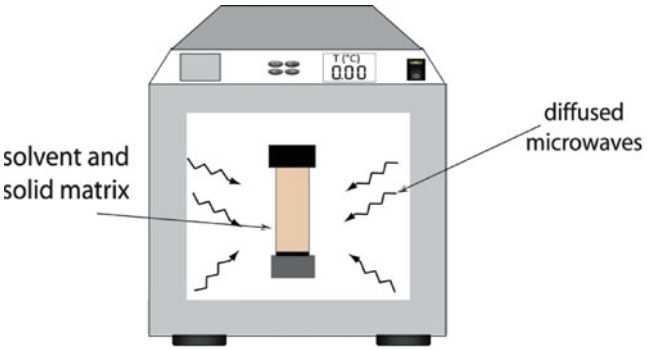



**Fig. 5.2** Microwave-assisted solvent extraction (MASE)/closed-vessel system

categories such as essential oils from plant material, coloring agents for the food and cosmetic industries, oil from oil seeds, etc. This approach is considered to support sustainable development as it requires less energy and solvent than conventional processes, while generating fewer wastes. The liquid-phase MAP extraction process is based upon the ability of a matrix to absorb microwave energy. The absorption efficiency is largely related to the moisture content of the material; the water molecules convert the microwave energy into heat, and the result is a sudden rise in temperature inside the material. According to Paré [15], when the plant cells are subjected to severe thermal stress and localized high pressures, the pressure buildup within the cells exceeds their capacity for expansion and causes their dislocation more rapidly than in conventional extraction, which leads to the release of their contents in the middle of extraction.

Microwave energy may be applied to the samples using two technologies: closed extract vessels under controlled pressure and temperature, and open vessels under atmospheric pressure.

Closed-vessel systems are generally advised for extractions under drastic conditions such as high extraction temperature. Most available closed-vessel systems are based on multimode microwaves; however, the advantages of high-pressure vessels combined with focused heating have led to the development of systems that combine both approaches and operate at a very high pressure and temperature. The solvent can be heated above its boiling point at atmospheric pressure, thus accelerating the mass transfer of target compounds from the sample matrix [16]. The MASE/closed-vessel system is schematized in Fig. 5.2.

In the so-called open systems, extractions proceed under atmospheric pressure. As a consequence, the maximum possible temperature is determined by the boiling point of the solvent at that pressure. A number of applications have reported the use of open-vessel systems with multimode and monomode microwave ovens. The solvent is heated and refluxed through the sample, and in this case the microwaves are focused on the sample placed into the vessel, allowing homogeneous and very efficient heating. This technique is called focused microwave-assisted solvent extraction (FMASE) [17]. Compared to closed-vessel extractions, open vessels offer increased safety in sample handling and, furthermore, they allow larger samples to be extracted.

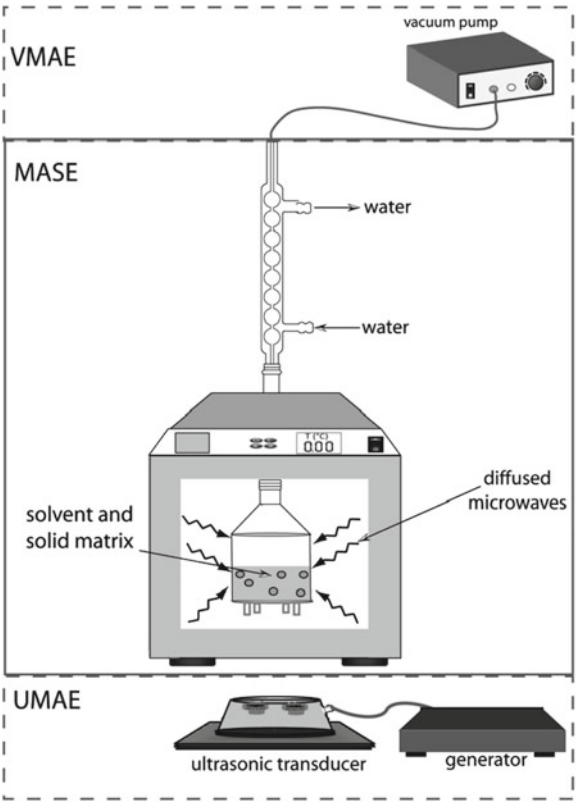



**Fig. 5.3** MASE/open-vessel system and innovative variants

MASE has been considered as a potential alternative to traditional solid–liquid extraction for the extraction of substances from natural matrices. It has been used for several reasons: (1) reduced extraction time, (2) reduced solvent usage and (3) improved extraction yield. After observing the potential of microwaves, scientists are continuously busy in inventing new techniques with assistance of microwaves. Along with the invention of new techniques, they have also derived different extraction techniques from MASE, such as microwave-integrated Soxhlet extraction (MIS), ultrasound and microwave-assisted extraction (UMAE), dynamic microwave-assisted extraction (DMAE), and vacuum microwave-assisted extraction (VMAE). A MASE/open-vessel system with innovative variants is represented in Fig. 5.3.

### *5.2.2 Microwave Solvent-Free Extraction (MSFE)*

Recent concerns about the natural and environmental hazards of organic solvents applied in extraction techniques have led to the development of solvent-free extraction techniques.

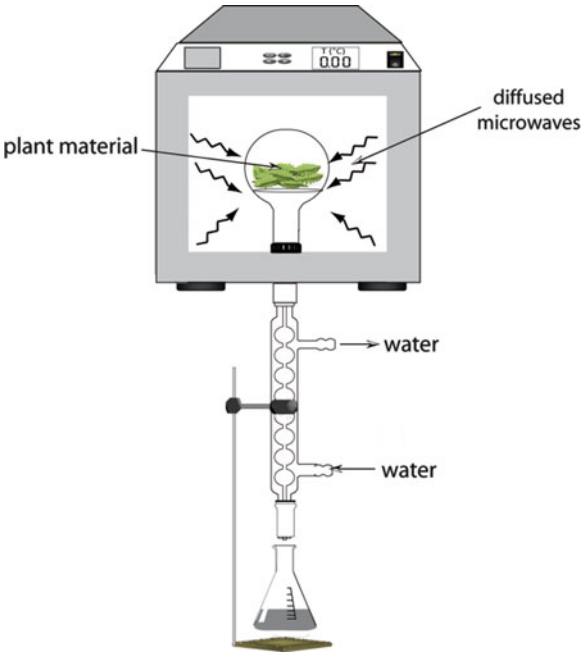



**Fig. 5.4** Microwave hydrodiffusion and gravity (MHG)

MASE, which had succeeded in the extraction of organic compounds from solid samples such as plant tissues, needs less organic solvent and shorter extraction times than traditional extraction methods, but we know that there are several disadvantages in using an organic solvent for microwave-assisted extraction.

Recently, a new and green technique for the extraction of natural products has been developed called microwave hydrodiffusion and gravity (MHG) (Fig. 5.4) [18].

This green extraction technique is an original "upside-down" microwave alembic combining microwave heating and earth gravity at atmospheric pressure. MHG was conceived for laboratory- and industrial-scale applications for the extraction of pigments, aroma components, and antioxidants from different kinds of plants. Based on a relatively simple principle, this method involves placing plant material in a microwave reactor, without adding any solvent or water. The internal heating of the in situ water within the plant material distends the plant cells and leads to the rupture of glands and oleiferous receptacles. The heating action of microwaves thus frees secondary metabolites and in situ water that are transferred from the inside to the outside of the plant material.

This physical phenomenon, known as hydrodiffusion, allows the extract diffused outside the plant material to drop by earth gravity out of the microwave reactor and fall through the perforated Pyrex disc. A cooling system outside the microwave oven cools the extract continuously. The crude extract is collected in a receiving flask.

## 5.3 Application of Microwave to Extraction of Antioxidants and Food Colors

Microwave-assisted extraction (MAE), which proved to be significantly faster, has sprung up and been developed into an alternative laboratory-scale extraction method in recent years. It has shown obvious advantages in terms of less use of solvents, high extraction efficiency, and antioxidant activity of extracts in comparison to conventional extraction techniques. As shown in Table 5.2, the majority of plant extracts by MAE are dietary polyphenols, including flavonoids (flavones, flavonols, isoflavones, catechins, flavanones, flavonones, and cinnamic acid derivatives), anthocyanins, and carotenoids (lycopene, lutein, carotenes, etc.), which are also considered the main natural pigments that are widely used in the food industry.

### *5.3.1 Recovery of Antioxidants by Microwave Assistance*

Microwave energy was introduced in the separation, extraction, and subsequent analysis of antioxidative compounds from various plants. MAE was proposed for recovery of flavonoids from soybeans under its optimal conditions [19, 20]. Moreover, Nkhili et al. have investigated that microwave water extraction (MWE) appeared to be more efficient than conventional water extraction (CWE) and could be used as an alternative method for extraction of green tea polyphenols, especially for extraction of flavanols and hydroxycinnamic acids [21]. MAE was also employed to extract other botanicals and showed remarkable advantages in short extraction time and high efficiency in extracting flavonoids when compared to other conventional (heat reflux, Soxhlet, maceration) and innovative (ultrasound-assisted) techniques [22, 23].

As microwave-assisted extraction has been more and more applied in antioxidant extraction, researchers have paid more attention to the improvement of this technique so as to develop and perfect it. Therefore, some derivative microwave-assisted extraction techniques have emerged as industry required. Dynamic microwave-assisted extraction (DMAE), which is considered continuous, rapid, and automated, has been described for extraction of flavonoids from different plants [24, 25]. The dynamic extraction system has the benefit that it can transfer the analytes extracted with flowing fresh solvent from the sample to the outside of the extraction vessel. DMAE has performed higher extraction yields without degradation of target components in comparison with the other methods (ultrasonic extraction, Soxhlet extraction, heat reflux extraction, and dynamic solvent extraction without microwave assistance). Another derivative technique called pressurized microwave-assisted extraction (PMAE) has also been mentioned for the extraction of flavonoids and could obtain a similar extraction yield to that obtained by DMAE [29].

Although microwave-assisted extraction and its derivative techniques have developed greatly with various advantages, no extraction technique can be considered a truly green, inexpensive, and easily carried out method for the extraction of antioxidants. Solvents of petrochemical origin are now highly regulated by European direc-

**Table 5.2** Antioxidant extracts

| Matrix | Analyte | Technique | Operating conditions and remarks | References |
|---|---|---|---|---|
| Yellow soybeans (finely ground) | Isoflavoids | MAE | P.atm, 600 W, acetonitrile/water (2 ml 80/20, v/v), sonicated with HCl 15 min, 1 min MAE. Excellent efficiency and low consumption (sample, solvent, time) | [19] |
| Soybeans | Isoflavones | MAE | P.atm, 500 W, T = 50 °C, 25 ml ethanol (50 %), 20 min. 75 % isoflavones extracted in 10 min and quantitative recoveries achieved in 20 min. High reproducibility without degradation | [20] |
| Green tea leaves (*Camellia sinensis* L.) | Flavanols | MWE | P.atm, 600 W, 80 °C or 100 °C, 120 ml milli-Q water, 60 min. Yield of flavanol extracts is higher than that in CWE, especially EGCG (*Epigallocatechin gallate*) concentration. More efficient at both 80 °C and 100 °C | [21] |
| Dried *Saussurea medusa* cells | Flavonoids | MAE | P.atm, 460 W, 10 ml ethanol (80 %), 6-min irradiation cycle: 15 s power-on, 30s power-off. Yield 4.1 % flavonoids, high selectivity, comparison among solvent, Soxhlet, heat reflux extractions, and ultrasound-assisted extraction | [22] |
| *Radix astragali* | Flavonoids | MAE | P.atm, 1,000 W, 110 °C, ethanol (90 %), 25:1[a], 25 min. No degradation. Yield of flavonoids is close to that of SOX with methanol and higher than that of UAE with methanol | [23] |
| *Platycladus orientalis* (book-leaf pine) | Flavonoids | DMAE | P.atm, 80 W, 5 ml methanol (80 %), 500:1[a], 5 min. Yield of flavonoids was found 1.72 % by DMAE coupled with on-line derivatization and UV-vis detection in closed and automated system, very short time and little solvent quantity required | [24] |
| *Saussurea medusa* Maxim | Flavonoids | DMAE | P.atm, 1,200 W, 2 l ethanol (80 %), 50:1[a], 60 min. Yield 4.97 % flavonoids. In comparison with the same dynamic system without microwaves | [25] |
| Longan peel | Total phenolic content (TPC) | MAE | P.atm, 500 W, 80 °C, 50 ml ethanol (95 %), 10:1[a], 30 min. Microwave extract possess abundant phenolic content (96.78 mg/g); excellent scavenging ability comparing to synthetic antioxidant BHT | [26] |
| Plants of Labiatae, Verbenaceae, and Styracaceae | Total phenolic compounds (TPC) | MAE | P.atm, 750 W, 20 ml acetone (60 %), 20:1[a], 4 min. Higher yield (23.8 mg gallic acid/g) of TPC found in *Rosmarinus officinalis* | [27] |

| | | | | |
|---|---|---|---|---|
| Dried roots of *Rhodiola* L. | Salidroside and tyrosol | MAE | P.atm, 400 W, 5 ml methanol (50 %), 5:1[a], 5 min. Good recoveries (94.4–123 %) of salidroside and tyrosol obtained from *Rhodiola* L. samples from five different growing areas | [28] |
| *Herba epimedii* | Flavonoids | DMAE | P.atm, 80 W, ethanol (60 %), 6 min. The extraction yield of flavonoids obtained by DMAE is higher than that obtained by PMAE, UE, HRE, and SOX. Time saving and less decomposition when extraction time increases | [29] |
| Onion (*Allium cepa* L.) | Flavonol | MHG | P.atm, 500 W, 23 min. Shorter extraction time, no solvent or water used, extraction of onion crude juice retaining fresh organoleptic properties with higher phenolic content (58.29 mg GAE/g DW); Significant yield (81.5 %) with 41.9 % flavonol content | [30] |
| Red, yellow, white, and grelot onion (*Allium cepa*) | Flavonol | MHG | P.atm, 500 W, 23 min. The highest antioxidant capacity was observed for red onion, followed by yellow, white, and grelot onion; MHG remained the preferred method for flavonoids extraction in comparison to CSE | [31] |
| Sea buckthorn (*Hippophae rhamnoides*) by-product | Flavonoids | MHG | P.atm, 400 W, 23 min. Shorter extraction time, no solvent or water used; MHG extract shows much higher phenolic content with greater antioxidant activity in comparison to CSE | [32] |
| Onion by-product | Flavonoids | VMHG | 0.7 bar, 500 W, 26 min. More moisture extracted and dry contents yield at reduced temperature; More antioxidants (total quercetin content) extracted in comparison to MHG and CSE; an efficient procedure for extraction of heat-sensitive plant components | [33] |
| Olive leaves | Biophenols | MAE | P.atm, 200 W, 8 ml ethanol (80 %), 8:1[a], 8 min. The main compounds ranged from 631 (verbacoside) to 23,200 mg/kg (oleuropein) | [34] |
| Grape skin and seeds | Phenolic compounds | MAE | P.(1–10 atm), 500 W, 65–140 °C, 20 ml methanol (100 %), 20 min. Flavanols was mostly found in skin but absent in grape seeds; catechin was abundant in seeds | [35] |
| Purple corn (*Zea mays* L.) cob | Anthocyanins | MAE | P.atm, 555 W, 1.5 M HCl–ethanol (95 %), (15:85, V/V), 20:1[a], 19 min. The highest total anthocyanin (185.1 mg/100 g) content is obtained by MAE method. More efficient and rapid than CSE. Six kinds of anthocyanins are identified | [36] |

(continued)

**Table 5.2** (continued)

| Matrix | Analyte | Technique | Operating conditions and remarks | References |
|---|---|---|---|---|
| Tomato paste | Lycopene | UMAE | P.atm, 98 W, 40 KHz ultrasonic processing, 10.6: 1[a], 367 s. Higher yield (97.4 %) of lycopene than that in UAE, more efficient | [37] |
| Noni plant roots (*Morinda citrifolia*) | Anthraquinones | MAE | P.atm, 720 W, 60 °C, 10 ml ethanol (80 %), 100:1[a], 15 min. Higher yield (95.91 %) obtained with higher antioxidant activity | [38] |
| Seeds, leaves, pulp, and fruits of sea buckthorn (*Hippophae rhamnoides*) | Phenolic constituents | MAE | P.atm, 150 W, 60 °C, 50 ml ethanol, 10:1[a], 20 min. Total phenolic content varies from 9.3 to 23.5 mg GAE/g, with highest amount of rutin compound (365 μg/g) | [39] |
| Aloe (Liliaceae) | Aloe-emodin | MAE | P.atm, 340 W, ethanol/water (20 ml 80/20, v/v), 15:1[a], 3 min. comparison among MAE, ultrasonic extraction, and Soxhlet method | [40] |
| Sweet grass leaves (*Hierochloë odorata*) | Polyphenols | MAE | P.atm, 200 W, 30 m; acetone, 10:1[a], 80 °C, 15 min. Best recoveries of antioxidants; 5,8-dihydroxycoumarin (0.42 %) and 5-hydroxy-8-*O*-β-D-glucopyranosyl-benzopyranone (0.11 %) obtained during one-step extraction | [41] |
| Green tea leaves | Polyphenols, caffeine | MAE | P.atm, 700 W, 100 ml ethanol (50 %), 20:1[a], 4 min. Yield 29.59 % polyphenols in very short time | [42] |
| Citrus mandarin peels | Phenolic acid | MAE | P.atm, 152 W, methanol (66 %), 16:1[a], 49 s. High extraction efficiency and antioxidant activity of extract within shorter extraction time than UE and RE | [43] |
| Chinese herbs (*Rhizma polygoni cuspidati*, *Myrica rubra* leaves) | Polyphenolic compounds | VMAE | P. 60 kPa, 50 °C, ethanol (50 %), 10:1[a], 10 min. The extraction yields of resveratrol, myricetin with VMAE were higher than that with MAE or HRE, no obvious difference among the extraction yields for emodin and quercetin with VMAE, MAE, and HRE. Less solvent consumption in VMAE | [44] |

[a]Solvent-to-material ratio (ml/g)

tives and REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals); hence, all the solvents used during extraction and any residues that the finished products may contain must be shown to be harmless. Therefore, industries are forced to turn to more environmentally benign alternative solvents and use new natural substance extraction processes. Microwave hydrodiffusion and gravity extraction (MHG), using innovative ideas and in accordance with six principles of Green Extraction, has been designed and implemented in extraction of antioxidants [30–32]. Zill-e-Huma et al. first proposed this green technique to evaluate and compare with conventional solvent extraction on recovery of antioxidant flavonoids from onions; this innovative MHG technique offers exciting advantages such as high yield (81.5 %) with 41.9 % of flavonol content, with better retention of remaining flavonoids (55.9 %) in onion residues, shorter extraction time (23 min), cleaner feature (no solvent or water used), and creation of by- and co-products (extraction of onion crude juice retaining fresh organoleptic properties with higher phenolic content) at optimized power (500 W). They then found that extracts obtained by MHG exhibited the highest antioxidant activities in all tests so as to further confirm this preferred extraction method in comparison to the conventional methods. Vacuum microwave hydrodiffusion and gravity (VMHG) extraction, modified from the MHG method, has also been studied by Zill-e-Huma et al. for extraction of onion by-products. In this work, reduction of pressure at optimized microwave power could help increase the antioxidant activity of onion extracts against those obtained at atmospheric pressure. This solvent-free VMHG extraction is shown to be a more efficient and green technique, allowing the extraction of flavonols at lower reactor temperature, when compared to MHG and conventional solvent extraction methods [33].

MAE has also effectively used in extraction of other antioxidative compounds [34, 35]. Yang and Zhai used a Box–Behnken design to obtain the best MAE conditions, and subsequent comparison with conventional solvent extraction under optimal conditions has proved that MAE was rapid and highly efficient in extracting anthocyanins from purple corn cob [36]. Moreover, a new coupling ultrasound–microwave extracting technology was developed for lycopene extraction from tomato paste that was considered more efficient than UAE [37]. Further comparisons with conventional and innovative techniques have been carried out to verify the superiority of MAE. Hemwimon et al. have made a comparison between MAE and other conventional extraction techniques (maceration and Soxhlet extraction) and ultrasound-assisted extraction (UAE) in antioxidative anthraquinones extraction from roots of *Morinda citrifolia*. Higher recovery of anthraquinones extracted by MAE under optimal conditions [720 W, 15 min, 60 °C, 10 ml EtOH:$H_2O$ (80:20)] was found than with other techniques [38]. The main reason for this improvement was the dipole rotation of the polar solvent (methanol, ethanol, acetone, etc.) in the microwave field. On the other hand, the extract from MAE showed slightly lower antioxidant activity than that of Soxhlet extraction but significantly higher than that from maceration and UAE [38, 39]; this is because the long extraction time of maceration lets the extracts be exposed to an environment that may cause an unfavorable reaction such as oxidation and photolysis. Wang et al. also reported the various advantages of MAE used in extraction of aloe-emodin from aloe with regard to cell disruption, extract yield, extraction time, and solvent consumption [40]. Some other

comparative studies had been carried out to present noteworthy benefits of MAE when compared to other common extraction methods (maceration, heat reflux, Soxhlet, rotary, supercritical fluid carbon dioxide, and ultrasound-assisted) [41–43]. Vacuum microwave-assisted extraction (VMAE), as a new modified MAE method, was described for extraction of phenolic compounds [44]. The extraction yield of part polyphenolic compounds (resveratrol, myricetin) with VMAE was higher than that with MAE and HRE by an increase of 6.4–9.4 % and 7.9–29.5 %, respectively. However, for emodin and quercetin, there was no significant difference among the yields extracted by the three extraction methods, with the exception of less solvent consumption in VMAE.

### *5.3.2 Recovery of Natural Pigments by Microwave Assistance*

Colorants have long been used in a wide range of food products with the aim of enhancing the aesthetic value of foods. Increasing market demand has sent more and more artificial colorants and dyes synthesized from petrochemical origins into our lives, which has resulted in a rapid decline in the use of natural colorant and dyes. However, people are inclined to use natural colors because of their health, safety, and environmentally friendly properties, although some technical problems (lack of knowledge of extraction, difficulty in plant collection, etc.) have prevented natural colors from succeeding commercially. Microwave-assisted extraction, one of the innovative extraction techniques, has been applied to overcome the aforementioned limitation of natural color extraction (as explained in Table 5.3 with different examples).

Sun et al. used the MAE method to extract anthocyanins (Acys) from red raspberries. Twelve kinds of Acys were finally extracted without any destruction of its chemical structure and the Acys compositions were similar to those obtained by conventional solvent extraction [53]. Moreover, Liazid et al. developed a new method for analysis of anthocyanins in grapes and found that the solvent used in MAE is the most important variable for the best yield of Acys extraction [46]. In addition, other natural colors such as curcumin and carotenoids have been studied with the MAE technique, providing similar conclusions [37, 45, 49, 50]. Dynamic microwave-assisted extraction was used in extraction of safflower yellow and flavonoids in comparison with conventional methods [25]. This extraction process could be easily monitored and continuously measured by this derivative technique. Pasquet et al. compared another derivative MAE technique called vacuum microwave-assisted extraction (VMAE) with MAE on extraction of microalgal pigments [51]. They discovered that microwave can greatly solve the mechanical barrier: it is hard to extract pigment from microalgae with a strong frustule. Wang et al. also tried this technique to extract safflomin A from Chinese herbs and they considered VMAE the better choice for extraction of thermosensitive compounds [47]. Another interesting study of coupling methods used in lycopene extraction described that ultrasound and microwave-assisted extraction (UMAE) did obtain higher lycopene yield (97.4 %) with less solvent in shorter time (367 s) than that (89.4 %) obtained by UAE in 29.1 min [38]. This combined UAME

**Table 5.3** Natural food colors extraction

| Matrix | Analyte | Technique | Operating conditions and remarks | References |
|---|---|---|---|---|
| Tomato paste | Lycopene | UMAE | P.atm, US (40 kHz, 50 W)/MW (98 W), 21.2 ml ethyl acetate, 10.6:1[a], 6.1 min. 97.4 % yield of lycopene proved it to be a more efficient and attractive extraction method | [37] |
| Dried rhizomes of *Curcuma longa* L., turmeric | Curcumin | MAE | P.atm, 140 W, 8 ml methanol (as modifier) followed by 40 ml acetone, 20:1[a], 4 min. Dual heating phenomenon of solvent and matrix resulted 27 % more efficient extraction | [45] |
| Safflower (*Flos carthami*) | Safflower yellow | On-line DMAE | P.atm, 60 W, 4 ml methanol (60 %), 1.33 ml/mg[a], solvent flow rate 1.0 ml/min, 4 min. 11.35 % yield of safflower yellow obtained; extraction can easily be monitored | [46] |
| Red raspberries | Anthocyanins | MAE | P.atm, 366 W, 240 ml 1.5 M HCl–95 % ethanol (15:85), 4:1[a], 12 min. 98.33 % yield was obtained; 12 kinds of anthocyanins extracted without any destruction of its chemical structure | [47] |
| Paprika | Carotenoids | MAE | P.atm, 50 W, 60 °C, 30 solvent mixtures; acetone, dioxane, ethanol, methanol, tetra-hydrofuran (15 %, 30 %, 45 %, 60 %, 75 %, and 90 %), 2 min. Extraction efficiency increases with organic solvents | [48] |
| Cape jasmine | Yellow pigment | U-coloumn MAE | 60 °C, 700 W, 70–95 °C, 30.7 ml $H_2O$, 1.6 min. 50 % higher yield obtained in comparison to conventional method | [49] |
| *Rabdosia serra* (Maxim.) Hara. | Yellow pigment | MAE | P.atm, 464 W, 120 ml alcohol (95 %), 60:1[a], 5.8 min. Extraction rate increases up to 90.6 % in lesser time | [50] |
| Rubiaceae plants | Alizarin and purpurin | MAE | P.atm, 600 W, 120 °C, 20 ml methanol (30 %), 15:1[a], 20 min. Relative recoveries was higher than 140 %; 14 samples extracted simultaneously | [51] |
| Purple corn (*Zea mays* L.) cob | Anthocyanins | MAE | P.atm, 555 W, 1.5 M HCl–ethanol (95 %), (15:85, V/V), 20:1[a], 19 min. The highest total anthocyanin (185.1 mg/100 g) content is obtained by MAE method. More efficient and rapid than CSE. Six kinds of anthocyanins are identified | [36] |

| | | | | |
|---|---|---|---|---|
| Marine microalgae (*Cylindrotheca closterium*, *Dunaliella tertiolecta*) | Chlorophyll *a*, chlorophyll *b*, fucoxanthine, β-carotene | MAE, VMAE | MASE: P.atm, 25–100 W, 56 °C, acetone (100 %), 3–15 min, magnetic stirring. VMASE: 27 kPa, 25–100 W, 56 °C, acetone (100 %), 3–15 min, magnetic stirring. MAE is considered as the best extraction process for microalgae with frustules. Rapidity, reproducibility, homogeneous heating, and high extraction yields | [52] |
| Chinese herbs (*Flos carthami*) | Safflomin A | MAE, VMAE | MASE: P.atm, 50 °C, ethanol (50 %), 10:1[a], 10 min VMASE: P. 60 kPa, 50 °C, ethanol (50 %), 10:1[a], 10 min. Under the same conditions, VMAE is a better choice for extraction of thermosensitive compounds than MAE and HRE because of its higher yields | [44] |
| Grape skins | Anthocyanins | MAE | P.atm, 500 W, 100 °C, 40 % methanol in water, 5 min. The extraction solvent is considered the most important variable for the anthocyanin extraction | [53] |
| *Platycladus orientalis* (L.) Franco (Chinese herb medicine) | Flavonoids | On-line DMAE | P.atm, 80 W, 5 mL aqueous methanol (80 %), 500:1[a], 5 min. Higher extraction yield, shorter extraction time and smaller quantity of extraction solvent in DMAE than the conventional method | [24] |
| Red, yellow, white, and grelot onion (*Allium cepa*) | Flavonol | MHG | P.atm, 500 W, 23 min. The highest antioxidant capacity was observed for red onion, followed by yellow, white, and grelot onion; MHG remained the preferred method for flavonoids extraction in comparison to CSE | [31] |
| Sea buckthorn (*Hippophae rhamnoides*) by-product | Flavonoids | MHG | P.atm, 400 W, 23 min. Shorter extraction time, no solvent or water used; MHG extract shows much higher phenolic content with greater antioxidant activity in comparison to CSE | [32] |

[a]Solvent-to-material ratio (ml/g)

method could avoid production of hydroxyl radicals by ultrasonic cavitation effect in extracts, which may decompose lycopene with the presence of water in extract. As we discussed in the antioxidant section, green extraction has been developed as the future trend. A solvent-free microwave hydrodiffusion and gravity extraction of flavonol from onion was studied by Zill-e-Huma et al. [31]. This new original technique not only kept the typical advantages of the previous microwave-assisted method but also improved them in terms of extraction time, solvent, efficiency, etc. It is interesting to note that the microscopic observations of extracted tissues showed microwave irradiation could incur a strong disruption of plant tissue structure (cell walls, vacuoles, etc.), so that target compounds could be efficiently extracted with the help of microwave assistance.

**Fig. 5.5** MASE (100 liters per hour)

**Fig. 5.6** Microwave extraction of polyphenols from wood

## 5.4 Application of Microwave Extraction in Industry

Although there are a significant number of companies using microwave heating for extraction, few announce that they are using this technology. Nevertheless, we can cite a number of them that apply microwave extraction as a tool for obtaining extracts or active compounds.

- CODIF Company (www.codif-recherche-et-nature.com/) uses microwaves for extraction of active compounds, mainly colors and antioxidants, from plants, flowers, algae, and also microalgae.
- CRODAROM Company (www.crodarom.com) has used continuous microwave solvent extraction for obtaining antioxidants used in phytosanitary products for more than 10 years (Fig. 5.5).
- Oleos Company (www.oleos.fr) produces vegetable oil rich in carotenoids by microwave maceration of plant material and vegetable oil and combined with other innovative techniques such as ultrasound and pulsed electric fields.
- Drywood Company (www.drywood.fr) produces polyphenol extracts during drying of wood at an industrial scale (Fig. 5.6).

## Abbreviations

**CSE** Conventional solvent extraction
**CWE** Conventional heating water extraction
**DMAE** Dynamic microwave-assisted extraction
**HRE** Heating reflux extraction
**MAE** Microwave-assisted extraction
**MHG** Microwave hydrodiffusion and gravity extraction
**MWE** Microwave-assisted water extraction
**PMAE** Pressurized microwave-assisted extraction
**RE** Rotary extraction
**SOX** Soxhlet extraction
**UAE** Ultrasound-assisted extraction
**UMAE** Ultrasound microwave-assisted extraction
**VMAE** Vacuum microwave-assisted extraction
**VMHG** Vacuum microwave hydrodiffusion and gravity extraction

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# Chapter 6
# The Role of Microwaves in Omics Disciplines

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## 6.1 Introduction

### *6.1.1 Definition of Genomics, Proteomics, and Metabolomics*

Genomics addresses the study of all genes and their interrelationships to identify their combined influence on the growth and development of an organism. Similarly, proteomics is defined as the study of the expression, localizations, functions, and interactions of all proteins expressed by the genetic material of an organism. Finally, metabolomics is concerned with the quantitative measurement of all low molecular weight metabolites (sugars, amino acids, organic acids, fatty acids, and others) in an organism's cells at a specified time under specific environmental/biological conditions.

Oliver et al. [1] coined the term "metabolomics" in their systematic functional analysis of the yeast genome and proposed the challenge of discovering what each gene product does and how genes in a living yeast cell interact to shape molecular and systems biology. Based on evidence gathered over the past few decades [2], the flow of information from genes to function is linear and translated through transcripts, proteins, and, finally, metabolites.

Microwaves have been used to a dissimilar extent to facilitate work on analytical platforms in the different omics. Thus, metabolomics has for several decades taken advantage of the large number of methods developed under the umbrella of the reductionist theory in molecular biology by using MWs to improve sample preparation steps. By contrast, the other omics have adopted MWs mainly to accelerate

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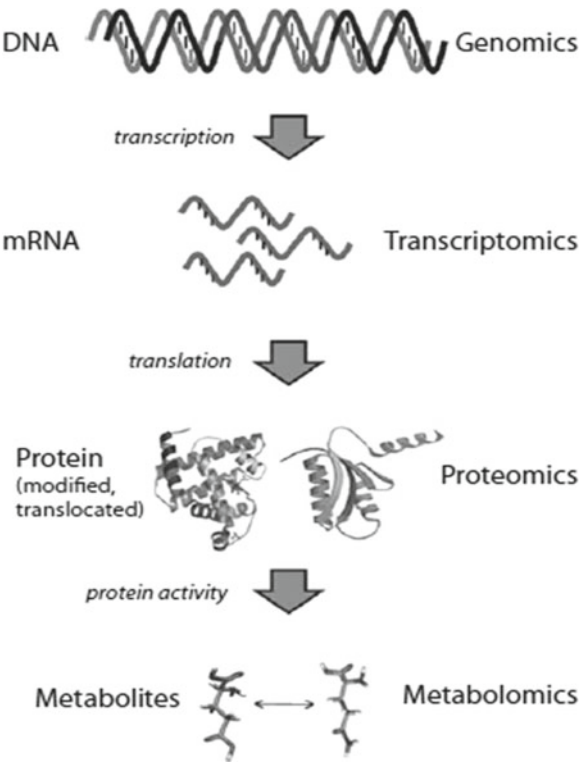


**Fig. 6.1** The 'omics cascade.' This cascade depicts the pathway from the gene to metabolism. The *grey arrows* indicate the biochemical processes, which communicate the genetic information to the next level of molecules (Reproduced with permission of Springer Verlag. From Michlmayr and Oehler [3])

sluggish operations such as sample preparation and detection, which are much slower when implemented with conventional methods without MW assistance.

### *6.1.2 The "Omics Cascade" and the Use of MWs Through It (Top-Down and Bottom-Up)*

The term omics dates from the early 1990s, when the Australian scientist Marc Wilkins was writing his Ph.D. thesis [3]. Tired of having to repeat the phrase "the entirety of all proteins expressed from a given genome at a specific time" dozens of times, he coined the term "proteome" to replace it and "proteomics" to designate the analysis of the proteome. Since then, the new terminology has expanded to various other fields of biomedical research including genomics, transcriptomics, metabolomics, lipidomics, glycomics, interactomics, and many more. The list is still growing. This chapter focuses on those "omics" that are directly connected to genes and their expression and constitute the so-called omics cascade (Fig. 6.1).

The specific methods used for omic analysis depend on the chemical properties of the analytes. Thus, the most common method in genomics uses DNA microarrays: single-chain oligonucleotides are spotted by a robotic printer onto a glass slide. The

oligonucleotides for mutation analyses are DNA stretches containing the wild type or the mutated sequence (DNA microarrays), whereas those for mRNA analyses are DNA stretches that are complementary to a specific mRNA (cDNA microarray). High-density oligonucleotide slides are manufactured by using a light-directed combinational chemical synthesis procedure to obtain thousands of different sequences in a highly ordered array on a small glass chip. For analysis of the genome or transcriptome in a tissue, nucleic acids (DNA or mRNA, respectively) are extracted from the sample, labeled with a fluorophore, and applied onto glass slides. Complementary DNA or mRNA can be hybridized to the corresponding feature on the glass slide while others are washed away. Hybridization is then viewed in a fluorescence scanner that provides a picture of dots of variable color intensity (Fig. 6.2).

Proteins possess a very complex chemical structure. In contrast to nucleic acids, which consist of only four distinct, but chemically very similar, building blocks, proteins are composed of 20 completely different amino acids. In addition to their highly diverse chemical properties, amino acids can bear post-translational modifications that further contribute to the complexity of protein analysis. All approaches to analyzing the proteome use an initial step intended to reduce such complexity, usually by using two-dimensional gel electrophoresis (2DGE) to separate the proteins as intact molecules according to isoelectric point and molecular weight (Fig. 6.2): this allows any modifications contributing to protein charge or molecular size to be identified. With the aid of fluorescently prelabeled proteins (DIGE technology), this method allows relatively small quantitative changes (20–30%) to be detected and affords sensitive quantitative analysis of disease-related proteome alterations as a result. Once identified, the protein concerned is cut out from the gel and digested into small peptides by proteases, the resulting peptide mixture being subsequently analyzed by mass spectrometry to obtain a highly accurate list of all masses (see Fig. 6.2).

Advances in omics technologies have resulted in the production of many biotherapeutics consisting of recombinant proteins or metabolites [4]. In proteomics, spectrometry is usually the tool of choice for the initial identification and subsequent complete characterization of proteins and their post-translational modifications (PTMs), and also of metabolites. Characterizing a single protein or a complex mixture of proteins by mass spectrometry (MS) typically involves (a) digestion of the protein(s) with a proteolytic enzyme or chemical followed by MS analysis (a protocol known as the "bottom-up" approach) [5]; or (b) fragmentation of the intact protein in the mass analyser of the spectrometer with no prior proteolytic digestion via high-energy dissociation (the so-called "top-down" approach) [6, 7]. Figure 6.3 depicts both approaches as applied to the mass spectrometric characterization of proteins. Obviously, only the bottom-up approach is possible with metabolites.

Bottom-up proteomics can be subcategorized into two types of analysis, namely, peptide mass fingerprinting (PMF) [9] and liquid chromatography (LC) coupled to MS/MS [10]. In PMF, peptides are analyzed in full (i.e., unfragmented), mainly by matrix-assisted laser desorption ionization (MALDI)–time-of-flight (TOF) MS analysis. Masses corresponding to the intact molecular weight of each peptide can be pieced together to identify a unique protein fingerprint, and spectra are interpreted visually or with the aid of a computerized search algorithm [11]. Alternatively,

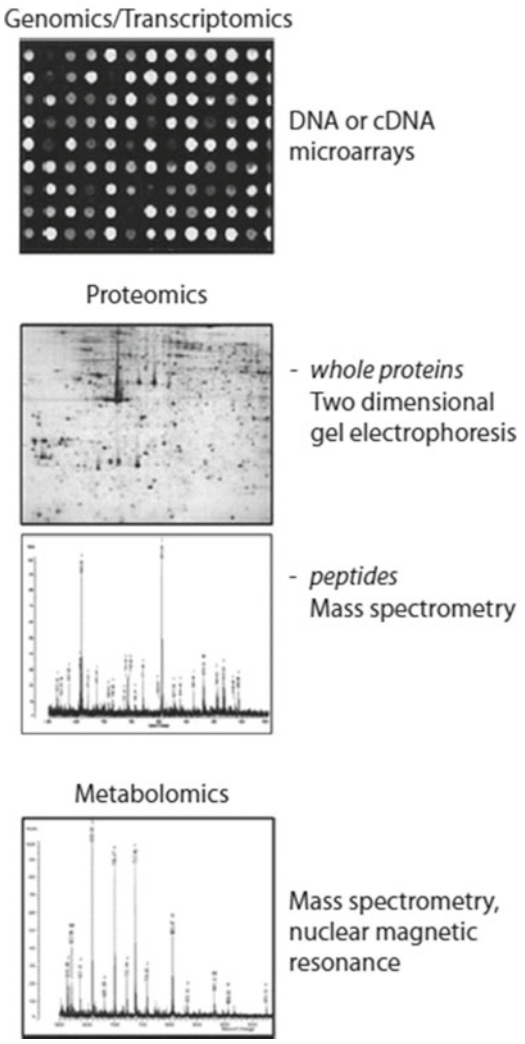


**Fig. 6.2** Technologies in omics analysis. For molecular profiling at different levels of gene expression different techniques are required. (Reproduced with permission of Springer Verlag. From Michlmayr and Oehler [3])

LC–MS/MS can be used to separate peptides by reversed-phase chromatography, which is typically coupled on-line to a mass spectrometer. Here, peptides are ionized as they are eluted from the chromatographic column and subsequently fragmented by tandem mass spectrometry (MS/MS) (see Fig. 6.2). Fragmentation can be induced by a number of processes occurring along the peptide backbone in the mass analyzer, which produce a series of characteristic fragment ions.

Although genomics and proteomics use a well-defined format imposed by both their characteristics and the longer time they have been around, metabolomics—the most recent of the great omics—uses less well defined formats, mainly as a result of its intrinsic features, namely: (a) the small molecules it involves are more difficult to categorize than are the objects of other omics; (b) in contrast to genes, transcripts,

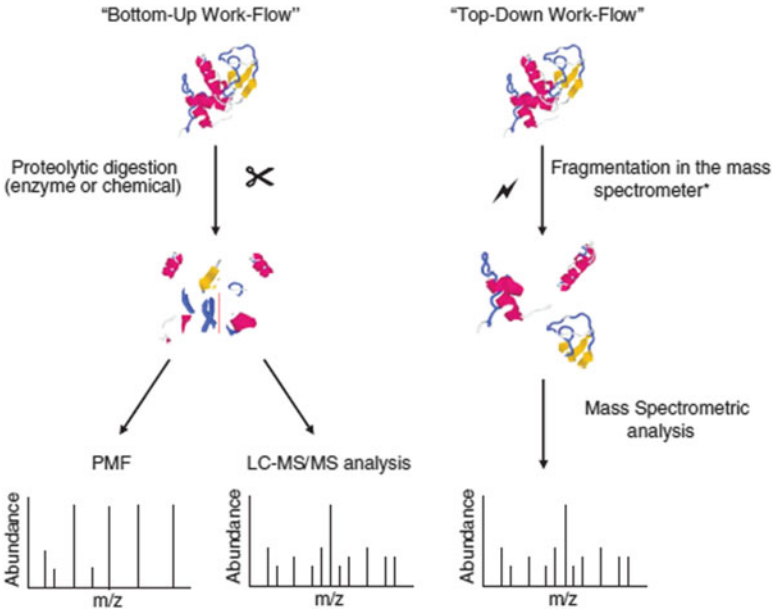


**Fig. 6.3** An overview of *bottom-up* and *top-down* proteomic work flows for the characterization of proteins. (Reproduced by permission of The Royal Society of Chemistry. From Lill [8])

and proteins, metabolites are not coded in the genome; (c) metabolites span widely different chemical classes (carbohydrates, amino acids, lipids) and physical states (solid, liquid, gas); (d) the techniques used to extract, separate, and analyze some metabolite classes are most often useless for others; (e) although nucleic acids and proteins can be detected with a single technique, metabolites usually require several such as capillary electrophoresis (CE), gas chromatography (GC), or liquid chromatography (LC) in combination with mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectrometry and, occasionally, a separation and/or preconcentration technique (Fig. 6.2); (f) plants contain more than 200,000 metabolites and humans about 10,000; (g) metabolite concentration ranges can easily span seven to nine orders of magnitude (from pmol to mmol). This work has and will continue to require the development of a variety of analytical platforms [12, 13] to meet the demands of metabolomics in its broad field of use.

### *6.1.3 The Superheating Theory and Other MW-Induced Phenomena*

The scientific literature abounds with examples where MWs have been used simply for rapid heating purposes. According to Stuerga et al. [14], the heating expeditiousness of MW can shorten the reaction times of chemical syntheses by

as much as 75%; however, this may simply be the result of superheating rather than increased nonthermal molecular perturbation [14, 15]. Many experts now believe that the effect results from the ability of MWs to superheat solvents beyond their normal boiling points. For example, water reaches 105°C before boiling in an MW oven, and acetonitrile boils at 120°C instead of its usual 82°C [16]. The increased boiling point obtained by superheating a pure solvent can be maintained while MW irradiation is applied. However, the presence of any ions or contaminants in the solvent can lead to the formation of so-called boiling nuclei, which aid the return of the overall solvent temperature to the "normal" boiling point [17]. Although this mechanism is more typical of organic chemistry reactions, it may also prevail in some omics reactions such as chemical cleavage or MW-assisted acid hydrolysis or digestion, which are conducted at higher temperatures.

The results of recent studies aimed at elucidating the exact mechanism of improved protein digestion by effect of MW irradiation suggest that MW energy causes polar molecules to align in a continuously changing electromagnetic field [18, 19]. This action results in permanent rotation of the molecules, which, in turn, produces heat that increases reaction rates. Trypsin autolysis, and chemical modifications such as deamidation, peptide degradation, and precipitation over longer digestion periods, may be behind this phenomenon. The catalytic effect of microwaves on proteolytic reactions is possibly a result of its enhancing dipolar rotation [20], boosting ion diffusion and heating. Vaezzadeh [21] assessed the potential of MW to cause molecular degradation, rearrangement, and alteration by irradiating a standard protein mixture without trypsin. The fact that no appreciable changes were observed suggested that MW energy is too low to break peptide bonds. Microwave irradiation raised the temperature of the water bath from 25°C to 55°C. The role of heating in the increased proteolysis efficiency was investigated by directly comparing MW-assisted and water bath-mediated digestion at 55°C, the former procedure being more efficient and revealing that nonthermal MW effects play a critical role in increasing the digestion efficiency. Therefore, direct heating of the sample in an MW oven may be more effective than indirect transfer of the heat from the tube walls in the water bath. It should be noted that no decrease in proteolysis specificity or significant post-translational modifications as a result of MW digestion were observed.

## 6.2 Microwave Equipment for Assisting Omics

A variety of MW devices have been used to perform analytical MW-assisted operations (particularly sample preparation) in the three major omics and their subdisciplines. Both monomode and multimode MW generation have been used for this purpose. Also, commercially available devices, laboratory-made designs, and household ovens have all been used to accelerate typical omics operations.

Monomode (also termed "single-mode" or "focused") MW devices are a standing-wave pattern by effect of interfering fields of the same amplitude but oscillating

in differing directions. A monomode MW system produces an array of nodes where the intensity of MW energy is zero and antinodes where it peaks [22]. One important consideration when using monomode MW systems is the distance of the sample from the magnetron. Thus, the sample should be placed within an appropriate distance from the anti-node of the electromagnetic wave path. During single-mode operation, usually only one vessel (or a cluster of small vessels) can be exposed to MW radiation at a given time. Consequently, monomode MW devices are typically used for small-scale drug discovery and specific omic studies [22]. However, monomode MW devices are especially useful whenever rapid heating is needed, a result of the sample being placed at the anti-node apex where the MW field density is maximal.

Multimode MW systems differ from their monomode counterparts in that they produce no standing-wave pattern, but rather induce chaotic MW dispersion. By inducing as much chaos as possible, an increased area can be subjected to effective heating; this allows multimode MW systems to accommodate much greater numbers of samples than monomode systems. As a result, multimode MW systems are typically used for the bulk/large-scale heating reactions typically involved in omic studies. Household MW ovens usually operate in this mode. One disadvantage of multimode MW-assisted heating is that temperature dispersion cannot be efficiently controlled and samples may be more susceptible to hot spots and uneven temperature distribution, a potential source of irreproducibility in the analytical results.

### *6.2.1 Dedicated Equipment*

A number of MW systems have been made commercially available with specific biological and biochemical applications in mind. For example, CEM markets a dedicated system for high-throughput digestion. The setup comprises a Discover system equipped with a screw-top container capable of holding multiple microvials or Eppendorf tubes together with an insert for a fiberoptic temperature probe. The fiberoptic probe is intended to help stabilize the temperature by monitoring the magnetron power while inducing simultaneous cooling to allow energy input while maintaining the relatively cool temperature required for the target reaction. This equipment is suitable for the digestion and/or extraction of metabolites (particularly from solid matrices).

A 45-ml vapor-phase hydrolysis vessel is available from CEM for use with the Discover MW unit that affords processing up to ten 300-ml samples in parallel. The system includes a valve panel to facilitate connection of the hydrolysis vessel to a vacuum and nitrogen source. The sealed sample vessel is alternately vacuum evacuated and purged with nitrogen to facilitate hydrolysis under inert, anaerobic conditions, and hence to prevent oxidative degradation of the sample components.

In addition, several companies have marketed systems with omics operations in mind. Such is the case with the CEM MARS 5, a microwave system equipped with polytetrafluoroethylene (PTFE) vessels for MW-assisted digestion. The MW-accelerated

reaction system has been designed for digesting, dissolving, and hydrolyzing a wide variety of materials in a laboratory setting. It uses MW energy to rapidly heat samples in polar or ionic solutions at high pressures. Its main use is for preparing samples for analysis by atomic absorption (AA), inductively coupled plasma emission spectroscopy (ICP), or gas or liquid chromatography.

The most frequently used model of laboratory MW generator in Japan is the "M-77" model. This and the MW-FISH model have proved more successful than other commercially available systems [23]. For example, they allowed <90% of formalin-fixed tissues and nearly 100% of alcohol-fixed tissues to be recovered irrespective of the time they were allowed to stand in a nonalcohol fixative.

A number of laboratories use the vacuum-assisted automatic MW histoprocessor MFX-800-3, which has an built-in vacuum system and temperature stabilizer to facilitate fast processing of tissues without damaging their original structure. This is an environmentally friendly, fast, highly economical, fully automatic microprocessor-controlled histoprocessor that can also be used in manual mode and is suitable for a wide range of uses. For example, it allows RNA extracted from tissue blocks to be readily used for polymerase chain reaction (PCR). Therefore, it may be useful to process tissues for molecular pathology applications.

Despite the proven efficiency of omics-dedicated MW-assisted devices, roughly one half of all peer-reviewed references to MW-assisted omics have used household multimode open-cavity MW systems such as those found in an everyday kitchen. Some modern cavity MW systems can deliver a very even field density, enabling MW heating to be used in a wide range of omics operations. However, these MW systems are prone to random dispersion of heat, which can lead to the generation of "hot spots." In the omics arena, it is imperative that samples be treated in a uniform manner and temperatures accurately controlled, especially when handling potentially heat-labile compounds. Some authors have suggested introducing beakers of cold water in household MW ovens to absorb excess thermal energy to minimize the effect of uneven temperature distribution. Others have proposed placing samples in pretested, fixed locations within the MW cavity to improve reproducibility between samples. However, application-specific MW systems provide more strict control and uniformity of heating for biochemical applications than do household MW ovens.

An unmodified household oven was recently used to irradiate Eppendorf containers for lysing in whole-blood samples; the samples were incubated with distilled water, boiled in the MW oven, and centrifuged [24], the supernatant being directly collected for PCR and restriction fragment length polymorphism (RFLP). For comparison, PCR and RFLP were performed on DNA from the same samples that was purified with the phenol–chloroform method and two commercial DNA extraction kits. The results obtained with MW assistance were qualitatively similar to those for DNA extracted with the other three methods, but the processing time was dramatically shorter with the former choice.

Before investing in an application-specific MW system, one should consider its technical and throughput benefits and limitations relative to a household MW oven. For reactions carried out at a high temperature, or reactions requiring no precise,

even distribution of dissipated heat (e.g., during staining and destaining of analytical gels or drastic digestions), it may be unnecessary to purchase a relatively expensive application-specific MW device. On the other hand, a commercial MW system is worth its price for high-throughput biochemistry laboratories conducting large numbers of proteolytic enzyme reactions daily.

### *6.2.2 Continuous/Batch Operation and High-Throughput Formats*

Although most MW devices work in a batch, discrete mode, some operate in a continuous manner in omics applications. In 2005, Comer and Organ reported a system consisting of a continuous-flow MW-assisted parallel capillary, which, although not designed with MW-assisted omics in mind, did show the potential for flow-based systems potentially useful for omics experiments [25]. The system included a reaction capillary of 200–1,200 mm in inner diameter and operated at flow rates of 2–40 ml/min, corresponding to a sample irradiation time of about 4 min. After leaving the reaction capillary, the reaction mixture flowed via Teflon tubing directly to a monitoring device or collection vessel. The capillary was irradiated with monomode MW of 2.45 GHz ranging from 0 to 300 W in power, and the reaction temperature was monitored with an internal infrared sensor.

In 2008, Hauser and Basile [26] reported an on-line MW system specifically designed for the cleavage of proteins at aspartic acid and also, optionally, for on-line reduction with the aid of dithiothreitol (DTT). A standard CEM reaction vessel was modified by drilling two threaded holes into the top cap and then fitted to the system with two adaptors and a 5-ml MW reaction loop made from fused silica capillary and connected to a tubing system. Because the actual MW heating unit itself was not modified, this allowed the system to remain operationally safe. The MW-assisted reactor was connected to an HPLC system; as a result, the length of time during which each sample was irradiated was dependent on the HPLC flow rate. The proteolytic products resulting from the cleavage reactions were either directly spotted onto a MALDI plate for MALDI–TOF/MS analysis or coupled to a reversed-phase LC column for further separation and analysis by electrospray ionization (ESI)-MS/MS. This setup has an enormous potential that may even afford further reductions in analytical protocols in the bottom-up analysis of proteins or other biochemical units.

High-throughput formats for simultaneously processing many samples are the most desirable for omic research. Many commercial MW systems designed for laboratory applications can be purchased with an autosampler to facilitate the sequential exposure of samples to MW-induced reactions. In omics, however, it is sometimes advantageous to prepare samples in a 96-well microtiter plate format, particularly for immunohistochemical analysis or proteolysis experiments, which are typically performed batchwise. Using a 96-well plate format during MW irradiation can in fact be highly advantageous and highly compatible with an automated

workflow. For many years, instrument manufacturers tried and failed to design an MW system compatible with 96-well plates until eventually, in 2007, the MARS open cavity multimode MW system from CEM was modified to accommodate this format. Indeed, this system can use a variety of turntables and vessels with the 96-well plate format for optimal high-throughput omics testing. In this format, the MARS MW system can be modified with a turntable to secure three individual 96-well microtiter plates. In addition, a temperature probe can be inserted into one of the wells for accurate temperature readout and control. This system was used by Zhu-Shimoni et al. [27] to develop and compare two enzyme-linked immunosorbent assay (ELISA) formats for measuring the amount of protein A leached from an immunoaffinity resin.

### *6.2.3 Solvents Used in MW-Assisted Steps in the Major Omics*

Microwave heating can cause a sudden rise in the internal temperature of a solution and lead to explosion. This potential requires checking the safety of all solvents to be used. In any case, explosions can be avoided by using a combination of low to moderate power with a longer exposure time.

The fact that solvents with a high dielectric constant (e.g., water) absorb increased amounts of MW energy makes their polarity a very important variable in MW-assisted omics extraction. Although polar solvents are widely believed to be better than nonpolar solvents for this purpose [28], there exists the opposite belief, based on the "broken cell-wall theory:" MW-transparent solvents are more efficient than MW-absorbing solvents.

The higher the dissipation factor is, the faster heat distributes through the extraction matrix and the faster it is transferred to the solvent [29]. Water has the highest dielectric constant ($\varepsilon \approx 80$) of all common solvents. However, its dissipation factor is lower than those for other solvents ($d \approx 1{,}500 \times 10^4$). Hence, the rate at which water absorbs MW energy is higher than that at which the system can dissipate heat. These phenomena account for the "superheating" effect observed with water as the solvent. Thus, intensely heating water may result in degradation of the analytes. Thus, it is better to use a solvent with a high dielectric constant and also a high dissipation factor to facilitate heat distribution through the matrix.

The possibility of introducing the extract directly into the analytical equipment to avoid the need to evaporate or redissolve the sample should always be considered, even though this requires the use of a solvent meeting the specific requirements of the analytical tool to be used. Metabolomic studies based on GC–MS require the use of volatile solvents and the derivatization of polar metabolites. LC–MS is subject to fewer limitations as regard the extraction solvent; however, the solvent used to inject the sample must be at least miscible with and, preferably, similar to the LC mobile phases, which, for typical reversed-phase separations, are usually aqueous eluents containing 5–50% of an organic solvent such as MeOH or ACN.

**Table 6.1** Sequence coverages (%) of protein digestions in various solvent systems with and without microwave irradiation [30]

| Proteins | $H_2O$ | 50% $CH_3OH$ | 30% $CH_3CN$ | $CH_3OH/CHCl_3/H_2O$ (49%/49%/2%) |
|---|---|---|---|---|
| Myoglobin | 100 (96) | 94 (100) | 94 (100) | 29(0) |
| Cytochrome *c* | 96 (100) | 95 (15) | 70 (14) | 39 (0) |
| Lysozyme | 36 (19) | 21 (6) | 30 (7) | 20 (4) |
| Ubiquitin | 42 (37) | 80 (15) | 53 (29) | 20 (20) |

Digestion efficiencies without microwave irradiation are indicated in parentheses
Digestion without microwave heating proceeded for 6 h at 37°C. All the reactions under microwave irradiation, except for those in the experiments that involved $CHCl_3$, proceeded for 10 min at 60°C. The experiments that involved $CHCl_3$ proceeded for 10 min at 50°C
*Source*: Reproduced with permission of Elsevier. From Lin et al. [30])

Some differences between omics also reflect in their most suitable solvents. For example, it has become increasingly popular to include a small amount of organic solvent in digestion buffers to partially denature the substrate protein and increase accessibility to the proteolytic enzyme with a view to accelerating proteolytic digestion. The amount of solvent added is usually small because too much solvent can denature the enzyme or induce precipitation of the substrate or enzyme. A study of MW-assisted tryptic cleavages in the presence of various organic solvents was undertaken to ascertain whether the solvent-enhancing effect was also present in MW-assisted digestion [30]. Using MW radiation in combination with an organic solvent such as methanol, acetonitrile, or chloroform was in fact found to boost tryptic digestions. The proportion of protein digested under MW irradiation increased with increasing amount of acetonitrile, methanol exhibiting the opposite trend. Table 6.1 shows the sequence coverages of protein digestion for various solvent systems in the presence and absence of MW radiation. The increased rates of protein digestion observed in the presence of an organic solvent were ascribed to denaturation of the protein and to differences in reaction temperature between solvent systems.

Sandoval et al. [31] investigated the effects of adding enzyme-friendly surfactants such as Rapigestt or an organic solvent (10–20% ACN) on the rate of MW-assisted PNGase F catalysis. Under these denaturing conditions in water bath-mediated incubations, enzymatic reactions were often accelerated by effect of increased accessibility of the active site of the enzyme to its substrate. The addition of an organic solvent had no substantial effect on the deglycosylation time; by contrast, that of Rapigestt reduced reaction times markedly. Thus, 0.1% Rapigestt shortened the MW-assisted reaction time to 10 min, albeit with considerable sample losses and precipitation; it was therefore chosen not to use this surfactant for high recovery of low-level materials [31].

Enzymes exhibiting catalytic action in nonaqueous media are often highly compatible with MW irradiation. For example, enzymes compatible with organic solvents tend to be extremely thermally stable; many retain their activity even after heating at 100°C over long periods [32]. The hypothesis behind this thermostability in nonaqueous environments—and hence of the compatibility with MW-assisted catalysis—is that, during freeze-drying, desiccation removes water molecules that

were hydrogen bonded to many surface residues and leads to the formation of a rigid structure by the enzyme side chains. The process can be reversed by reconstituting the freeze-dried enzyme in an aqueous medium.

Selecting an appropriate solvent for metabolomic applications is more difficult owing to the wide range of polarities spanned by metabolites. Each solvent has a different profile of compounds it can contain depending on its polarity and specific interactions. Also, no ideal, universal solvent for extraction exists. Therefore, in choosing an extraction method, one should previously consider what types of compounds are to be extracted. For example, plants can contain three different types of compounds as concern extraction, namely:

1. Nonpolar compounds, which are constituents of cell membranes, the cuticula or specialized cells (e.g., those of glandular hairs); most are either terpenoids or fatty acids and their derivatives.
2. Medium-polar compounds, which include most secondary metabolites. These compounds are involved in the interaction of plants with the environment (e.g., in defense mechanisms against pests and diseases) and possess biological activity, so they can be expected to have drug-like properties such as the ability to cross cell membranes.
3. Polar compounds. Much of the primary metabolism concerns water-soluble compounds such as sugars and products involved in the biosynthesis of amino acids and production of energy.

None of the available metabolomic methods for sample preparation can contend with such a broad variety of compounds; also, usually, only part of the compounds are contained in the analytical sample (i.e., in the solution obtained after sample preparation but before insertion into the instrument for analysis) [33].

pH is an important factor in aqueous solvents because acidic and basic conditions may lead to all types of artifacts. For example, the common metabolite chlorogenic acid (5-cinnamoyl-quinic acid) may be converted into its 3- and 4-isomers [34], or into a lactone. With methanol as the extractant, it is difficult to ascertain whether a methoxy group is naturally occurring or comes from the solvent. Ethanol does not have this problem because ethoxy groups are rare in nature.

Despite the previous assertion that a high dielectric constant together with an also high dissipation factor facilitate heat distribution through the matrix, acetone has been found to extract polyphenols more efficiently than methanol when, in fact, the latter has a higher dielectric constant and dissipation factor than the former. These results can be interpreted in the light of the aforementioned broken cell-wall theory, based on which MW-transparent solvents are more efficient than MW-absorbing solvents. For example, the amount of phenols obtained by MW-assisted extraction for 4 min were comparable to those of conventional extraction for 2 h. With the exception of water as solvent, the amounts of total phenolic compounds obtained with MW-assisted extraction were equal to or greater than those obtained by refluxing, thus indicating a reduction in extraction time and an increase in extraction efficiency in terms of relative amounts of phenolic compounds extracted.

## 6.3 Microwave-Assisted Steps in Genomics

Both sample preparation and detection in genomics have been found to benefit from MW assistance, with dramatic shortening of operating times and/or improvements in sensitivity in most cases.

### *6.3.1 Microwave Assistance to the Key Tool in Genomics: The Polymerase Chain Reaction (PCR)*

Two indispensable tools in molecular biology are the polymerase chain reaction (PCR) and a variation of this technique known as "rolling circle replication." Both methods allow nucleotide material to be amplified by several orders of magnitude and have revolutionized the fields of diagnostics, forensics, and biomedical discovery since their inception.

The vast majority of PCR-based procedures use a heat-stable DNA polymerase; this assembles new strands of DNA from free nucleotides, which are aligned along a single-stranded DNA template (a primer). These reactions require high temperatures to ensure efficient separation of DNA double-helix strands, and these are typically provided by thermocyclers. Heating is alternated with cooling to allow DNA synthesis. The ramping time (the time taken by the thermocycler to raise the temperature to the required level) has been optimized many times during the past decade to reduce the time needed for each PCR cycle. The slow dissipation of transferred heat from a thermocycler typically limits useful reaction volumes for adequate heat penetration within a reasonable time to about 0.2 ml.

In 2003, Fermer et al. [35] assessed the usefulness of MW radiation as the heat source for the PCR. They used a single-mode MW cavity for preliminary tests. The lack of control of the monomode system meant that the critical cooling period could not be applied with MW control and also that there was no means of accurately measuring the temperature in the reaction mixture. To offset these shortcomings, Fermer et al. performed the MW treatment in transparent polypropylene tubes that were transferred by hand to a temperature-controlled heating block for each cycle of primer annealing and determined the energy content of each irradiation pulse empirically. Both plasmid and chromosomal DNA were thus successfully amplified in what were the first of a series of MW-assisted PCR developments; Taq polymerase remained intact and fully functional even after 25 PCR cycles with MW irradiation [35].

Fermer et al. [35] subsequently developed an effective method for milliliter-scale PCR using highly controlled MW thermocycling [36]. They found high-density in situ MW heating to be in many ways superior to traditional heating-block heating as it avoided large temperature gradients and hot walls in the reaction vessel, all of which led to more precise control of the reaction. By refining the method, the authors obtained a concentration of PCR product of 10–30 nM after 33 s and an amplification

efficiency of 92–96% in 94 min [32]. Neither Taq polymerase nor the nucleotide sequences used were destroyed by prolonged MW irradiation; also, the PCR reaction benefited from MW assistance.

Rolling circle replication is a nucleic acid replication method whereby one can rapidly synthesize multiple copies of circular molecules of DNA or RNA such as plasmids, the genomes of bacteriophages, and the circular RNA genome of viroids [37].

In 2006, Yoshimura et al. [38] published a paper on MW-assisted rolling circle amplification. The reaction was performed in a volume of 25 ml, using 1 ml of primer template mixture and continuous MW irradiation at 120–160 W while keeping the reaction mixture at 65°C. Based on the results, a PCR rolling circle reaction under MW irradiation was more effective than one under conventional heating-block heating.

### *6.3.2 Other MW-Assisted Sample Preparation Steps in Genomics*

Samples for genomic studies are often prepared by cell fixation or cell lysis.

#### 6.3.2.1 Cell Fixation

Cell fixation is used primarily to reduce DNA (and protein) alterations by preserving the samples, as well as for embedding. Archived formalin-fixed, paraffin-embedded tissue (FF-PET) is the usual source of DNA available for procurement by virtue of its easy storage and transport. In general, DNA isolation from FF-PET involves three unique steps, namely, (1) tissue deparaffination, (2) tissue digestion, and (3) DNA purification.

*p*-Formaldehyde is the reference fixative for flow cytometry (FCM) and in situ hybridization analyses. However, this compound can cause nucleic acid alterations by forming methylene bridges between functional groups in nucleic acids [39] and make amplification of target DNA sequences in *p*-formaldehyde-fixed cells more difficult [40]. Also, aldehydes are incompatible with proteomics because they result in protein cross-linking, which reduces protein recovery and complicates analyses [41]. Hence, the urgent need to develop a suitable fixation procedure to overcome the problems inherent in the use of aldehydes. Bödör et al. [42] set out to demonstrate that RNA isolated with the help of MW energy is suitable for quantitative expression analysis. Starting from the reported description of some alternative fixatives causing reduced nucleic acid fragmentation [43, 44], they planned to integrate these reagents into their MW-assisted histoprocessing procedure to obtain more intact DNA or RNA samples. Their results not only confirmed previously reported data but also showed that the entire fixation and embedding process as performed in a vacuum-assisted MW apparatus provided samples amenable to DNA and RNA isolation for PCR and RT-PCR tests [42]. In addition, histoprocessing by

use of MW-based sample preparation reduced processing times relative to conventional methods; also, it decreased chemical costs by a factor of ten and allowed perfect preservation of tissue and cellular structures. The templates obtained by isolating DNA and RNA sufficed for PCR and RT-PCR studies. These results confirmed the previous data of Hsu et al. [45], who obtained high quality of DNA for Southern blot hybridization from MW-fixed, paraffin-embedded liver tissues. The reagent- and cost-effectiveness of embedding techniques using MW acceleration in one or more histoprocessing steps have expanded their use to an increasing number of laboratories. There have been claims that tissues processed in this way should also be suitable for molecular pathology analyses.

A comparison of template preparation by using a household MW oven or a boiling procedure revealed that aqueous solutions of DNA exhibited no increased absorption of MW energy [46]. Low-level 2,450-MHz pulsed-wave MW exposure induced no DNA damage detectable by the alkaline comet assay [47]. Also, no mutagenic effects were detected after 2,450-MHz radio frequency exposure of various tissues of lacZ-transgenic mice exposed in utero [48].

Fixing microbial cells in a manner as rapidly as possible is the key to maintaining a low sample temperature and hence to reducing alterations to proteins and DNA, a major hurdle to molecular research into natural (particularly oceanic) microbial cells because proper preservation is the key to facilitating further land-based molecular analyses. With this aim, Mary et al. [49] examined the use of MW for cell fixation before high-speed flow cytometric (FCM) sorting to define the metaproteomes and metagenomes of key microbial populations. Their results showed that cells fixed under the optimal MW fixation conditions had flow cytometric signatures similar in light scatter, DNA, and pigment contents to unfixed control cells. Microwave-assisted fixation of a natural sample provided cell recoveries ranging from 88% for pico-eukaryotes to 100% for *Prochlorococcus* and a loss of bacterial activity in fixed cells. The study demonstrated that MW irradiation is a powerful method for in situ proteomic and genomic studies requiring sample preservation before analysis. Moreover, its combination with flow-cytometric sorting can facilitate a wide range of applications in environmental microbiology and should improve existing understanding of natural microbial communities and their functioning. So far, this method has only been applied to pigmented cells. For microbial cells, which lack fluorescent pigments, it could be combined with in situ hybridization with specific rRNA probes [50, 51]. However, one should be aware that MW energy changes with time; also, the equipment should always be calibrated to standardize the fixation protocol before use, and the efficiency of MW fixation should be experimentally confirmed. Further study with dedicated MW equipment, preferably based on focused rather than multimode MW, is needed [49].

#### 6.3.2.2 Microwave-Assisted Lysis (MAL)

The most common preliminary operation in genomic studies is lysing of cells to release DNA. A number of methods for DNA extraction have been reported in the

past few years. These methods are often labor intensive, time consuming, costly, or limited to a small number of samples per run. Most available protocols include growing mycelia in a liquid culture and subsequent freeze-drying or maceration in liquid $N_2$ and grinding of the frozen material to break cell walls [52]. Other methods require the use of specific equipment such as grinders or filters [53–55]; also, they use excessive amounts of starting material. Many use toxic chemicals such as phenol or chloroform [56], which are hazardous and can cause the loss of DNA, an especially undesirable outcome when attempting to isolate DNA from a small number of cells [57]. In addition to being sluggish, these methods use much glassware, which is inconvenient when handling a large number of samples [52].

A number of methods have recently been developed to isolate fungal DNA for PCR that afford the simultaneous treatment of many samples. These methods use minute amounts of starting material and are quite fast. The method reported by Griffin et al. [57] uses tissue from freshly grown fungal isolates and subjects it to seven rounds of freezing/thawing in a crushed dry-ice/ethanol bath and a boiling-water bath. Rapid freezing and boiling cycles were also applied by Manian et al. [58] to mycorrhizal fungi, albeit with liquid nitrogen. Other methods use salt extraction buffers to weaken cell walls [59] and are applicable to a host of substrates including plant tissue, fruits, vegetables, fungi, and insects. Cold acetone treatments or glass beads (occasionally in combination with LiCl) have also been used to weaken cell walls before lysis or DNA extraction [60–62].

Some methods using a household MW oven have been shown to alter cell walls and membranes to an extent facilitating the action of lysis buffers in further breaking open cells and organelle membranes. These methods, which are applicable to mycelia and spores, can be implemented with inexpensive equipment and reagents [63, 64]. The DNA extracts obtained are pure enough for fragments about 0.5–1 kb to be amplified. Microwave irradiation of samples was found to denature tissue DNA when used for in situ hybridization [65] or as a pretreatment step for DNA extraction from fungi, plants, and animals [66, 67]. In addition, it destroyed cell structures and exposed DNA [67].

Poorly planned and executed comparative research into conventional lysis protocols and MW-assisted procedures has led to spurious conclusions about the suitability of MW for facilitating lysis. Such is the case with work by De Maeseneire et al. [68], who compared the usefulness of ten DNA extraction methods for *Myrothecium* and *Aspergillus*, and assessed the suitability of the resulting DNA for PCR, using the *Aspergillus* strain as reference. In nine of the methods, DNA was extracted from mycelium before PCR. A final assay used mycelium directly in the PCR mixture. All methods used tiny amounts of mycelium grown on solid medium. A comparison of the nine methods used to screen fungal transformants for specific genetic modifications for a variable time (45 min to 6.5 h) revealed that the best was the protoplast method proposed by van Zeijl [69]. The methods were implemented as described in the literature, with no changes for the given sample and using a household oven in methods assisted by MW irradiation. The duration of the MW-assisted method (1.5 h) suggests that neither the best possible MW equipment nor the most suitable MW operating conditions were used.

In any case, MW irradiation dramatically shortens lysing, which has been applied to widely different types of cells by using a household oven in most instances. By way of example, whole-blood samples were pretreated in 10-μl aliquots that were first mixed with 490 μl distilled water in 1-ml Eppendorf tubes; then, the mixture was incubated at room temperature for 2 min, the tube cap punctured with a needle, and the tube placed in a heat-resistant jar containing 500 ml water that was heated in a household oven at 700 W for 7 min. Finally, the Eppendorf tubes were centrifuged at 14,000 rpm for 30–60 s and the clear supernatant used directly for PCR [24].

Bacteria were lysed with gold bowtie deposits on a glass slide that was heated in an MW device for 13 s. Bacterial suspensions (2 ml) were placed in wells previously sterilized by rinsing with 70% ethanol and air dried. A variety of configurations of gold deposited on glass slides was tested, and the gold bowtie was found to be the best for efficiently lysing *Salmonella* with the aid of MW irradiation. Overnight bacterial cultures subjected to MW-assisted lysing with gold triangles were found to be fragmented into a range of sizes. Examination of lysed bacteria by electron microscopy showed bacteria with blurred edges surrounded by clumps of lysed debris and bacteria from unlysed samples to exhibit distinct edges against a clear background [70].

The aforedescribed and various other sample preparation methods reported so far testify to the usefulness of MW irradiation for accelerating and/or improving lysis in genomics and similar studies. For example, Man and Burgar developed a novel antigen unmasking protocol for immunohistochemistry and subsequent PCR amplification also using, at least partly, MW oven radiation [71]. Also, Lou et al. used MW and thermal cycler boiling methods to prepare cell samples before PCR for human papillomavirus detection [72], and Ekuni et al. [73] found MW-accelerated demineralization to preserve RNA integrity and facilitate RTPCR amplification in dento-alveolar tissues.

### *6.3.3 Microwave-Assisted Detection in Genomics*

Microwave energy dramatically increases the sensitivity and expeditiousness of two detection techniques used in genomic studies, namely, MW-assisted fluorescence in situ hybridization (MW-FISH) and MW-accelerated metal-enhanced fluorescence (MAMEF).

#### 6.3.3.1 Microwave-Assisted Fluorescence In Situ Hybridization (MW-FISH)

Fluorescence in situ hybridization (FISH) is an essential tool of diagnostic pathology for identification of amplifications and translocations of genomic components in human tumors [74], especially in hematological malignancies [75], childhood tumors [76], and sarcomas [77].

The additional effort required to collect and handle samples usable for FISH in poorly preserved formalin-fixed paraffin-embedded tissues led, a decade ago, to using microwave irradiation and developing MW-assisted fluorescence in situ hybridization (MW-FISH). Microwave irradiation had previously been shown to provide more accurate and efficient signal detection in lymphocyte cyto-smear and cancer cell stamp preparations [78, 79]. There are now several variants of MW-FISH protocols that have been standardized by adaptation to particular laboratory requirements [80].

The MW-FISH protocol is especially powerful for arrays consisting of specimens that have been fixed in various ways, stored, and, occasionally, even neglected for a long time. At present, MW-FISH dramatically increases the efficiency of signal retrieval from each of hundreds of tissue specimens in arrays (from 40% with a conventional protocol to 95% with MW-FISH). Also, MW-FISH is compatible with most bacterial artificial chromosome probes and can be implemented in various ways, as follows.

*FISH with intermittent MW irradiation.* The steps of the FISH protocol with intermittent MW irradiation are depicted in Fig. 6.4. Sections of tissue blocks that have been stored in formalin for a month or longer usually yield no signals when subjected to the ordinary FISH protocol without MW irradiation. Also, only a modest improvement is obtained with a protease treatment. Some pathologists have used various protocols including MW-FISH and had difficulty in obtaining consistent results. By contrast, the MW-FISH protocol yielded acceptable signals in 95% of blocks [81]. Although the mechanism by which MWs improve signal sensitivity is unknown, scanning electron microscopy analysis of formalin-fixed paraffin-embedded tissues revealed a looser intranuclear matrix after MW exposure [81].

The need for MW-FISH depends on the particular material and its condition. Thus, there have been a few reports [82, 83], mainly in regard to brain tumor samples, of adequate FISH signals being obtained with commercially available protocols, but it is common knowledge among histotechnologists that no commercial protocols requiring stringent protease treatment are readily feasible [84–86].

*Repeated FISH application.* As in rehybridizing of the probe on the membranes used for Southern or Northern blotting, histological sections for multiple hybridizations with FISH probes by stripping the dyes used for the first hybridization have been reused and should be useful for multiple probings of rare sections or small microscopic lesions. However, repeated FISH has only been successful when implemented in accordance with the MW-FISH protocol.

*Double immunohistochemical staining.* Microwave-assisted double staining with an immunohistochemical protocol allows the localization of gene products such as membrane proteins and their genomic signals in the same cells. A detailed description of the procedure can be found elsewhere [87].

The MW-FISH protocol has proved especially useful in retrospective investigations of tissues fixed and preserved over long periods. Its success rate with randomly selected pathology archives (70–95%) exceeds that of the conventional protocol (~40%). The MW-FISH protocol and current availability of human genome information, together with information on a variety of other histopathological attributes, have paved the way to the exploration of specific, large-scale genomic changes in

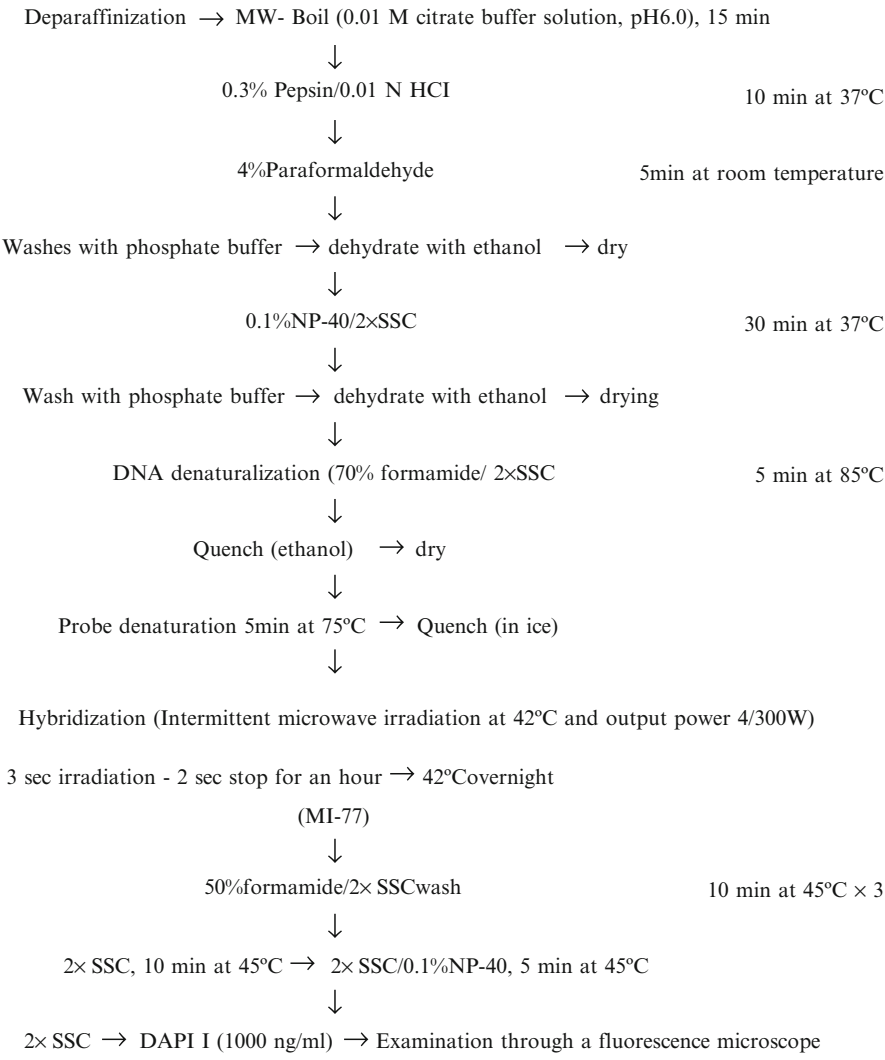


**Fig. 6.4** Flowchart of the microwave-fluorescence in situ hybridization (MW-FISH) protocol. (Reproduced with permission of Oxford University Press. From Sugimura [81])

human tumor tissues, even at an incipient stage. In practice, this protocol is very useful for retrospective surveillance of amplicons in tumor tissue by use of hundreds of bacterial artificial chromosome clones and many specimens in the form of a tissue microarray. The MW-FISH protocol holds promise for expanding the range of treated materials, harvesting more genetic information, and shortening the overall procedure [86].

#### 6.3.3.2 Microwave-Accelerated Metal-Enhanced Fluorescence (MAMEF)

Malicka et al. [88] introduced a new approach to DNA hybridization assays based on metal-enhanced fluorescent (MEF) whereby changes in fluorescence intensity allow one to track the hybridization of a complementary target on a solid surface. These authors obtained an increase of approximately 12 fold in fluorescence intensity from the DNA hybridization assay on silver island films relative to the fluorescence intensity from the same assay but under free solution conditions. In addition, the photostability of the tagging fluorophore was significantly improved. These achievements were made possible by the MEF phenomenon, which soon became a powerful tool for fluorescence-based assays [89–91]. A dramatic improvement in MEF was achieved with a new platform technology, MW-accelerated metal-enhanced fluorescence (MAMEF), which combines the benefits of MEF with the use of low-power MW to kinetically accelerate bioaffinity reactions. In MAMEF technology, the MEF phenomenon allows much more sensitive assays to be developed, and low-power MW heating ccan to be used to kinetically accelerate assays within seconds, thereby significantly reducing bioassay run times. In this way, MAMEF provides enables ultrafast and ultrabright immunoassays; for example, a model DNA hybridization assay spanning the concentration range 50–1,250 nM was thus kinetically completed within 20 s. The hybridization kinetics was accelerated more than 60 fold as compared to an identical hybridization assay run at room temperature. The MW-induced temperature jump in the bulk medium was calculated to be about 5°C by using a thermally responsive fluorophore. Fluorescence lifetime and fluorescence resonant energy transfer tests recently showed that MW irradiation induces no structural or environmental changes in proteins [92]. The temperature of the bulk medium above the silver island films was calculated to increase from 23°C to 28°C during a 20-s DNA hybridization assay. However, the temperature rise around silver nanoparticles is believed to be much higher by the effect of localized heating around/on the nanoparticles [92], resulting in faster hybridization kinetics. Silver island films serve three purposes in hybridization assays: as a platform for immobilizing the anchor probe on a solid surface; as enhancers of the emission intensity; and for localizing heat delivery.

MAMEF technology holds promise as an inexpensive, ultrafast, ultrasensitive alternative to DNA target detection using relatively inexpensive equipment.

Example applications of this new, rapid diagnostic technique include the detection of DNA from *Bacillus anthracis* spores and vegetative cells within 1 min (spore lysing for 30 s and sample preparation included) [93, 94]. The target was a highly conserved region within the gene encoding protective antigen (PA) [93]. MAMEF has also been used to successfully detect DNA from less than 100 colony-forming units (CFU)/ml in *Chlamydia trachomatis* within 40 s [95]. In addition to being an ultrafast, sensitive, specific assay using relatively simple but cost-effective technology, MAMEF can be implemented in 96-well plates [96] and hence affords multiplexing [97].

One very recent application of MAMEF to the *Salmonella* assay was capable of lysing and detecting 1 CFU suspended in 1 ml bacteriological medium. The time to

detection (processing time excluded) was only 30 s. These levels of expeditiousness and detection limit greatly surpass those of all currently available assays. Thus, Nga et al. [98] recently reported a multiplex real-time PCR assay targeting *Salmonella typhi* and *Salmonella paratyphi* A. The sensitivity of the assay on blood samples was low, with only 42% sensitivity for *S. typhi* and 39% for *S. paratyphi* A. Thus, low sensitivity was most likely the result of the poor detection limit of the assay. The sensitivity of nucleic acid-based detection can be markedly increased by introducing an incubation step, albeit at the expense of markedly increasing the duration of the assay and hindering adaptation for use as a point-of-care diagnostic test. For example, Zhou and Pollard [99] overcame low sensitivity from small sample volumes by including a 3-h incubation step in tryptone soya broth containing 2.4% ox bile before detection of *S. typhi* by PCR. They obtained a detection limit of 0.75 CFU/ml in blood; however, the overall protocol took almost 8 h to complete. Moreover, the need for an incubation step in a culture medium excluded its use as a point-of-care diagnostic test. Similarly, a fluorescence in situ hybridization (FISH) method for the detection of *Salmonella* spp. using a novel peptide nucleic acid (PNA) probe and including an overnight enrichment step, provided 100% sensitivity and 100% specificity and was able to detect 1 CU per 10 ml blood [100]. The length of the enrichment step (overnight) makes this assay neither rapid nor adaptable as a much-needed point-of-care diagnostic test for invasive *Salmonella* disease. One molecular test requiring no enrichment is that of the Lightcycler SeptiFast Test MGRADE kit (Roche Diagnostics, Germany), which provides a commercial real-time PCR assay. This kit detects and identifies the 25 most common pathogens known to cause bloodstream infections directly from whole blood in 6 h but does not target *Salmonella* [101]. Hit rates of 70–100% were achieved for 23 of 25 organisms at 30 CFU/ml, but only 15 of 25 at 3 CFU/ml, in serial experiments on ethylenediaminetetraacetic acid (EDTA)–blood samples spiked with variable concentrations of bacterial and fungal reference organisms. These results suggest that the assay may not be as sensitive as blood culture, which has a theoretical sensitivity of 1 CFU. However, two studies indicate that the Lightcycler SeptiFast is more sensitive than blood culture; thus, it detected target DNA in some samples that were negative by blood culture [102, 103]. This method has the disadvantages that it includes a sample preparation step requiring the use of a centrifuge and that the time needed for detection is rather long. On the other hand, it affords (a) sensitive, specific MAMEF-based detection of *Salmonella*; (b) detection in only 1 ml of sample, which is a lower volume than those used in previously reported methods (and provides proof-in-principle that MAMEF can process large volumes); (c) lysing and detection of *Salmonella* without centrifugation or washing; and (d) detection in blood. These features can be expected to facilitate the development of a multiplex MAMEF-based *Salmonella* assay for efficient detection of the chromosomal oriC from blood-borne *Salmonella* and for further determining whether the serovar is *Salmonella typhimurium* or *Salmonella enteritidis*, the two non-typhoidal salmonellas (NTS) most commonly isolated from invasive sites.

Other recent applications of MAMEF have shown its usefulness for detecting targets in complex biological samples such as blood by biotinylated BSA (b-BSA) [101]. Although detecting protein and DNA targets in blood in this way is possible, the sample must be diluted before detection. Ongoing research has led to the

development of various methods for lysing red and white blood cells to reduce the viscosity of the liquid and facilitate mass transport of biological components to the surface during MW-accelerated processing, and hence overall fluorescence detection, as well as the release of bacteria from white blood cells.

The key assets of MAMEF technology for detection of pathogens in blood are as follows: (a) a high expeditiousness and sensitivity; (b) the procedure affords multiplexing (e.g., up to three DNA or protein targets can be identified in a single sample well within 20–40 s); (c) well fluorescence can be detected with a variety of standard, inexpensive sample well-reader technologies; (d) the assay platform requires no washing to remove excess fluorescent probe or labeled DNA/antibody; (e) chambers are disposable, which minimizes the risk of cross-contamination; (f) no centrifugation is needed; and (g) the assay can be made quantitative by comparing levels of fluorescence to a standard curve. These attributes make MAMEF amenable to development into a point-of-care device usable by people with minimal training.

## 6.4 Microwave-Assisted Steps in Proteomics

### *6.4.1 General Overview of Proteomic Steps*

The first step in proteomic research work is the separation of proteins, the electrophoretic mobility of which is dictated by the length of the polypeptide chain or molecular weight, as well as by higher-order protein folding, post-translational modifications, and various other factors. After proteins are separated on the gel, the sample is usually fixed with a reagent to mobilize the gel and stop migration or dispersion. Fixation is typically performed with highly purified methanol to clean up the gel from any residues of sodium dodecyl sulfate (SDS) running buffer. After fixation, the gel is stained with, for example, Coomassie blue or silver stain. Traditional staining protocols involve immersion of the gel or membrane into the stain solution for a long time, often overnight incubation. After staining, the gel is destained to remove background stain and allow the bands for the target proteins to be visualized. Proteins can also be electroblotted from the gel onto polyvinylidene fluoride (PVDF) membranes to facilitate long-term storage of samples and render them more compatible with some analytical techniques such as Edman degradation. PVDF membranes can be stained and destained in the same manner as SDS-polyacrylamide gel electrophoresis (PAGE) membranes.

Whichever the origin (total protein or subcellular fractionation), biological samples to be subjected to two-dimensional electrophoresis require a pre-treatment to release all protein components present: these are essentially lipids, low molecular mass fatty nucleic components of organic and inorganic nature, and cofactors such as vitamins, salts, and inorganic ions. Removing these components is an art, the efficiency of which depends on the quality of the preparation and hence on the success or failure of the procedure. Any technique used to remove these components must meet certain conditions: (a) it should not alter the protein profile so as to avoid irretrievable losses of

certain proteins; (b) nor should it alter proteins (e.g., by using conditions where endogenous proteases are active and can proteolyse some sample components); and (c) it should involve as few operations as possible, and the resulting preparation should be compatible with the first-dimension gel (targeting) or any procedure alternative to 2DE analysis. One critical point here is the presence of salt buffers used to obtain the preparation. For example, Tris, phosphate saline (PBS), and HEPES, which are often used in biochemical applications, focus on one region of the gel, if present in the final preparation, because this region appears "empty" of proteins. The total concentration of ions in the sample should not exceed 40 mM; above this limit, the sample conductivity will be too high and will lead to overheating and deterioration of the gel.

Sample preparation usually includes the following steps:

1. Extraction or solubilization of proteins
2. Removal of lipids by extraction with an organic solvent
3. Removal of nucleic acids by nuclease digestion, coprecipitation with basic compounds, or ultracentrifugation

In some protocols, salts are removed by changing the buffer sample solution or by precipitation targeting total proteins with trichloroacetic acid in acetone and redissolution in the sample solution.

### *6.4.2 Microwave-Assisted Fixation, Staining, and Destaining*

Microwave irradiation has been successfully used to accelerate fixation, staining, and destaining since the early 1990s; however, the first citation on record appears to be that of Nestayy et al. [104], who conducted an in-depth study into the effect of MW irradiation on the staining of proteins in gels or membranes with a variety of common stains. Microwave-assisted staining with Coomassie blue, SYPROs ruby, silver stain, and colloidal gold protocols was evaluated. These authors found that the traditionally time-consuming process of staining and destaining gels was significantly accelerated by the use of MW. They used a household MW into which the gels, held in Petri dishes or microwavable containers, were placed for irradiation. They hypothesized that the faster staining and destaining observed was mainly caused by heat produced by the MWs, maximizing the efflux and influx of solvent and solutes from the gel or membrane. After separation by SDS-PAGE, proteins were identified by in-gel tryptic digestion, followed by tandem mass spectrometric characterization. Nestayy et al. went on to monitor the effect of MW-assisted staining on subsequent mass spectrometric analyses. They observed increased proteolytic cleavage after MW-assisted staining compared to conventional methods (e.g., room-temperature incubation) and ascribed the effect to increased denaturation of proteins embedded in the gel or membrane matrix after MW exposure. Note that the digestion itself was not performed in the presence of MW radiation, which was only used to stain and destain the gel or membrane. Denaturation increased the accessibility of the substrate proteolytic sites to the enzyme. Heat generated from the MW process may also have contributed to gel or membrane expansion,

thereby enhancing exposure of the protein to enzymatic cleavage and boosting extraction of peptides from the gel or membrane after digestion.

It was concluded that, overall, MW irradiation of proteins separated by SDS-PAGE or blotted onto PVDF membranes often significantly improves proteolytic coverage relative to traditional gel-staining techniques. In addition, there were seemingly no detrimental effects such as the loss of post-translational modifications or increased deamidation or oxidation. An MW-enhanced dye-based blue-black ink staining method for quantitative visualization of proteins spotted onto a nitrocellulose membrane by incubation in a domestic MW oven was subsequently reported [105]. The total staining time was reduced from more than 30 min to less than 3 min by the use of MW. Also, a 500-fold expansion in dynamic range (from low-nanogram to mid-microgram total protein amounts were detected) was obtained, and the method allowed samples in complex buffers and chaotropes to be quantified.

This MW-assisted operational mode is the key to overcoming a major obstacle to the molecular investigation of natural (especially oceanic) microbial cells: proper preservation for further land-based molecular analyses. Traditionally, proteomic studies have been performed on unfixed cells; however, working with natural samples collected during fieldwork (e.g., during oceanic cruises) requires either immediate analysis or efficient sample fixation and storage: the latter is intended to preserve cells for further processing in specialized laboratories. Samples for flow cytometry (FCM) are generally flash-frozen in liquid nitrogen and stored at −80°C until analysis after fixation [106–108]. However, when a nucleic acid-specific stain is used to discriminate, count, and sort bacterioplankton clusters or viruses, the bacterioplankton cells must previously be fixed [109, 110]; this additionally allows proteome profiles to be preserved intact over long sorting runs. In contrast to genomic analyses, where whole-genome amplification is feasible, this is a requirement for collecting enough protein material from a natural sample for proteomic analysis. As a result, the fixation conditions are critical toward preserving cell integrity with minimal alteration of cellular macromolecules.

The use of MWs for cell fixation before high-speed flow cytometric sorting to define the metaproteomes and metagenomes of key microbial populations has shown that MW treatment is a potentially powerful technique for in situ proteomic and genomic studies requiring sample preservation before analysis. Moreover, its combination with flow cytometric sorting can provide wide-ranging applications in environmental microbiology and improve our understanding of natural microbial communities and their functioning.

### *6.4.3 Enzymatic Proteolysis: Digestion by Trypsin and Other Enzymes*

Despite major advances in “top-down” MS methods for characterizing proteins, the traditional “bottom-up” approach involving digestion of a protein into smaller peptides and subsequent peptide mass fingerprinting or reversed-phase separation and tandem mass spectrometric identification remains the most widely used analytical

methodology for protein characterization. However, proteolysis is still a rate-limiting step in sample preparation, and a number of traditional protocols recommend overnight incubation of the substrate and enzyme at physiological temperature. In any case, some recent studies have shown that many proteolytic enzymes can tolerate temperatures above 37°C.

Rapid, efficient protein digestion is crucial toward assuring accuracy in bottom-up proteomic applications as this is one of the most important steps of the pipeline. In shotgun experiments, complete, thorough digestion of all proteins in a complex mixture is commonly assumed. The digestion efficiency can be affected by a host of variables including (a) a low solubility, (b) marked protein folding, (c) inadequate reaction times, and (d) enzyme degradation. Surprisingly, little work has been conducted on the properties of peptides from complex digestion-resistant samples subjected to shotgun methodology.

In-gel digestion and in-solution digestion are two of the most widely used tryptic digestion techniques. In an in-gel digestion, proteins are run on a one-dimensional (1D) or 2D gel to separate the proteins. Gel bands are excised and then digested with trypsin [111]. With in-solution digestion, proteins are digested directly in specific buffers or solvents such as ammonium bicarbonate or acetonitrile [112]. One of the main advantages of using in-gel digestion is that it allows removal of any compounds potentially harmful to mass spectrometers such as detergents and salts [111], and one of its greatest disadvantages is the loss of peptides (especially during extraction, where some peptides may remain trapped in the gel matrix) [111, 113]. One other disadvantage of in-gel digestion is the long time needed to complete most protocols, which leads to increased costs [113]. New technologies such as scientific MW and barometric pressure cycling may help alleviate many of these problems [114–116]. These new technologies have the potential to significantly reduce the time required for digestion and hence to increase throughput and save money.

#### 6.4.3.1 Microwave-Assisted Tryptic Digestion

Trypsin is a 24-kDa protein belonging to the serine hydrolase enzyme family and usually obtained from porcine pancreas. Trypsin is perhaps the most commonly used proteolytic enzyme in bottom-up proteomics as it converts most protein mixtures into more readily analyzable peptide populations. This enzyme cleaves at the carboxyl termini of arginine (Arg) and lysine (Lys), except when sterically hindered by a neighboring proline residue.

Tryptic digestion is conventionally carried out at physiological temperature (37°C) in a water bath or convection oven for lengthy periods (8 h to overnight) to ensure complete hydrolysis. Such a long digestion time is a serious limitation for sample preparation and has led researchers to develop a number of protocols to reduce it. These protocols include adding a small amount of an organic solvent such as acetonitrile or methanol, a detergent (urea) or an acid-labile surfactant (RapiGestt) to the digestion buffers to raise the digestion efficiency and decrease incubation times, usually by enabling further denaturation of proteins for better exposure of

cleavage sites to the proteolytic enzyme [117–119]. Immobilization protocols have also been explored to further accelerate proteolysis by using enzymes immobilized on solid supports [120, 121] or proteins immobilized on PVDF membranes followed by incubation with non-ionic surfactants and proteolytic enzymes [122]. More recently, methods using alternative energies such as ultrasonic vibrations (from a sonoreactor or ultrasonic probe) [123] or MW irradiation have been used to boost proteolytic catalysis [124, 125]. Microwave-assisted tryptic proteolysis has been used in many studies and adopted as a standard protocol by many laboratories in recent years [126].

Accelerated proteolytic cleavage of proteins under controlled MW conditions (i.e., a set temperature, pressure, and power) in a scientific monomode MW system was first reported by Pramanik et al.[124], who demonstrated accelerated MW-assisted digestion with the proteolytic enzymes endoproteinase Lys-C (Lys-C) and trypsin. MALDI–TOF/MS allowed detection of both the intact protein (starting material) and the resulting proteolysis products. Sequence coverage (i.e., how much of the protein sequence was identified by characterizing the proteolysis products) was determined and quantitation accomplished by LC–MS/MS. This method facilitated assessment of the rate of completion of proteolytic cleavage and allowed direct comparison between the MW-assisted method and traditional tryptic digestion. The initial study was conducted on bovine cytochrome *c*, a globular protein known to be relatively resistant to traditional proteolytic cleavage methods. After a mere 10-min MW exposure at 37°C, peptides accounting for a high proportion of the protein were detected by MALDI–TOF/MS analysis. Complete digestion of several nonreduced, tightly folded proteins was completed within 12 min in the presence of MW radiation; by contrast, no proteolysis was observed when using a water bath for the same time. The level of proteolysis achieved by tryptic digestion of cytochrome *c* with MW irradiation at 37°C for 12 min was comparable to that observed after a 6-h incubation at the same temperature in a water bath. Additional proteins were used to evaluate MW-assisted tryptic digestion and show that MW-assisted conditions were universally applicable to accelerated in-solution tryptic digestions. Pramanik et al. also showed that these protocols can be successfully applied to in-gel tryptic digestion because MW irradiation facilitates digestion of proteins embedded in a semi-immobile matrix. Destaining of the gel before enzymatic incubation was also accomplished in the MW system, and in-gel MW-assisted digestion for 15 min provided high peptide recovery and protein coverage for bottom-up protein characterization.

The results of this initial study afforded several interesting conclusions: (a) when the enzyme was excluded from MW-assisted incubation, the substrate protein remained intact and was not cleaved by heat-induced proteolysis or self-induced degradation, which indicated that accelerated proteolysis was not a random phenomenon; (b) proteolysis sites remained specific and, even when samples were incubated with MW assistance at 60°C, cleavage occurred specifically at predictable Lys and Arg residues for tryptic cleavage; (c) the observed kinetics differed significantly between water-bath and MW-assisted incubations; (d) sampling aliquots for 5–30 min revealed that, in contrast to water-bath incubation, MWs accelerated

proteolysis within the first few minutes but their effect dropped after 30 min, suggesting that the enzyme was rapidly denatured and inactivated upon MW exposure [124]; and (e) no artifactual effects such as significantly higher deamidation or oxidation were observed as a direct result of MW-assisted incubation. Overall, Pramanik et al. showed 60°C to be the optimum proteolysis temperature, and that proteolysis was greatly enhanced by MW radiation and tightly folded proteins, which usually require hours of incubation with conventional methods to benefit the most from MW-assisted proteolysis [124].

Alvarado et al. [127] recently compared traditional overnight digestion to MW and pressure-assisted digestion by using a CEM Discover MW system in conjunction with modified and unmodified trypsins. Four replicates of each digestion method were conducted, and each digestion method was labeled with a specific color. The temperature was monitored by means of a fiberoptic probe and held constant at 50°C throughout. The protocol yielding the largest number of proteins on average was that involving reduction/alkylation and extraction in the MW system, but digestion proper in an incubator overnight; a total of 600 and 554 proteins were identified with modified and unmodified trypsins, respectively. A similar protocol excluding overnight incubation allowed 332 and 446 proteins to be identified with modified and unmodified trypsins, respectively, whereas overnight digestion followed by extraction in a Barocycler afforded 177 and 418 proteins with modified and unmodified trypsins, respectively. The results obtained with the other methods fell in between the previous data, thus reflecting the variability inherent in the particular conditions used, but also that using MWs invariably boosted digestion.

Most studies on MW-assisted digestion have focused on mixtures of known proteins and the use of SDS-PAGE or protein sequence coverage with MS identification. Very few have investigated the characteristics of the peptides produced by different digestion methods. In particular, complex protein mixtures including digestion-resistant membrane proteins have not been extensively investigated. Zhong et al. [128] used acid hydrolysis in combination with MW digestion, but their approach lacked the specificity required to analyze a membrane-enriched fraction of cell lysate. Vaeezadeh et al. [21] developed three different digestion protocols and tested them on crude *Staphylococcus aureus* N315 membrane protein extracts prepared by using the hydrolytic enzyme lysostaphin as described previously [129]; thus, the procedure involved the following steps: (1) conventional digestion with trypsin at a protease/protein ratio of 1:25 and overnight (18 h) incubation at 37°C, the reaction being stopped by addition of 1 M formic acid; (2) digestion for a further 1 h under identical conditions, the reaction being again stopped with 1 M formic acid; and (3) MW-assisted digestion by using the same reduction and alkylation conditions, and an identical trypsin ratio, as before. A household MW oven was used for heating. Samples were placed in Eppendorf tubes in a holder that was in turn placed in a beaker containing 500 ml water at 25°C, irradiated for 6 min, and quenched with 1 M formic acid. The exact amount of energy transferred by the MW oven could not be measured, but the final water-bath temperature immediately after MW irradiation was 55°C. After digestion, peptides were concentrated and desalted, and then subjected to isoelectric focusing, purified, dry evaporated, resuspended in 25 μl HPLC buffer, and stored at −20°C until

MALDI–TOF/TOF and LTQ Orbitrap analyses. Digestion under MW irradiation for 1 h allowed the largest numbers of proteins and peptides to be identified. However, many proteins and peptides were identified uniquely in a specific digestion protocol. Each procedure had specific advantages, but that using MW assistance was the most efficient; thus, it allowed the identification of 35% of the putative *Staphylococcus aureus* N315 proteome in a single experiment. In addition, MW irradiation excluded the presence of concentrated salts, urea, and detergents used for protein solubilization, which might have interfered with mass spectrometry. The improved digestion efficiency of membrane and hydrophobic proteins obtained with accelerated digestion was ascribed to increased recovery by effect of shorter digestion times, which probably helped avoiding precipitation or sticking of proteins to the tubes. The increased temperatures reached with MWs may also have facilitated unfolding of the proteins and increased their digestion efficiency as a result. However, the greatest differences were those in trypsin specificity. Probably because of the shorter reaction time, the average number of peptides identified on the two platforms revealed that more peptides with missed cleavages were identified with accelerated digestion than with conventional digestion. Although an increase in the number of missed cleavages can be predicted by bioinformatics, half-cleaved peptides cannot be predicted in silico. One interesting finding accounting for inconsistency between the results of some authors was that only 50% of peptides in the same sample analyzed on the TOF/TOF and the LTQ Orbitrap were similar. As stated by Stapels and Barofsky [130], and Yang et al. [131], MALDI and ESI tend to favor the ionization of different sets of peptides. Combining the two approaches to analyze the same sample was shown to increase the number of proteins identified and the confidence of identification.

Microwave-assisted tryptic digestion on immobilized surfaces is performed according to Walkeiwicz et al. [132], who found magnetite beads to provide the optimal material among 150 substances examined for MW absorption. Chen and Chen [133] expanded on this concept by using magnetite beads to accelerate MW-assisted enzymatic digestion. Multifunctional magnetite beads accelerated MW-assisted digestion by virtue of their ability to absorb MW radiation more efficiently than with conventional solution-based proteolysis. The beads acted as “trapping probes” whereby their negatively charged functionality allowed proteins to be adsorbed through electrostatic attraction on their surfaces, thereby increasing the surface area of the proteins and leading to their concentration in the vicinity of the MW-sensitive material. In addition, proteins were denatured, which rendered them easier to proteolyse after adsorption on the beads [133]. Digestion by MW-assisted proteolysis on magnetite beads (150–600 mg) was dramatically increased, with complete proteolysis in as little as 30 s. Magnetite beads proved multifunctional; in fact, they were successfully used for MW-assisted proteolysis by trypsin immobilized on bead surfaces rather than absorbed for digestion. In 2008, Lin et al. conducted novel MW-assisted digestions with trypsin immobilized on magnetic silica microspheres [134] or nanoparticles [135]. The latter nanomaterial proved useful with individual proteins in an extract from rat liver. The proteome, which required no preparation of prefractionation, was digested in 15 s and revealed 313 proteins by LC–MS/MS analysis, thus showing that immobilized trypsin MW-mediated methods are effective for large-scale, high-throughput proteomic analysis.

#### 6.4.3.2 Microwave-Assisted Digestion with Other Proteolytic Enzymes

Although trypsin is the most commonly used enzyme for bottom-up proteomic analysis, a plethora of other enzymes are also useful for deeper analyses (e.g., to ensure complete coverage of a protein for de novo sequencing projects [136, 137] with a view to characterizing post-translational modifications or determining protein isoforms). Also, the increased popularity of top-down methodologies for handling larger polypeptides has led to the development of a new technique often referred to as "middle-down" proteomics, which uses Lys C, Glu-C, endoproteinase Asp-N (Asp-N), and other enzymes producing larger peptide fragments (15–50 amino acids) [138]. Vesper et al. [18] examined the potential of MW-mediated Glu-C proteolytic digestion, which, however, was found to yield fewer proteolytic products than conventional convective heating. No autolytic peaks from the Glu-C enzyme were detected, so it was concluded that inactivation of the enzyme was caused by MW-induced denaturation resulting from its instability at high temperatures rather than by autolysis [18].

Lill et al. [15] investigated whether MW-assisted digestion boosted proteolysis by various enzymes for mass spectrometric identification. Bovine serum albumin and myoglobin (50 pmol) were subjected to either (a) tryptic digestion in a water bath at 37°C (or a CEM Discover MW system at 37°C at 2–5 W of applied power) or (b) Asp-N digestion with an MW system or a water bath. Incubations lasted 5, 10, or 30 min, were stopped by addition of 0.1% trifluoroacetic acid, and followed by storage at −20°C. Microwave-assisted tryptic proteolysis was completed within 1 h (or 30 min at 60°C with the MW system). Microwave-assisted Asp-N digestion exhibited no significant reduction in proteolysis time over conventional water-bath incubation; however, proteolysis was not decreased as was previously observed with the heat-labile enzyme Glu-C [15]. Lys-C is the most commonly used enzyme in middle-down proteomics; as does trypsin, its cleavage leaves a basic residue at the C-terminus that can promote increased ionization and informative fragmentation. Lys-C MW-assisted digestion was also investigated by Pramanik et al. [124], who found the enzyme to behave identically with trypsin; thus, MW-assisted Lys-C digestion at high temperatures provided by MW radiation rapidly provided proteolytic fragments for mass spectrometric analysis. The MW irradiation conditions were optimized and comparisons with non-MW-mediated proteolysis methods conducted on a case-by-case basis because each enzyme might behave differently, even from other members of its own family. In addition to the heat lability of some enzymes such as Glu-C, some substrate proteins or PTMs may be altered by high temperatures or vibrational energies. This difference between substrate proteins may explain the slight differences in optimal temperature for MW-assisted enzymatic digestion reported in the literature.

Although increased incubation temperatures can equate to increased proteolytic digestion of proteins by several enzymes, it has been shown [15, 124, 126] that a high temperature in itself does not boost catalysis under MW irradiation. Collins and Leadbeater [139] hypothesised that MW-mediated effects on proteolysis might be caused by increased dipole movements of the α-helices of proteins. Most proteins possess α-helices and β-sheets as part of their tertiary structure; also, the polypeptide

backbone in α-helices is coiled around the protein axis, the side chains of the amino acids pointing outward and downward from the backbone as a result. This helical structure leads to stacking of peptide bond dipoles by effect of massive hydrogen bonding in the protein structure. In turn, hydrogen bonding produces a large overall net dipole effect across α-helices which, being susceptible to vibrational and structural rearrangements, may boost catalysis in the presence of MW radiation. If MW energy induces perturbation of the three-dimensional structure of a protein, then digestion may be facilitated by exposure of previously enclosed or buried regions of the protein to a proteolytic enzyme. This action may explain why some authors have seen more dramatic effects of MW-assisted proteolysis on nondenatured or reduced proteins with complex tertiary structures than on less structurally complex proteins.

### *6.4.4 Chemical Proteolysis: Acids and Other Reagents*

In the absence of an appropriate enzyme, acid-mediated cleavage affords proteolysis at specific amino acid residues or motifs. In addition, chemical proteolysis is an attractive method when available enzymes are not strong enough for efficient cleavage. Several acid-mediated digestion methods have recently been adopted and optimized by incorporating MW radiation to increase yields and decrease reaction times for more efficient digestion of proteins. Using MW assistance in these reactions markedly shortens the time required for their development. The two most frequently used approaches in this context are trifluroacetic acid (TFA) proteolysis and aspartic acid-specific proteolysis.

Zhong et al. [140] demonstrated the use of MW-assisted acid hydrolysis (MAAH) for rapid protein degradation at acid-labile sites for the purpose of protein identification (especially, for the analysis of membrane proteins resistant to conventional tryptic digestion). Using controlled acid hydrolysis to proteolyse proteins has several advantages over conventional enzyme-mediated catalysis including that the protein can be dissolved in any solvent (e.g., directly in an acid) and also that no special buffer is required for catalysis. This step is particularly useful for the analysis of membrane-associated proteins, which are often solubilized in concentrated urea or harsh detergents and salts. Zhong et al. [140] used a 25% TFA aqueous solution containing 20 mM dithiothreitol (DTT) to minimize oxidation of methionine, tryptophan, and other amino acids under MW irradiation for the proteolysis of proteins or protein mixtures. A household 900-W (2,450 MHz) MW oven containing a beaker of water to absorb excess heat was used. TFA MW-mediated proteolysis was demonstrated on the hydrophobic protein bacteriorhodopsin and the resulting peptides were separated by reversed-phase HPLC for analysis by MALDI–MS/MS. TFA-mediated proteolysis of proteins was generally completed within 10 min; by contrast, only one peak corresponding to the N-terminus was observed in a comparative control experiment involving hydrolyzing proteins in 25% TFA in a conventional oven at 110°C for 4 h. Protein aggregation during conventional heating prevented further hydrolysis and led to minimal protein cleavage as a result. Using MW irradiation prevented aggregation and afforded efficient proteolysis. The extent

of proteolysis was easily adjusted by changing the concentration of TFA (1–3 M) and irradiation time (1–10 min). Lower acid concentrations (e.g., 0.3 M TFA) and shorter irradiation times (e.g., 2 min) produced fragments preferentially containing the N- and/or C-terminus of the protein. Higher acid concentrations and shorter irradiation time produced more internal fragmentation ions in addition to N- and C-terminal fragment ions. MAAH of the hydrophobic protein bacteriorhodopsin in the presence of 25% TFA produced a labile cleavage site on both sides of glycine residues, thus suggesting that this might be a specially acid-labile residue motif.

Hua et al. [141] further optimized a method using an acid medium for aspartic acid (Asp)-specific proteolytic cleavage by applying MW radiation to boost the catalytic effects on Asp-specific residues in proteins. Incubation times were thus reduced from 8 h at room temperature to 30 s with MW irradiation and the presence of 2% formic acid, MALDI–TOF/MS analysis revealing the presence of similar cleavage products. Further increasing the MW irradiation time to 6 min resulted in fewer missed cleavage products and lower mass fragment ions for peptide mapping. Zhong et al. [140] had previously examined the effect of acid type on MAAH and found formic acid to alter some peptides during the hydrolysis process. Also, Goodlett et al. [142] found the use of formic acid to catalyze proteolytic reactions to cause formylation, mainly at side chains or in serine and threonine residues. No similar finding was reported by Hua et al. [141]. Chemical digestion provided increased flexibility by effect of the harsh digestion conditions used causing specific cleavage of aspartic acid residues.

Swatkoski et al. further explored the utility of MW-assisted acid proteolysis and its integration into proteomic workflows in a preliminary study of the yeast ribosome proteome [143] that was followed by another using ovalbumin and other polypeptide standards [144]. Most of the peptides identified were the result of either N- or C-terminal Asp-specific cleavage, or degradation products of these peptides, which exhibited N- and C-terminal "clipping." Post-translational modifications such as phosphorylation and N-terminal acetylation were found to be stable under MW-assisted acid cleavage conditions, which afforded expanding the use of this method to more global proteomic projects. No artifactual acetylation was observed with acetic acid; whatever the acid, however, N-terminal pyroglutamate formation occurred at high frequencies when the N-terminal amino acid residue consisted of a glutamine. A pH about 2 and a temperature of 140°C in the MW system were recommended for Asp-specific cleavage [142].

Sandoval et al. [145] investigated MW-assisted acid-mediated cleavage of proteins for bottom-up protein characterization and as a tool for N- and C-terminal sequencing, and showed MAAH to be a useful tool for rapid protein identification.

No use of MW in CNBr, asparagine/glycine, or other chemical cleavage protocols has been reported to date. One suggestion as to why these reactions have been omitted is that they are often difficult to quantify; in fact, these proteins can react very rapidly, even in the absence of MW radiation. Microwave-mediated chemical digestion is highly suitable for any proteins seemingly resilient to oven-mediated digestion or, indeed, for which no proteolytic enzyme for digestion at "convenient sites" is available. Special safety precautions should be adopted when performing these chemical digestions because some reagents (particularly CNBr) are carcinogenic or highly toxic.

In summary, MW-assisted chemical proteolysis has proved a useful tool for protein characterization and global proteomic research. Most chemical cleavages result in peptides longer than the average tryptic peptide; also, the peptides are easily resolved chromatographically [141–146] and may carry higher charge states leading to more efficient and informative fragmentation patterns than significantly shorter peptides. In addition, chemical-mediated MW-assisted digestion provides an attractive solution for proteins that are resilient to traditional proteolytic digestion techniques.

### *6.4.5 Microwave-Assisted Hydrolysis of Proteins*

The first step in an amino acid analysis assay (AAA) involves hydrolyzing the protein to its constituent amino acids, usually by breaking amide bonds with 6 N HCl at 110°C for 24 h, a method first described by Hirs et al. [147] more than 50 years ago. The resulting hydrolysates are analyzed and quantified against standard amino acids injected in preset amounts. AAA methods can be categorized as pre- or post-derivatization methods depending on whether the amino acids are labeled before or after chromatographic separation.

Conventional hydrolysis in the vapour phase with 6 N HCl at 110°C for 24 h can be replaced with elevated temperatures for just 1 h at the expense of compromising the hydrolysis vessel [145]. Because analysis times for hydrolysates typically range from 12 to 90 min, hydrolysis is often the rate-determining step in AAA. This limitation has promoted research into MW-assisted acid hydrolysis (MAAH) and its adoption by many groups to allow the rapid hydrolysis of samples and significantly reduce analysis times as a result.

Protein hydrolysis for AAA was one of the preliminary protein chemistry methods shown to benefit from MW assistance. The benefits of using MW in this field have been the subject of several comprehensive reviews [115, 148–150]. The first study on the potential of MAAH showed 8–12 min MW-assisted vapor-phase hydrolysates to be equivalent to conventional 24-h, 110°C heating-block hydrolysates. One problem with conventional vapor-phase protein hydrolysis is that the vessel may leak. Leakage can lead to lower recoveries of hydrolysis products (e.g., amino acids) and often goes unnoticed until the final analysis of the resulting hydrolysate products. With MW-mediated hydrolysis in a CEM apparatus, leaking can be easily detected from a drop in the monitored pressure before the final analysis step; this simplifies troubleshooting in the event of leakage [148].

Concomitantly with the previous investigations, Chiou and Wan [149] proposed an MW-assisted protein hydrolysis method that takes only 4–12 min. The risk of explosion from acid hydrolysis at a high pressure and temperature was demonstrated, and Teflon/Pyrex custom-made vials were used as safer vessels for MAAH.

Other authors [115, 150] further optimized the MAAH protocol for the complete hydrolysis of proteins into their constituent amino acids to ensure thorough, uniform hydrolysis on batches of samples. Similar amino acid recoveries were obtained for proteins hydrolyzed under MW irradiation at 175°C for 10 min or with two common protocols (non-MW-mediated incubation at 110°C for 24 h or 150°C for 1 h [145]).

This MW-assisted approach has been validated with the quantitation of thousands of recombinant proteins and proved an invaluable tool for the rapid quantitation of proteins in the biotechnology industry.

### *6.4.6 Other MW-Assisted Sample Preparation Steps in Proteomics*

#### 6.4.6.1 N- and C-Terminal Sequencing Using MW-Assisted Acid Hydrolysis

In the biotechnology industry, it is important to verify that correct transcriptional and intracellular processing of a recombinant protein has occurred. The most common protocol for N-terminal sequencing uses chemistries first reported by Edman, who derivatized the free N-terminal amine with phenylisothiocyanate [151]. The long process (Edman degradation cycles typically take 30–60 min) is repeated to allow the sequential amino acid characterization of the protein N-terminus [151].

In 2004, Zhong et al. [152] used MAAH as an alternative method for the N- and C-terminal characterization of proteins. MAAH involves exposing proteins to high acid concentrations under MW irradiation, which causes denaturation and cleavage at acid-labile sites. The initial protocol exposed proteins in 6 N HCl to MW irradiation for increasing times from 30 s to 2 min and analyzed the resulting hydrolysis products by MALDI–TOF. MAAH of intact proteins over short periods facilitated the identification of ions corresponding to incremental ladders of amino acid chains from the N- and C-termini of the proteins.

Sandoval et al. [145] explored MAAH as an alternative method for N-terminal sequencing and found one terminus from the protein to appear preferentially in the MALDI–TOF data over the other; in some cases, however, both termini were seen in equal amounts. Myoglobin exhibited preferential cleavage at the N-terminus, whereas lysozyme exhibited C-terminal peaks. MAAH therefore proved a useful tool for characterizing blocked N-termini and provided higher throughputs than traditional deblocking or "bottom-up" methods alone.

#### 6.4.6.2 Microwave-Assisted Identification and Characterization of Post-Translational Modifications (Glycopeptides and Glycoproteins, Enzyme-Mediated N-Linked Deglycosylation, Methods for Phosphorylation Mapping)

The vast diversity of the proteome has been ascribed to various post-translational events (particularly, the presence of more than 200 covalently attached PTMs). These modifications play a critical role in controlling interactions at both the molecular and the cellular level. It is therefore important to be able to characterize them to gain insight into the mechanisms involved in intracellular and extracellular pathways. A variety of tools are available for the analysis or characterization of PTMs;

however, many can benefit in terms of decreased reaction times or increased biochemical efficiency from MW assistance.

The characterization of glycopeptides and glycoproteins is one other application greatly benefiting from the use of MW radiation. Microwave-assisted techniques for the characterization of oligo- and monosaccharides (as well as fatty acids and sphingoids) were first reported by Itonoria et al. [153]. Methanol hydrolysis under alkaline conditions was performed on glycosphingolipids by using a 2-min MW-assisted reaction to obtain a by-product-free lysoglycosphingolipid intermediate. Subsequent 45-s MW exposure to 1 M HCl in methanol followed by extraction completed the hydrolysis. Using MW radiation shortened the typically long time for hydrolysis from hours to minutes. In 2005, Lee et al. [154] reported a method based on MW-assisted partial acid hydrolysis for the characterization of monosaccharides obtained from glycopeptides, which required 30–120 s exposure for partial cleavage of the oligosaccharides compared to 1 h with conventional thermal heating. This method proved particularly useful for identifying glycopeptides and rapidly determining the monosaccharide composition of glycopeptides. Enough cleavage was observed in many cases to obtain monosaccharide composition information from a wide range of glycopeptides. Lee et al. [155] extended this protocol to the analysis of oligosaccharides from intact glycoproteins.

Microwave-assisted enzyme-mediated N-linked deglycosylation was first investigated by Sandoval et al. [31], using enzyme-friendly surfactants such as Rapigestt or an organic solvent (10–20% ACN) for MW-assisted PNGase F catalysis. The addition of 0.1% Rapigestt decreased the MW-assisted reaction time to 10 min, albeit at the expense of substantial sample losses and precipitation; it was therefore decided not to further pursue the use of such surfactants to obtain high recoveries from low-level materials [31]. Tzeng et al. [15–157] further developed the MW-assisted PNGase F-mediated deglycosylation protocol previously reported by Sandoval et al. for the facile MALDI–MS analysis of neutral glycans. In-solution MW-assisted tryptic digestion and MW-assisted deglycosylation in the presence of PNGase F were followed by the use of carboxylated/oxidized diamond nanoparticles for selective solid-phase extraction to remove proteins and peptides from the released glycans. MALDI mass spectral signal suppression effects caused by cation adducts such as KI and NaI were minimized by mixing the acidic matrix with an NaOH solution. In addition to suppressing the formation of potassiated and sodiated oligosaccharide ions, this method suppressed the spectral signals of peptides not being fully retained by the diamond nanoparticles. A combination of these methods afforded the analysis of neutral glycans from proteins in less than 2 h, in contrast to the 2 days typically required by conventional methods [157].

Microwave-assisted methods for phosphorylation mapping were investigated by Sandoval et al. [31] as an alternative to the precise mapping of phosphorylation sites. Traditionally, such nucleophilic derivatization reactions are performed in a water bath at 60°C for 1–3 h. Phosphoserine residues undergo ready β-elimination within 1 h, but phosphothreonines typically require longer incubation times for complete reaction, and, even then, often after extended incubation, the reactions are incomplete. These authors found equivalent results to those obtained after 3 h

incubation of phosphopeptides/proteins β-eliminated with propanethiol to be provided by MW-assisted incubation at 100°C for 2 min. They also investigated whether MW irradiation would accelerate the on-membrane β-elimination reaction relative to traditional water-bath incubation. Heating for 2 min in a water bath failed to start the reaction; after 1 h, the results were similar to those obtained with only 2 min of MW irradiation.

Enrichment of phosphopeptides by use of IMAC probes is another application of magnetite beads for accelerated MW-assisted enzymatic digestion [133]. Chen et al. [158] further explored the utility of magnetite beads by coupling zirconia to the beads for phosphopeptide enrichment. Proteins for characterizing phosphorylation were denatured and mixed with a suspension containing magnetic particles coated with zirconia by pipetting up and down for 1 min. After rinsing the particles, trypsin in ammonium bicarbonate was added to the mixture and the sample heated in a household MW oven for 1 min to effect particle-mediated tryptic digestion. Samples were then acidified and the resultant peptides mixed vigorously with the beads by pipetting, which caused phosphorylated peptides to be adsorbed on the zirconia. The particles were then rinsed and phosphopeptides eluted by using 0.15% TFA mixed with 2,5-DHB (30 mg/ml) containing 0.5% phosphoric acid for MALDI–TOF analysis with excellent results.

Microwave-assisted enzymatic removal of N-terminal pyroglutamyl with pyroglutamyl aminopeptidase (PGAP) was made possible by the high thermal stability of this enzyme; the procedure exhibited increased deblocking efficiency over thermocycler-mediated incubation [133].

Other MW-assisted methods for the characterization of PTMs include those for (a) O-linked enzyme-mediated MW-assisted deglycosylation [145]; (b) the characterization of metal-catalyzed reaction sites on proteins [159]; (c) that of lipase selectivities [160, 161]; and (d) that of dissociation in protein complexes.

### *6.4.7 Microwave-Assisted Protein Quantitation (ICAT$^R$, iTRAQ$^R$, and Fluorescence and Chemiluminescence)*

Isotope-coded affinity tags (ICATt) were first reported by Gygi et al. [162], who used them for the accurate quantitation and simultaneous sequence identification of individual proteins from complex proteomic mixtures. ICATt technology uses a pair of tags consisting of two isotopically labeled sulfydryl reactive groups, one composed of an eightfold deuterated linker with a biotin affinity tag and the other of an identical tag except for its having a non-isotopically labeled linker. The side chains of cysteine residues from a reduced protein from sample A were reacted with the light version of the ICATt reagent, whereas sample B was treated with the deuterated form of the tag. The two samples were combined and digested with an appropriate enzyme, the biotinylated tagged (cysteine-containing) peptides being isolated from the complex mixture by avidin affinity separation. Peptides were released from the affinity resin, separated by reversed-phase chromatography, and analyzed by

tandem MS. A second generation of ICATt reagents incorporates a cleavable linker to minimize the tag mass addition and $^{13}C$ rather than deuterium for the isotopic tag to provide closer reversed-phase chromatography retention times between light and heavy labeled peptides. Another set of isobaric tags specifically labeling free amines is available for multiplexing quantitation experiments. iTRAQt reagents were designed for the identification and quantitation of up to four different samples simultaneously. Although ICATt is limited to cysteine-containing residues, iTRAQt reagents are more universally applicable and can be added to the free N-terminus and lysine residue of any peptide. The iTRAQt tags consists of (a) a charged reporter group that is unique to each of the four reagents, (b) a peptide reactive group, and (c) a neutral balance portion.

Rutherford et al. explored the utility of MW-assisted labeling of proteins with both ICATt and iTRAQt reagents [163]. The ICATt labeling protocol used cleavable ICATt reagents, and the traditional reaction protocol was run in parallel to a modified MW-assisted version whereby the tag labeling reaction was developed in a Discover system at a maximum temperature of 60°C and followed by cooling for 10 min. Alternative conditions for the biotin cleavage step using MW radiation were also explored. The total amount of time required to perform the ICATt labeling protocol was reduced from more than 10 h to a mere 30 min with identical modification and cleavage of the peptides. The same conditions were also used for peptides reacted with the iTRAQt reagents and reaction times decreased from 2 h to 10 min as a result. Although NHS-esters (the amine chemistry used to couple iTRAQt reagents to free amines) usually degrade at high temperatures, using MWs to assist labeling had no detrimental effect, possibly as a result of the very short incubation time used (30 min).

Fluorescence and chemiluminescence protein quantitation can also benefit from the accelerating effect of MW. Immunoassays are commonly used to detect and quantify a wide variety of substrates. In its typical format, an immunoassay uses antigen–antibody binding for analyte recognition and, most often, fluorescence-based readouts [92]. The two rate-limiting factors of a typical immunoassay are the slow antigen–antibody binding kinetics and the quantum yield of the tagged fluorophore that is used to generate a fluorescence signal readout. Geddes and coworkers [164, 165] assessed MW-accelerated protein detection and quantitation methods using MW-accelerated metal-enhanced fluorescence and chemiluminescence.

Initially, Aslan and Geddes used a combination of metal-enhanced fluorescence (MEF) and low-power MW radiation to kinetically accelerate assays and dramatically increase the quantum yield and photostability of weakly fluorescing species [92]. The effects of MW radiation on metal-related reactions facilitated the joint use of metallic nanoparticles, fluorophores, and MWs to develop a kinetically accelerated, optically amplified immunoassay and were shown in preliminary work to provide a more than tenfold increase in signal and hence increased assay sensitivity. In addition to this dramatic increase in sensitivity, the assay run time was reduced approximately 90 times. Overall, Geddes et al. showed silver nanostructures to dramatically increase the quantum yield of proximity fluorophores and also that using low-power MW facilitated rapid, uniform heating. They found microwave radiation

to disturb neither the silver nanostructures nor the proteins being assayed; rather, it simply boosted mass transport of proteins to the silvered surface [92].

By using an improved version of their original method (termed MW-triggered metal-enhanced chemiluminescence, MT-MEC), Geddes et al. [164] showed the use of low-power MW in combination with enzymes and chemiluminescent species to afford significantly faster total quantitative protein detection than conventional methods. To further develop this technology, they went on to combine the principles of MW circuitry and antenna design with their work by using MT-MEC to demonstrate the potential of "triggering" chemically and enzyme-catalyzed chemiluminescence reactions [165]; in this way, they demonstrated the utility of MW-triggered chemiluminescence assays to dramatically improve signal-to-noise ratios in surface assays.

## 6.5 Microwave-Assisted Steps in Metabolomics

As in genomics and proteomics, developing a universal method for metabolomics is a difficult task that is further complicated in the latter by the extremely varied nature of metabolites.

As in other omics, analytical processes in metabolomics involve three distinct steps: (1) sample preparation, (2) data acquisition by use of analytical chemical methods, and (3) data mining with appropriate chemometric methods. Although other omics have benefited from MW assistance in the former two steps, metabolomics has taken advantage of it only in the first. In any case, metabolomics is the omics discipline with the greatest number of uses of MWs for sample preparation. In fact, many MW-assisted steps for the preparation metabolite-containing samples were developed and routinely in use even before the term "metabolomics" was coined.

Most samples used in metabolomic analyses come from plants; also, those from animals are largely liquid. Plant metabolomic analyses usually involve one or more of the steps depicted in Fig. 6.5, all which have profited from MW assistance, albeit to a rather different extent.

The sample preparation steps most commonly performed with MW assistance include drying, digestion (or solid–liquid extraction), liquid–liquid extraction, steam distillation (for volatile metabolites), and derivatization.

### *6.5.1 Microwave-Assisted Drying*

Freeze-drying (FD) is a useful technique for removing water from biological tissues in plant and food samples. Cellular components freeze at once, and ice sublimates under conditions of high vacuum and low temperatures. Because FD restricts biological activity, it is often believed that cellular metabolites are degraded to a limited extent only by this technique. However, cell structure is known to be altered by some

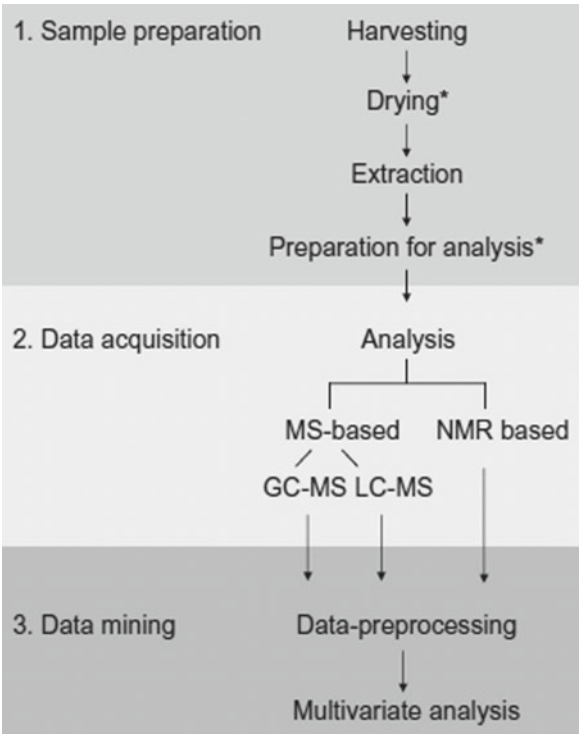


**Fig. 6.5** Flowchart of the metabolomic study in plants. Sample preparation steps can be changed depending on the analytical methods; however, in general many steps are common. (*This step can be omitted in some analyses.) (Reproduced with permission of Wiley Interscience. From Kyong Kim and Verpoorte [165])

factors including the increase in cell volume during freezing, which has serious effects on the levels of some cellular metabolites. Oikawa et al. [166] studied the effects on metabolite levels of FD for sample preparation in metabolomic analysis and found a substantial decrease in some metabolites such as succinate and choline in *Arabidopsis* and pear, respectively. Also, they found the effects of FD on certain metabolite levels to differ between *Arabidopsis* plants and pear fruits. These results suggest that it is necessary to confirm metabolite recovery in each sample species when FD is used for sample preparation. Microwave treatments have the advantage that enzyme activities are destroyed, and metabolomic changes caused by enzymes such as peroxidase and glycosidase are avoided. For instance, only one major anthocyanin glycoside was detected in a flower sample after a short microwave treatment, whereas several glycosides were identified in the same fresh flowers subjected to a classical extraction procedure [167]. Microwave-assisted drying warrants in-depth study with a view to expanding its use for sample preparation in plant metabolomic analyses.

### *6.5.2 Microwave-Assisted Solid–Liquid Extraction or Leaching*

Microwave-assisted metabolomic leaching has been more frequently used with plants than with animal samples. The latter (especially those for clinical metabolomics)

are usually liquid (blood, urine, saliva, tears) or, less commonly, gaseous (e.g., exhaled air). Metabolites in leaves, branches, roots, and fruits are usually dissolved by solid–liquid extraction of the sample. The greatest shortcoming of leaching in metabolomics is the presence of a wide variety of compounds at very different levels and spanning a broad range of polarity in the samples. No single extractant can remove the whole range of compounds potentially present in a solid metabolomic sample. The choice of extractant has thus strongly limited the view of the metabolome. In fact, obtaining a full view of the metabolome probably requires several extractions with different solvents. Whatever solvent is used, each metabolite will partition between it and the sample matrix. Also, leaching is hindered by physical and chemical interactions between metabolites and the sample matrix, which can be regarded as an adsorbent (e.g., lignin and cellulose provide a support for the aqueous phase in the presence of nonpolar extractants and the lipid phase with aqueous solvents. The dissolution rate is governed not only by physical and chemical forces, but also by the diffusion rate from the sample particles into the extractant. Also, solubility and dissolution rate are two different concepts; thus, a low dissolution rate requires prolonged extraction, the use of external energy (conventional heating, MWs) to raise the temperature, or ultrasonication. Microwave-assisted extraction (MAE) has proved an advantageous choice in this context. As noted earlier, however, there are some limitations to the types of organic solvents that can be used with MAE. In general, metabolomic studies are designed to detect as many metabolites as possible in an organism, which entails using extractants capable of removing different metabolite groups.

Phenolic compounds constitute a large group of widely studied secondary plant metabolites with multiple biological effects including antioxidant [168, 169] and antimicrobial activity [170]. The food industry is very interested in phenols because they improve the quality and nutritional value of foods [171–173]. The two major classes of polyphenols [namely, phenolic acids and flavonoids (flavonols, flavanols, isoflavones)] play a central role in the prevention of human pathologies [174]. Conventional leaching of these metabolites is usually performed at a refluxing temperature of 90°C for at least 2 h. This method, which has been used for many decades, is very time consuming and requires relatively large amounts of solvents. As shown by the following examples, extraction at the same temperature under MW irradiation provides increased yields in shorter times and by using less solvent.

Proestos and Komaitis [175] used a household MW oven to develop a fast method for isolating phenols from aromatic plants that they compared with the conventional reflux method (90°C for 2 h). The total phenolic content of all plant extracts obtained with the four extractants used (acetone, methanol, water, and ethyl acetate–water) was determined with the Folin–Ciocalteu assay and found to depend on the particular plant and extracting solvent. Polar solvents are usually believed to be more efficient than nonpolar solvents. Based on the results of the total phenolic content determination with MW-assisted extraction, the amount of phenolic substances extracted from most of the plants decreased significantly ($p<0.05$) in the following solvent sequence: acetone > methanol > water > ethyl acetate/water. With the conventional method, however, phenolic levels decreased significantly ($p<0.05$) with decreasing polarity of

the solvent: water > methanol > acetone > ethyl acetate/water. The results obtained by HPLC analysis revealed that MW-assisted extraction provided significantly higher concentrations of phenols ($p < 0.05$) than did conventional extraction; by exception, a few compounds (especially with water as the solvent) exhibited the opposite trend, possibly as a result of a "superheating" effect. Although the phenolic content of plants extracted under MW irradiation was more or less similar to that obtained by heat-reflux extraction in most instances, MWs obviously reduced the extraction time (from 2 h to 4 min). The main conclusions of the study were that the use of MWs reduced both extraction time and extractant volume, and increased extraction yield. Concerning the nature of the extractant, only water was found to provide reduced or similar amounts of phenolic compounds relative to the conventional method, possibly as a result of localized superheating. Acetone, an MW-transparent extractant, proved the best solvent for extracting phenolic compounds from plant tissues in the presence of MW radiation; this can be ascribed to its efficient absorption of MW energy, which raised the temperature inside plant cells to a level causing their walls to break and their constituent compounds to be released into the solvent.

Properly understanding whole metabolic patterns in both wild and genetically modified organisms is becoming increasingly important toward understanding the biological function of a genome. The inorganic phosphate concentration in soil, usually in the micromolar range, is the key to proper development of several plant functions such as efflux (or extraction) of organic acid from roots, accumulation of phosphate to vacuoles, and activation of phosphate uptake. The mechanisms through which plants control the phosphate concentration of cells to regulate the metabolism of this anion were investigated by using boiling water to extract phosphorus compounds from crushed *Arabidopsis* samples that were immediately irradiated with MW (600 W for 15 s). Subsequent determination of phosphate by ion chromatography–MS/MS showed that the potentially dirt extract did not interfere with high-resolution detectors. A previous study had exposed the difficulty of determining some sugar phosphates in plants by HPAEC–PAD owing to the interference of the sample matrix, which was incompletely suppressed by a cleanup step on a titanium dioxide column [176].

The greatest concern with green chemistry recently led to the use of green solvents for MW-assisted extraction in a closed-vessel system under controlled temperature and pressure conditions for the extraction of different classes of active biomarker compounds (flavonoids, organic acids, and alkaloids) in *Uncaria sinensis*. Figure 6.6a, b illustrates the influence of the extraction temperature and time on the different target metabolites and testifies to the difficulty of quantitatively extracting all metabolites in sample under identical working conditions. Selective extractants, and strict control of the extraction temperature and time, are therefore required for subsequent development of target analyses for the different compound classes or families when sensitive determination is needed; alternatively, a compromise in the working conditions can be adopted to accomplish partial extraction of the different metabolite classes or families for coverage metabolic analysis.

The first method for the simultaneous ultrasound-assisted emulsification–extraction of polar and nonpolar compounds from solid plant material with two immiscible extractants, developed by authors of this chapter to extract phenols and

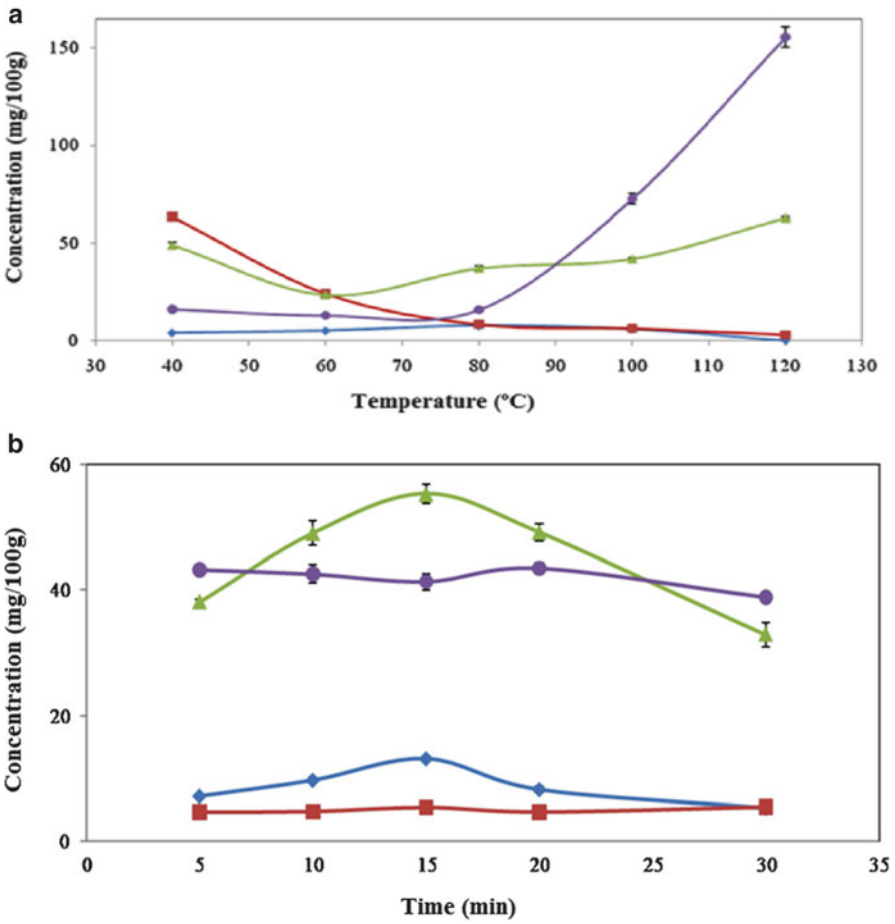


**Fig. 6.6** (**a**) Effect of different extraction temperatures on the recovery of biomarker compounds from *Uncaria sinensis* by microwave-assisted extraction (MAE) at 20 min ($n=3$): caffeic acid and rhynchophylline (**a**) and epicatechin and catechin (**b**). The decrease of catechin at 40°C and 80°C compared to 60°C and the decrease of caffeic acid at 80°C and 120°C compared to 100°C was found to be significant based on a two-tailed Student's *t* test ($p<0.05$). (♦) Catechin, (■) caffeic acid, (□) epicatechin, (●) rhynchophylline. (Reproduced with permission of Elsevier. From Ngin Tana et al. [177]. (**b**) Effect of extraction time on the recovery of biomarker compounds from *U. sinensis* by MAE at 100°C ($n=3$). The difference in the means of catechin at 5, 10, 15, 20, and 30 min and the difference in the means of rhynchophylline at 15, 20, and 30 min were found to be significant based on a two-tailed Student's *t* test ($p<0.05$). (♦) Catechin, (■) caffeic acid, (□) epicatechin, (●) rhynchophylline (Reproduced with permission of Elsevier. From Ngin Tana et al. [177])

lipids from acorns, alperujo, and grape seeds [178], was followed by the use of MAE for the same purpose for the first time; the MAE method took advantage of the emulsion formed with an immiscible system of two extractants under MW irradiation [179]. Boiling of the extractant with the lowest boiling point promoted the formation of an emulsion that facilitated mass transfer of the analytes from the solid

matrix to the extractants to an extent dependent on their nature and with a high efficiency as a result of the high contact surface for exchange and the temperature created in the whole system. The method in question, which required 14 min for quantitative extraction, was implemented in a Microdigest 301 digestor and allowed leaching of polar and nonpolar compounds (phenols and lipids) from alperujo with ethanol–water and hexane as extractant. Following leaching and separation of the two phases by centrifugation, the polar and nonpolar fractions were analyzed by HPLC–MS/MS and GC–ion-trap MS. The proposed method compared favorably with the reference method for isolation of each fraction (the Folch method for lipids and the stirring-based method for phenols).

### *6.5.3 Microwave-Assisted Digestion: Sample Preparation for Ionomic Analysis*

Digestion is an uncommon step in metabolomic analysis because the drastic conditions it generally promotes usually alter metabolic profiles. Most often, this treatment is used for elemental determinations; therefore, it is usually connected with ionomic studies.

The ionome is defined as the mineral nutrient and trace element composition of an organism and represents the inorganic component of cellular and organic systems. This definition extended the previously used term "metallome" [180, 181] to include biologically significant non-metals [182]. The ionome also includes both essential and nonessential elements.

Ionomics (the study of the ionome) involves the quantitative and simultaneous determination of the elemental composition of living organisms and also of changes in such composition in response to physiological stimuli, developmental state, and genetic modifications [183]. Ionomics requires the use of high-throughput elemental analysis technologies and their integration with both bioinformatic and genetic tools. Ionomics has the ability to capture information about the functional state of an organism under different conditions driven by genetic and developmental differences, as well as by biotic and abiotic factors. By virtue of its relatively high throughput and low cost, ionomic analysis has the potential to provide a powerful approach to not only the functional analysis of the genes and gene networks directly controlling the ionome, but also to the more extended gene networks that control developmental and physiological processes affecting the ionome indirectly.

The ionome can be regarded as the inorganic subset of the metabolome. This definition captures and highlights several critical concepts in the study of the ionome. Firstly, the study of the ionome is predicated on the fact that it should provide a snapshot of the functional status of a complex biological organism; this information is held in both the quantitative and qualitative patterns of mineral nutrients and trace elements in the various tissues and cells of the organism.

The inception of ionomics coincided with the blending of ideas from both metabolomics and plant mineral nutrition [184]. Sample preparation for ICP techniques typically involves acid digestion and dilution. Open-air or MW-assisted digestion

can be used for this purpose. Following are discussed some examples illustrating how MW can accelerate and improve this step.

The extensive metabolic cross-talk in melon fruit recently developed by Moing et al. [185] using spatial and developmental combinatorial metabolomics is an excellent example of the improvement in digestion promoted by MWs. Multi-elemental analysis performed by ICP–MS was preceded by digestion of freeze-dried melon samples in a microwave oven at 210°C for 50 min, a very short time relative to conventional digestion, using a maximum pressure of 40 bar and 5 ml 65% $HNO_3$ and 5 ml 15% $H_2O_2$ as digestion medium. Also, multi-elemental analysis (32 elements) in tomatoes and tomato paste was preceded by digestion with 4.5 ml $HNO_3$, 1 ml $H_2O_2$, and 0.5 ml of HF for each sample in this case. The operating conditions used for microwave digestion were as follows: 1,000 W over 10 min and holding of the power for 8 min. An Anton Paar Multiwave 3000 digestor with programmable power control was used in both cases.

The metabolic profiling of the cadmium-induced effect on the pioneer intertidal halophyte *Suaeda salsa* was studied by nuclear magnetic resonance (NMR)-based metabolomics by digesting dried tissue with concentrated nitric acid in a CEM microwave digestor where the samples were heated in an MW oven (program: heating to 200°C in 15 min and holding at 200°C for 15 min). All completely digested samples were appropriately diluted with ultra-pure water for quantitation of Cd by ICP–MS. The dose- and time-dependent metabolic responses induced by environmentally relevant concentrations of cadmium (2, 10, and 50 μg/l) were characterized in the homogeneous aboveground part of *S. salsa* by using NMR-based metabolomics. Significant cadmium-induced metabolic differences were observed in amino acids (valine, leucine, glutamate, tyrosine), carbohydrates (glucose, sucrose, and fructose), intermediates of the tricarboxylic acid cycle (succinate, citrate), and osmolytes (betaine) in *S. salsa*. The presence of these metabolic biomarkers was suggestive of elevated protein degradation and of disturbances in osmotic regulation and energy metabolism. Overall, this study showed that NMR-based metabolomics is useful for detecting metabolic biomarkers induced by contaminants in the pioneer plant *S. salsa* in intertidal zones.

An approach based on MW-assisted digestion followed by size-exclusion chromatography (SEC) coupled on-line with ultraviolet (UV) detection and off-line with graphite furnace atomic absorption spectrometry (GF–AAS) detection and MALDI–TOF/MS was developed to estimate molecular weight distribution in water-soluble Cu, Fe, Mn, and Zn species in Brazil nuts, cupuassu seeds, and coconut pulp. Samples were digested with a dilute oxidant mixture (2.0 ml $HNO_3$, 1.0 ml $H_2O_2$, and 3.0 ml water) in a closed-vessel microwave oven. The heating program consisted of four steps by which the temperature was raised from 8°C to 200°C in 20 min. The combined information obtained with SEC–UV, GF–AAS, and MALDI–TOF/MS confirmed the association of Cu, Fe, Mn, and Zn with water-soluble compounds in the target samples. This work improved existing understanding of the chemical and biochemical reactions involving these species, and of their differential action and behavior in relation to toxicity, mobility, or bioavailability.

### 6.5.4 Liquid–Liquid Extraction

Liquid–liquid extraction (LLE) has not been extensively used with MW assistance, neither in general nor in metabolomics in particular. The sample type most often subjected to MW-assisted LLE is urine. Kouremenos and coworkers [186] used LLE in combination with MW radiation to determine the metabolic profile of infant urine by comprehensive two-dimensional gas chromatography for subsequent application to the diagnosis of organic acidurias and for biomarker discovery. Sample preparation involved using 1 ml diluted urine supplied with 100 µl of 1 mmol/l solution of internal standard (3,3-dimethylglutaric acid). The mixture was placed in an MW CEM device at 450 W for 90 s and, after cooling and saturating with solid sodium chloride, 50 µl 6 mol/l hydrochloric acid was added and the solution extracted with 5 ml ethyl acetate on a rotary mixer for 5 min. The upper organic layer was separated by centrifugation and transferred to clean glass tubes containing 10 µl 25% ammonia to minimize evaporative losses of volatile organic acids and dried under $N_2$ at 60°C. The liquid–liquid extraction step was in fact performed in the absence of MW radiation.

### 6.5.5 Steam Distillation

One less frequent, but interesting microwave-assisted sample treatment is steam distillation, also known as solvent-free microwave extraction. This treatment, which is specially indicated for the removal of essential oils from aromatic plants, has been applied to hard, dry plant materials such as bark, roots, and seeds [187]; aromatic plants such as basil (*Ocimumbasilicum* L.), garden mint (*Mentha crispa* L.), thyme (*Thymus vulgaris* L.) [188], and oregano [189]; and, mainly, flowers [190–192]. Therefore, it is discussed at length in Chap. 4. As shown next, the use of MW radiation has led to a dramatic shortening of extraction times relative to conventional steam distillation.

### 6.5.6 Microwave-Assisted Derivatization (MAD)

Derivatization is a common step in analytical chemistry in general and metabolomics in particular. Derivatization can be implemented for very different purposes, the most common of which is to increase the volatility and/or thermal stability of metabolites for gas chromatographic separation. Other, less common purposes in metabolomics studies include facilitating the detection of metabolites and improving chromatographic separation. Conventional derivatization methods may take a long time (more than 70 min in some cases) at high temperatures (up to 120°C for complete silylation of amino acids, for example) [193]. Conventional derivatization uses heat, which is transferred from the vessel wall to the reactants; in microwave-assisted derivatization, energy is directly

distributed evenly and directly to the solvent and sample by MW heating. In general, MAD involves the effective heating of materials via "MW dielectric heating" effects [194]. The overall efficiency depends on the ability of MW to heat the material (whether a solvent or reagent) and increase the reactivity of the target compounds.

Most types of derivatization have been dramatically improved, both in efficiency and in rapidity, when assisted by MW energy. Therefore, metabolomics and MAD constitute an excellent association.

Derivatization before GC chemically modifies a compound to increase its volatility or improve its stability; also, it boosts separation performance and sensitivity [195]. The most popular method for GC is silylation, which reduces sample polarity and replaces active hydrogens with trimethylsilyl (TMS) groups. In fact, MW-assisted silylation of organic acids, alcohols, carbohydrates, steroids, and amino acids is commonplace in metabolomics [196]. Microwave-assisted silylation of amino acids with BSTFA is frequently required to simultaneously silylate amino and carboxyl groups in amino acids in a single step [197–202] with a view to reducing the long time required for conventional derivatization (more than 1 h at 100°C). This method affords the rapid determination of amino acids in blood and urine, a frequent need in metabolomics because their abnormal accumulation in the body is a symptom of a deficiency of enzymes associated with an amino acid metabolic pathway. Other derivatization reactions benefiting from MW assistance before GC separation and MS determination are acylation and alkylation [196].

A study compared the effect of MW irradiation, ultrasonication, ultracentrifugation, and conventional heating on the derivatization to dinitrophenyl derivatives of nine amino alcohols for their subsequent enantioseparation on α1-acid glycoprotein and β-cyclodextrin columns; microwave-assisted derivatization (MAD) proved the best choice, with shorter derivatization times and higher efficiency than the others [203].

Although the aforedescribed MAD methods involve targeting metabolomic analysis (i.e., the determination of individual compounds or compound families), metabolomic coverage is the most desirable approach in metabolomics. Konstantinos et al. [204] developed a method for the simultaneous microwave-assisted metoximation and silylation of sugars, amino acids, organic acids, and fatty acids in a commercial MW device. The derivatization products were individually separated and determined by comprehensive two-dimensional gas chromatography–TOF/quadrupole-MS. Special care was required when adding the derivatization reagents, a large excess of which produced a number of artifactual peaks, mainly at low masses or retention times.

Microwave radiation has been used to assist multiple steps in metabolomic sample preparation including derivatization [205]. One-step extraction–derivatization–concentration before GC–MS analysis of 20 phenols and 10 phenolic acids was successfully accomplished within 2 min in a household 900-W microwave oven set at 40% of its total power. A compromise solution of catalysts, organic solvents, derivatization reagents. and pH was required to determine all metabolites in different types of samples (environmental, commercially available pharmaceutical dry plants).

Miniaturization is a highly desirable goal and a growing trend in MW-assisted sample preparation in metabolomics. Damm et al. [206] have reported MW-assisted derivatization

protocols for use before GC–MS that utilize a silicon carbide-based microtiter plate platform fitted with screw-capped GC vials. They selected three standard derivatization protocols (acetylation for morphine, pentafluoropropionylation for 6-monoacetylphorphine, and trimethylsilylation for $\Delta^9$-tetrahydrocannabinol) and achieved complete derivatization within 5 min at 100°C in a dedicated multimode MW device equipped with on-line temperature monitoring. The ensuing platform allowed the simultaneous derivatization of 80 reaction mixtures under strictly controlled temperature conditions.

One typical derivatization reaction for improving detection is the formation of fluorescent compounds from nonfluorescent or poorly fluorescent analytes. Metabolites such as histidine, and 1- and 3-methylhistidine, in human serum were individually separated by capillary electrophoresis after MAD, using fluorescein isothiocyanate and a household MW oven for 150 s. The use of an MW system not specifically designed for research purposes introduced irreproducibility problems that were easily solved by using a commercial dedicated device [207].

Measurements of extracellular metabolites have several advantages over the analysis of microbial cultures for intracellular compounds (metabolic fingerprinting). Villas-Bôas et al. [208] developed and optimized a method for high-throughput analysis of metabolites resulting from the breakdown of natural polysaccharides by microorganisms. The simple protocol used enabled simultaneous separation and quantitation of more than 40 different sugars and sugar derivatives, in addition to several organic acids in complex media, all by using 50-μl samples and a standard GC–MS platform that was fully optimized for this purpose. Sample derivatization was based on the protocol proposed by Roessner et al. [209] except that the incubation procedure was modified to increase the reaction throughput substantially. The dried samples were resuspended in 80 μl methoxyamine hydrochloride solution in pyridine, and incubated in a household microwave oven for 2.8 min with multimode irradiation set to 400 W and 30% of exit power. A volume of 80 μl of (N-methy-N-(trimethylsilyl) trifluoroacetamide) (MSTFA) was then added to each sample, followed by 3.0-min incubation in the microwave oven under conditions identical to those used in the previous step. The final incubated mixture was transferred to a GC–MS vial that was tightly capped and analyzed. The metabolic footprinting profile allowed sample types to be distinguished. Also, differential metabolite-level data provided insight into the specific fibrolytic activity of the different microbial strains and lay the groundwork for integrated proteome–metabolome studies of fiber-degrading microorganisms.

## 6.6 Foreseeable Trends in MW-Assisted Steps in Omics

However rapidly it may be growing, the use of MWs to assist analytical omics is still in its infancy. A number of questions remain unanswered as to the exact mechanisms of action of MW radiation as compared to traditional heatin, and the actual utility and potential of this emerging field. So far, the kinetics and specificity of MW-assisted incubations and reactions in genomics and proteomics have only been examined in a very small number of areas and on a limited number of systems; by

contrast, MW-assisted steps involving metabolites have been developed almost since the inception of MW devices in the analytical laboratory.

This chapter describes a variety of methods profiting from MW-assisted heating and catalysis. Many researchers may already have formed an opinion on whether MW-assisted methodologies would benefit their particular laboratories. Past research and present needs suggest some foreseeable trends in the use of MWs to assist omics, namely:

(a) The use of magnetite beads for accelerated MW-assisted enzymatic digestion and other sample preparation steps. The acting effect of beads as "trapping probes" with electrostatic attraction can induce a concentration effect near MW-sensitive material. Magnetic beads of materials other than zirconia, iron, gallium and metal oxides are bound to be designed, tested, and marketed for this purpose.
(b) Quantum dots (QDs), which are extensively used as fluorescence reporters in biomedical research, are likely to grow in use in various labelling applications in preference over conventional labeling methods. The recent inception of QDs in the omics arena [210] will foreseeably be followed by technical modifications based on MW assistance.
(c) The use of nanostructured materials, widely introduced in the clinical field [211, 212], and in the omics area as a result, will take advantage of MWs to improve the target processes, particularly in integrative omic studies [213].
(d) Microfluidic technologies (e.g., microsphere-based flow cytometry [214]), of growing presence in omics [215] and in nanomedicine in general [216], and nanoscale platforms [217], can be expected to benefit from MW assistance.
(e) Bioinformatic methods [218] including nanoparticle ontology [219] and nano-informatics [220] can be expected to help interpret the interaction of micro- and nano-omics systems with MWs.

An important, final consideration is what type of MW device to use for MW-assisted omic reactions at both microscale and nanoscale. New commercially available miniaturized MW devices improving on existing laboratory-specific MW systems and household MW ovens can be expected to emerge. Although laboratory-specific MW devices are expensive, they provide substantial advantages in the form of increased throughput and time savings.

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## Abbreviations

**2DGE** Two-dimensional gel electrophoresis
**AA** Atomic absorption
**AAA** Amino acid analysis assay
**b-BSA** Biotinylated BSA
**CE** Capillary electrophoresis

| | |
|---|---|
| **CFU** | Colony-forming unit |
| **CNBr** | Cyanogen bromide |
| **CU** | Colony unit |
| **DIGE** | Differential gel electrophoresis |
| **DTT** | Dithiothreitol |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **ESI** | Electrospray ionization |
| **FCM** | Flow cytometry |
| **FD** | Freeze-drying |
| **FF-PET** | Formalin-fixed paraffin-embedded tissue |
| **FISH** | Fluorescence in situ hybridization |
| **GC** | Gas chromatography |
| **GF–AAS** | Graphite furnace atomic absorption spectrometry |
| **HEPES** | Hydroxyethyl piperazineethanesulfonic acid |
| **HPAEC–PAD** | High performance anion-exchange chromatography with pulsed amperometric detection |
| **ICAT$^{R}$** | Isotope-coded affinity tags |
| **ICP** | Inductively coupled plasma |
| **IMAC** | Immobilized metal affinity chromatography |
| **iTRAQ$^{R}$** | Isobaric tag for relative and absolute quantitation |
| **LC** | Liquid chromatography |
| **LTQ** | Linear trap quadrupole |
| **MAAH** | MW-assisted acid hydrolysis |
| **MAMEF** | MW-accelerated metal-enhanced fluorescence |
| **MEF** | Metal-enhanced fluorescence |
| **MS** | Mass spectrometry |
| **MT-MEC** | MW-triggered metal-enhanced chemiluminescence |
| **NMR** | Nuclear magnetic resonance |
| **MW** | Microwaves |
| **PCR** | Polymerase chain reaction |
| **PGAP** | Pyroglutamyl aminopeptidase |
| **PNA** | Peptide nucleic acid |
| **PNGase F** | Peptide:N-glycosidase F |
| **PTFE** | Polytetrafluoroethylene |
| **PTMs** | Post-translational modifications |
| **PVDF** | Poly(vinylidine difluoride) |
| **QDs** | Quantum dots |
| **RFLP** | Restriction fragment length polymorphism. |
| **RT-PCR** | Reverse transcription polymerase chain reaction |
| **SDS** | Sodium dodecyl sulfate |
| **SEC** | Size exclusion chromatography |
| **Taq** | Thermus aquaticus |
| **TFA** | Trifluroacetic acid |
| **TMS** | Trimethylsilyl |
| **TOF** | Time-of-flight |

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# Chapter 7
# Pharmaceutical and Nutraceutical Compounds from Natural Matrices

**Pedro Cintas, Emanuela Calcio-Gaudino, and Giancarlo Cravotto**

## 7.1 Introduction

Microwave-assisted extraction (MAE) has been successfully applied, in various forms, to the isolation of biologically active compounds that lend themselves to pharmaceutical and nutraceutical applications [1]. Despite the fact that classic liquid–liquid and solid–liquid extraction methods (maceration, Soxhlet extraction, etc.) present several drawbacks, modern science is still far from fully replacing them with MAE or other nonconventional techniques [2]. Microwave (MW) radiation is currently used for the rapid extraction of several classes of bioactive compounds, phytonutrients, functional food ingredients, and pharma-active substances from biomass [3–5].

The term "nutraceutical," coined in 1989, indicates natural products that are often obtained from edible plants and which provide health benefits by their physiological or metabolic functions. The name, therefore, refers to both the nutritional and pharmaceutical properties that a compound may posses [6]. Nutraceuticals include dietary fiber, a number of types of phenolic compounds and antioxidants, polyunsaturated fatty acids, amino acids, proteins, and minerals. Benthin et al. [7] published in 1999 one of the earliest studies on herbal nutraceuticals, which included phenolic compounds, lignans, carotenoids, oils and lipids, essential oils, and other bioactive compounds.

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The main advantages of MAE are reduced solvent consumption, minimal sample manipulation, shorter operational times, and good selectivity, recovery yields, and reproducibility [8]. The method enables up to 10–15 samples to be run in a single extraction and so gives rise to high sample throughput. GLP (good laboratory practice) requirements can be fulfilled by MAE, and the fact that it can be automated makes this technique suitable for pharmaceutical applications. Dielectric volumetric heating is particularly suitable for thermolabile constituents (Chee et al. [9]).

It has been reported that MAE of phenolics in water is not as efficient as conventional methods because water has a higher dielectric constant and a lower dissipation factor than other solvents. In MAE it is better to use solvents with both a high dielectric constant and a high dissipation factor. Extractability also depends on the type of plant material extracted and the solvents used for the extraction (Proestos and Komaitis [10]). In the presence of polar molecules or ionic species, MAE provides rapid heating that leads to collisions with the surrounding molecules and so does not need to be carried out at high pressure. Power and extraction time for natural products are in the range of 25–750 W and 30 s to 10 min, respectively [1]. MAE has been used for the extraction of polyphenolics from a number of plant sources, such as tea leaves, flax seeds, radix, and vanilla among others [11–14]. MAE causes the compounds of interest to desorb from the plant matrix because the free water molecules present in the gland and vascular systems are heated, which leads to localized heating and dramatic expansion during which plant cell walls are ruptured, allowing the extracted molecules to flow toward the organic solvent. The effect of MW energy is strongly dependent on the dielectric susceptibility of both the solvent and solid plant matrix. Most of the time the sample is immersed in a single solvent or mixture of solvents that absorb MW energy strongly, so that the elevated temperature increases solvent ability to penetrate the matrix, ready to dissolve the molecules of interest [15, 16]. The main disadvantages of MAE are its high capital cost and possible need to filter the sample if fine particles are used for the extraction of compounds. The advantages and disadvantages of the main extraction methods are reported in Table 7.1.

## 7.2 Main Families of Pharmaceutical and Nutraceutical Compounds Obtained with MAE

### *7.2.1 Anthraquinones*

Dàvid et al. [17] developed a new, simplified open-vessel MW extraction (OVME) method that also uses solid-phase extraction (SPE) for the preparation of aqueous extracts of senna leaves (*Senna folium*) for the specific investigation of sennosides A and B (Fig. 7.1). Senna is a medicinal plant for short-term use in cases of occasional constipation and is present on the list of the World Health Organization's essential medicines. Its main active ingredients are sennosides A and B [18]. OVME was

**Table 7.1** Advantages and disadvantages of different extraction methods

| Method | Advantages | Drawbacks | Suggestions |
|---|---|---|---|
| Solid liquid extraction (SLE) | Commonly used, simplest procedure | Chances of impurities; introduction of analytical errors | Accurate choice of solvents |
| Pressurized liquid extraction (PLE) | Low solvent amount, fast procedure | Unsuitable for thermolabile compounds | Optimal setting of several parameters: solvent ratio, temperature, pressure, extraction time |
| Supercritical fluid extraction (SFE) | Eco-friendly and efficient method (GRAS[a] solvents) | High capital investment requirement of high pressure | Optimal setting of pressure may reduce operation costs |
| Ultrasound-assisted extraction (UAE) | Highly efficient and fast procedure (room temperature), useful for thermolabile compounds | Volume limit for batch production, need of flow-reactors | Optimal setting of frequency/power and uniform distribution of energy |
| Microwave-assisted extraction (MAE) | Low solvent amount; high extraction rate and yield | High capital cost | Suitable particle size (crushing/grinding) to improve efficiency |

[a]GRAS, generally recognized as safe
*Source*: Reproduced in part from Ajila et al. [2]

performed in a Whirlpool VIP34 1,650-W MW oven. The MW power was set at 160, 350, and 500 W nominal energy levels for 1, 3, and 5 min, respectively. After preparation, all the aqueous extracts were purified by SPE (LiChrolut RP-18).

### *7.2.2 Benzoquinones*

Embelin (Fig. 7.2) is a benzoquinone derivative from *Embelia ribes* that is endowed with several pharmacological properties which include antibacterial, antiinflammatory, and analgesic activity [19]. Latha [20] described a rapid and efficient MAE process for the selective extraction of embelin from *E. ribes*, with a significant reduction in solvent quantity: 92% (w/w) embelin recovery was observed (purity 90%) in only 80 s (Fig. 7.3).

### *7.2.3 Extraction with a MW Pre-Treatment*

The following examples show the advantage of carrying out sample pre-irradiation before the actual extraction. The main family of compounds studied are phytosterols

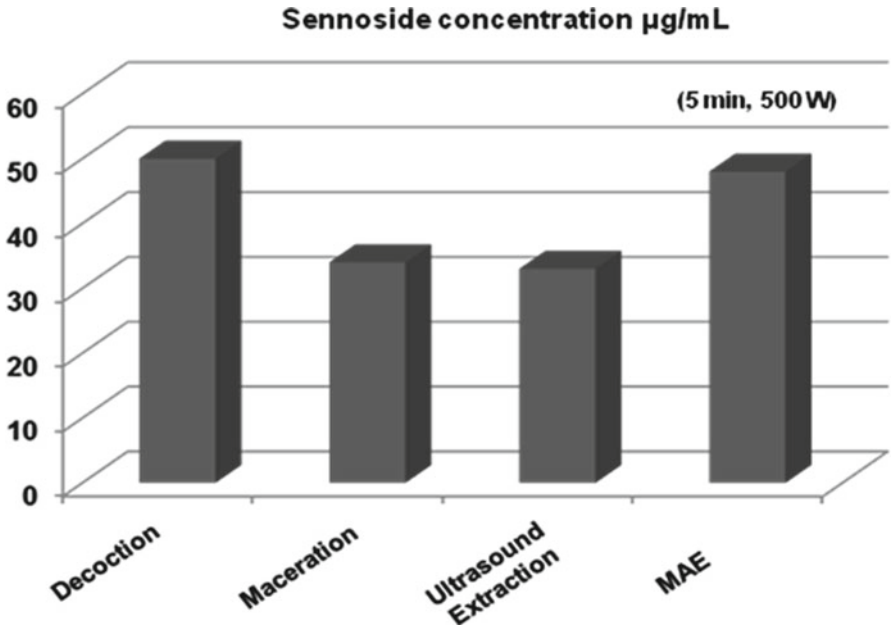
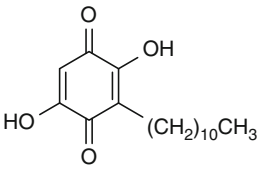



**Fig. 7.1** High pressure liquid chromatography-mass spectrometery (HPLC-MS) analysis of sennosides A and B in aqueous extracts of *Sennae folium* under different techniques. (Reproduced in part from *WHO Model List of Essential Medicines for Children*, 3rd edn. [18] Copyright 2009)

**Fig. 7.2** Chemical structure of embelin




(brassicasterol, stigmasterol, campesterol, sitosterol, and Δ5-avenasterol) and tocopherols (α- and γ-tocopherol).

Damirchi et al. [21] irradiated rapeseed with MW before extraction to investigate the influence of this preheating on the oil yield, its oxidative stability, and composition profile. Rapeseed was preheated for 2 or 4 min and oil was then extracted with solvents or with a press. Rapeseed MW pre-treatment can increase oil extraction yield (by 10%) and phytosterol and tocopherol oil content (by 15 and 55%, respectively). The oil extracted from untreated rapeseed using the press had the lowest oxidative stability (1 h), this was increased to 8 h when the rapeseed was pretreated with MW. Therefore, basing our claims on the obtained results, it would appear advisable to treat rapeseed with MW before extraction by oil press, because it gives a relatively good oil recovery with a higher amount of nutraceuticals, and can produce oil with a longer shelf life and enhanced value.

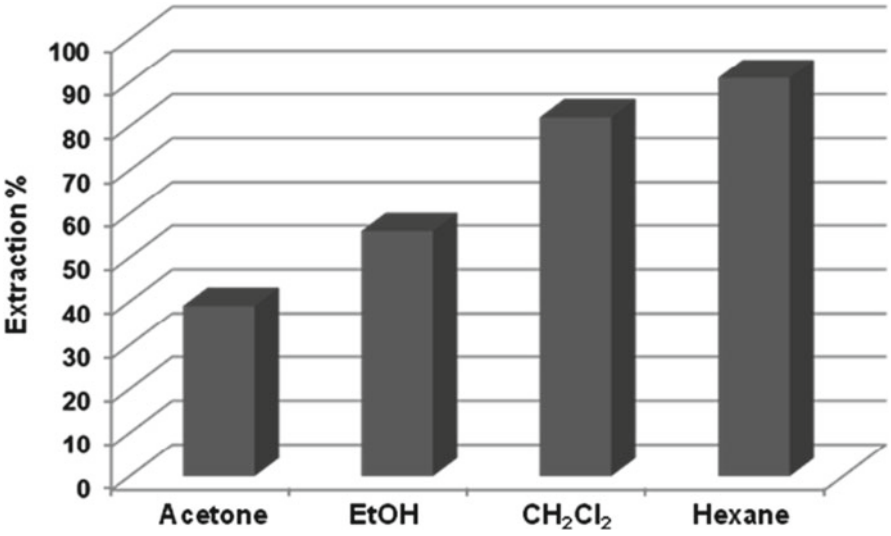



**Fig. 7.3** Microwave-assisted extraction (MAE) (150 W) of embelin using different solvents (ratio 1% w/v). Reproduced in part from Damirchi et al. [21])

### *7.2.4 Continuous Microwave-Assisted Extraction (CMAE)*

Terigar et al. [22] studied soybean and rice bran oil extraction in a continuous MW system, starting from the laboratory up to pilot scale. These oils are widely used in the food, cosmetic, and pharmaceutical industries because of the high amount of antioxidants and other valuable nutrients they contain (gamma oryzanol, tocotrienols, and tocopherols) and the well-balanced fatty acid profiles they present. The oils were extracted from soy flour and rice bran at various time–temperature combinations using ethanol (feedstock ratio 3:1) with a CMAE system. An analysis of oil quality indices (IV, AV, FFA content, wax, and phospholipids) indicate that it meets prescribed quality standards, further justifying use of MW as a rapid tool for oil extraction.

Asghari et al. [23] extracted a series of bioactive compounds from different medicinal plants. The MAE of *E*- and *Z*-guggulsterone (1) from *Commiphoria mukul*, and tannic acid (2) from the galls of *Quercus infectoria* and cinnamaldehyde from *Cinnamomum verum* J.S. Presl, was compared with conventional extraction (Fig. 7.4).

Guggul, or guggulsterone, is a recognized hypolipidemic, antioxidant, and antiinflammatory compound. It has been established that guggulsterone is an antagonist at the farnesoid X-receptor (FXR), a key transcriptional regulator for the maintenance of cholesterol and bile acid homeostasis [24]. Tannic acid is known for its antibacterial properties.

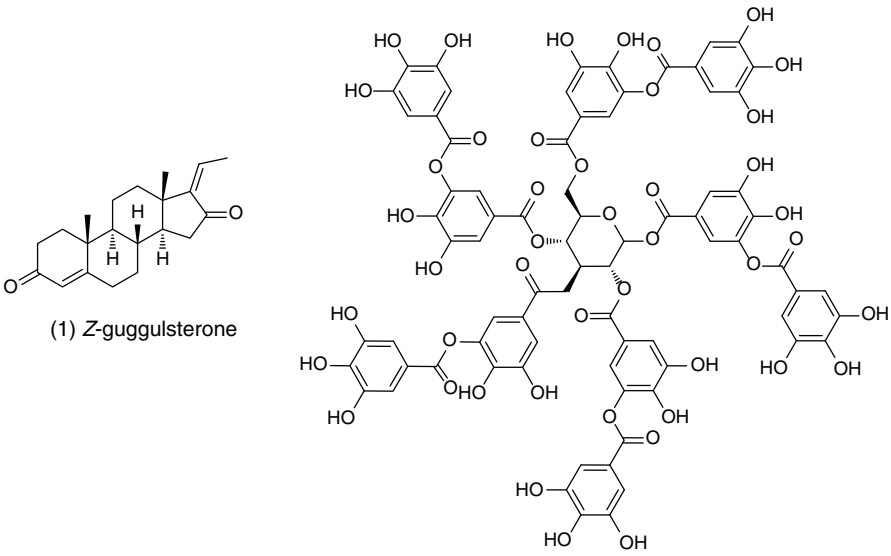



**Fig. 7.4** Chemical structure of guggulsterone and tannic acid

**Table 7.2** Comparison of microwave-assisted extraction (MAE) with conventional procedure (conventional Soxhlet extraction, CSE)

| Plant | *Commiphoria mukul* | | *Quercus infectoria* | | *Cinnamomum verum* J.S. Presl | |
|---|---|---|---|---|---|---|
| Characteristics | MAE | CSE[a] | MAE | CSE[a] | MAE | CSE[b] |
| Sample (g) | 13.3 | 50 | 15 | 50 | 3 | 15–20 |
| Solvent volume (ml) | EtOAc | EtOAc | MeOH 90% | Aq. MeOH 50% | Aq. EtOH 80% | $H_2O$ |
| | 130 | 500 | 125 | | 40 | 500 |
| Temperature (°C) | 80 | bp | 67 | bp | 120 | 97 |
| Time | 1 h | 3 h | 30 min | 24 h | 1 h | 2 h |
| Press (bar) | 1 | Ambient | 1 | Ambient | 1 | Ambient |
| Yield (%) | 2.5–3 | 2 | 10–20 | 10 | 0.84–1.0 | – |

Extraction of bioactive chemical compounds from the medicinal Asian plants by microwave irradiation

*Source*: Reprinted from Deng [24]; available online at http://www.academicjournals.org/JMPR, ISSN 1996–0875 ©2011 Academic Journals)

[a]Soxhlet

[b]Steam distillation

[c]mg isolated active compound/g dry plant material

Ordinary solvent extraction at room temperature was carried out to compare MAE with traditional extraction methods (Table 7.2). The main advantages of the use of MAE are the considerable reduction in time and solvent consumption and the

increased purity of crude extracts over conventional extraction techniques. Furthermore, it is usually simpler to separate the crude extracts from the plant matrix in the MAE method.

### *7.2.5 Polyphenols*

Phenolic compounds are one of the main classes of secondary metabolites and offer several health benefits (antioxidants, free radical scavengers) [25], and so are commonly used as functional foods and in the prevention of chronic diseases [26].

Sutivisedsak et al. [27] suggested that the extraction yield of phenolic compounds, from eight bean types, and their antioxidant power could be enhanced using MAE. The effects of extraction temperature and solvent were evaluated, and a comparison was made between conventional extraction and MAE. Deionized water, ethanol/water 50%, and pure ethanol (49 ml each) were used as solvents; 15 min irradiation was performed in a professional MW oven (Ethos 1600; Milestone) at three different temperatures (50°C, 100°C, and 150°C). Extraction with 50% ethanol/water at 150°C was most effective. The total phenolic content obtained in water at 100°C under MAE was two to three times higher than the conventional extraction with water at the same temperature.

Singh et al. [28] developed a means to exploit potato peel as a source of phytonutrients such as phenolic antioxidants [29]. These antioxidants have free radical scavenging effects and decrease the risk of coronary heart diseases [30] by reducing cholesterol in blood serum and by enhancing the resistance of vascular walls [31]. The authors used a response surface method to optimize MAE parameters and conditions (extraction time, solvent ratio, and MW power). Higher levels of phenolics were recovered using less solvent and very short extraction times.

Ajila et al. [32] tested the solid-state fermentation of apple pomace using *Phanerocheate chrysosporium* to release phenolic antioxidants. The extraction of polyphenols from apple pomace and fermented apple pomace was carried out using ultrasonic-assisted extraction and MAE methods. The effects of various solvents, temperature, time, and detergents were investigated in the extraction of polyphenols for both techniques. Optimized conditions were MW for 10 min at 60°C at a pressure of 692 kPa and power rating of 400 W in a professional oven (Mars; CEM, Matthews, NC, USA). The extraction yield of polyphenol from apple pomace was higher under MW or US irradiation, which also improved antioxidant activity.

Song et al. [33] employed an efficient MAE technique to extract total phenolics (TP) from sweet potato (*Ipomoea batatas* (L.) Lam.) leaves. The optimal MAE conditions were determined using the response surface methodology, which provided large benefits in terms of yield and extraction time (Fig. 7.5).

The use of natural antioxidants in the food industry has increased in recent years, and there is a growing interest in improving the extraction processes using GRAS (generally recognized as safe) solvents. In their work Rodríguez-Rojo et al. [34] studied the extraction of antioxidants from rosemary using different extraction

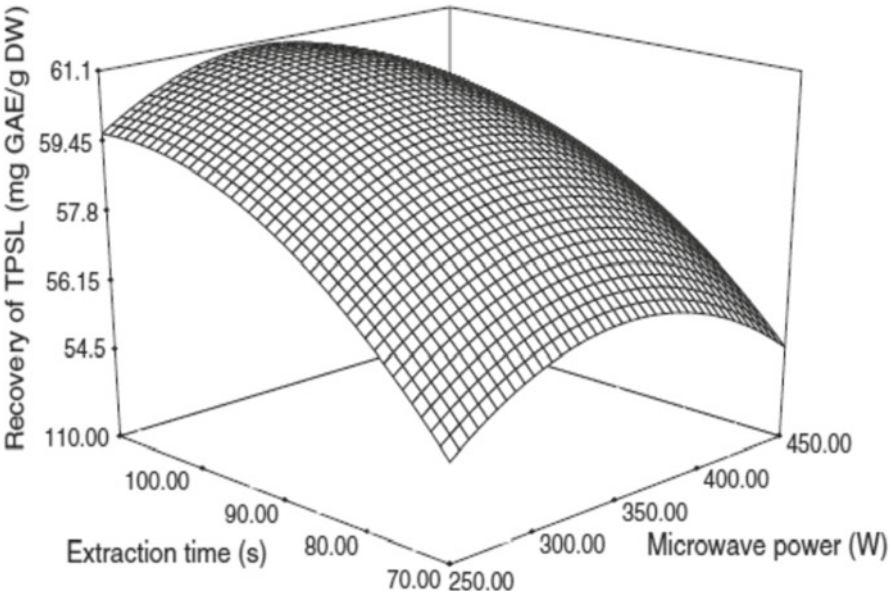



**Fig. 7.5** Response surface plots showing effects of MW power and extraction time on the recovery of TPSL and their interaction. The ethanol proportion was constant at 70% (v/v). (Reprinted from Rodríguez-Rojo et al. [34], copyright (2011), with permission from Elsevier)

processes [conventional, MAE, and ultrasound-assisted extraction (USAE)], solvents (ethanol and water), and plant pre-treatments (de-oiled and milled, de-oiled and fresh plant). The double pre-treatment, de-oiling using solvent-free MW extraction (SFME) and milling, proved to be essential to overcoming inner mass transfer limitations. The proposed extraction procedure, solvent-free oil extraction and grinding followed by an assisted solvent extraction with a benign solvent (water or ethanol), provides a rosemary extract of equal or higher antioxidant content than those produced using other extraction techniques or different procedures within the same processes (MAE and USAE). The amount of rosmarinic acid was between 50 and 140 mg/g dried extract, carnosic acid content in ethanolic extracts about 80 mg/g dried extract, and total phenolic content between 110 and 180 mg GAE/g (gallic acid equivalents/g) dried extract. Moreover, the process is very fast (less than 15 min) and more efficient in terms of yield and energy consumption.

### *7.2.6 Stilbenes and Minerals*

*Vitis coignetiae*, a wild grapevine, found between 100 and 1,300 m above sea level in Korea, deserves great importance as a source of nutraceuticals. *V. coignetiae* contains more organic acids and water-soluble vitamins than *Vitis vinifera* and has

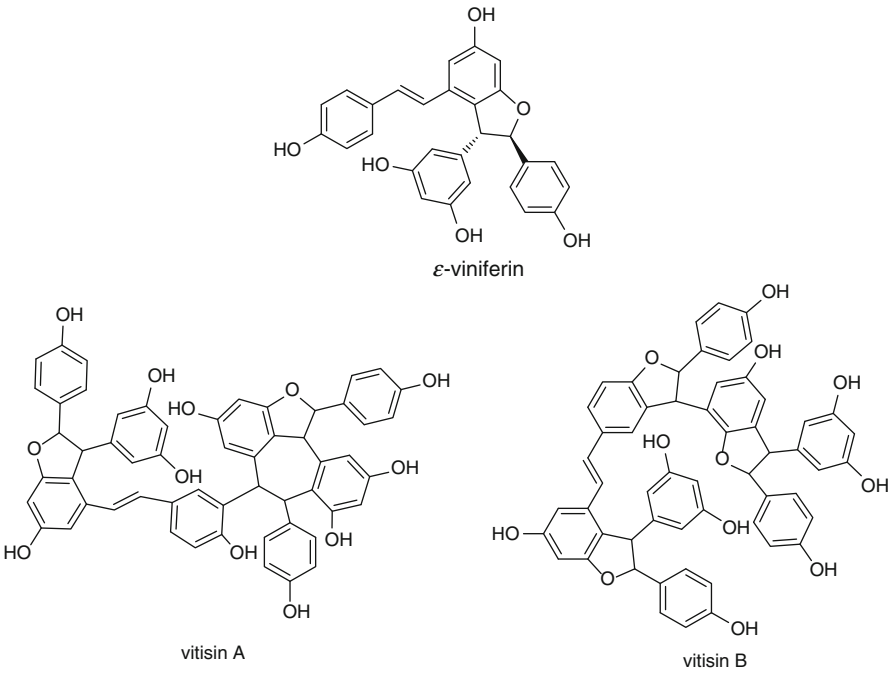



**Fig. 7.6** Chemical structure of new oligostilbenes from *Vitis coignetiae*: ε-viniferin and vitisin A and B

a mineral (e.g., K, Ca, Fe, P) content that is ten times higher. New oligostilbenes from *V. coignetiae* were identified as ε-viniferin and vitisin A and B (Fig. 7.6). The methanolic extract of *V. coignetiae* showed hepatoprotective activity in an in vitro assay using primary cultured rat hepatocytes. Activity-guided fractionation of the extract afforded ε-viniferin as an active component. The protective effect of ε-viniferin against carbon tetrachloride-induced hepatic injury in mice was shown by serum enzyme assay as well as by pathological examination. Recently, Kim et al. [35] extracted pterostilbene using MAE and found it to be a potent chemopreventive agent. Kim et al. [36] described optimized MW extraction conditions for viniferin from *Vitis coignetiae.* To improve total extract yield, they established MW power at 70–150 W for 8–18 min, using 30–50% ethanol concentration.

### *7.2.7 Lignans*

Lignans are phytoestrogens abundant in the bran layer of cereals and the seed coat of several oil seeds. Lignans have antioxidant and weak estrogenic or antiestrogenic effects, thus providing protection against cardiovascular diseases, metabolic syndrome, and certain tumors.

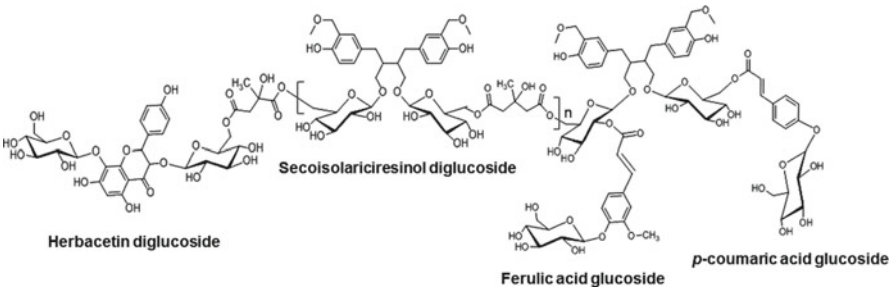



**Fig. 7.7** A sketch of the lignin macromolecule in flaxseed. (From Gao et al. [38])

Nemes and Orsat [37] described the efficiency, repeatability, and reliability of an optimized MAE method for the analytical quantification of lignans in plant materials (flaxseed) [37]. The MAE experiments were carried out in a monomode (focused) MW apparatus (Star System 2; CEM) with a nominal power of 800 W and MW frequency of 2.45 GHz. The MW power was applied intermittently (30 s on/off) for 3 min. The temperature of the extracts rose from room temperature (22–23°C) to about 67°C over the 3-min span. The recovery of lignans throughout the extraction, preparation, and analysis steps is 97.5% with a coefficient of variation <1%. The MAE method is efficient for extracting lignans from the plant matrix, and it achieves significantly higher extraction yields than the two established reference methods (Fig. 7.7).

Gao et al. [38] defined an on-line continuous sampling dynamic MAE coupled with HPLC separation for the determination of lignans in Wuweizi and naphthoquinones in *Zicao* (Fig. 7.8).

Podophyllotoxin (lignan) displays a range of activities that include cathartic, purgative, antiviral, vesicant, and anti-helminthic properties. Additionally, lignan and its derivatives are exciting leaders in the field of antitumor agents. In fact, podophyllotoxin is the pharmacological precursor for the important anticancer drug etoposide [39, 40].

### *7.2.8 MAE with Ionic Liquids (ILs-MAE)*

Yuan et al. [41] reported the use of ILs-micelles for the ILs-MAE extraction of podophyllotoxin from three herbal medicines (Fig. 7.9), *Dysosma versipellis*, *Sinopodophyllum hexandrum*, and *Diphylleia sinensis*, employing IL aqueous solution as an alternative and effective surfactant (Table 7.3).

### *7.2.9 Flavonoids*

Flavonoids exhibit a wide range of biological effects, including antibacterial, antiinflammatory, antiallergic, and antithrombotic properties. Epidemiological studies point to a possible role in preventing cardiovascular diseases and cancer.

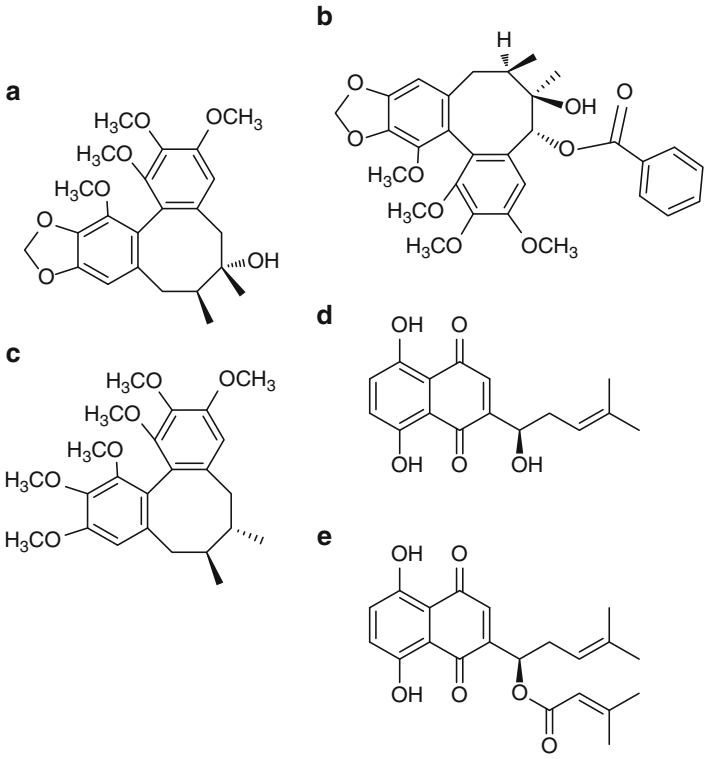
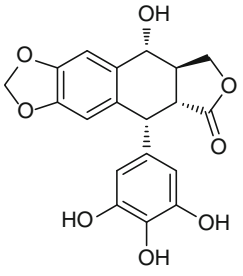



**Fig. 7.8** Chemical structure of naphthoquinones components in *Zicao*: structure of (**a**) Schisandrin A, (**b**) Schisantherin A, (**c**) deoxyschizandrin, (**d**) Shikonin, and (**e**) β,β'-dimethylacrylshiconin

**Fig. 7.9** Chemical structure of podophyllotoxin




Flavonoids behave as antioxidants via a variety of mechanisms that include the direct trapping of reactive oxygen species, the inhibition of the enzymes which are responsible for superoxide anion production, the chelation of the transition metals involved in processes that form radicals, and prevention of the peroxidation process via the reduction of alkoxyl and peroxyl radicals [42].

Biesaga [43] used a LC-MS/MS method to evaluate the stability of flavonoids extracted from maize samples using four different methods: heating under reflux, sonication, maceration, and MAE. The 11 flavonoids belong to different groups:

**Table 7.3** Comparison of ionic liquids (ILs) extraction efficiencies using different extraction techniques42

| | | Proposed technique (%) | Reference techniques (%) | | |
|---|---|---|---|---|---|
| Samples | ILs | MAE (10–15 min) | Maceration (2 days) | Heat (5 h) | Ultrasound (45 min) |
| *D. versipellis* | [bmim][$BF_4$] | 96.8 | 60.6 | 85.7 | 84.8 |
| *S. hexandrum* | [denim][$BF_4$] | 89.1 | 58.1 | 75.8 | 80.5 |
| *D. sinenis* | [amim][$BF_4$] | 82.4 | 52.4 | 70.1 | 73.7 |

[bmim][$BF_4$] 1-Butyl-3-methylimidazolium tetrafluoroborate
[denim][$BF_4$] 1-decyl-3-methylimidazolium tetrafluoroborate
[amim][$BF_4$] 1-allyl-3-methylimidazolium tetrafluoroborate
*Source*: Reproduced in part from Veitch et al. [42]

**Table 7.4** Total phenolic and flavonoids content of the leaves of three varieties of *Labisa*45

| Plant | Phenolics[a] | Flavonoids[b] |
|---|---|---|
| *L. alata* | 3.48 | 2.49 |
| *L. pumila* | 3.37 | 2.77 |
| *L. lanceolata* | 3.23 | 2.29 |

[a]mg gallic acid equivalent/g
[b]mg rutin equivalent/g
*Source*: Reproduced in part from Zhou et al. [45]

flavonols (kaempferol, myricetin, rhamnetin, quercetin, rutin), flavanones (naringenin, naringin, hesperedin), flavones (apigenin, luteolin), and isoflavones (genistein).

Karimi and Jaafar [44] used MAE to extract flavonoids, isoflavonoids, and phenolic compounds from the leaves of three varieties of *Labisia pumila* Benth. to then determine their antioxidant properties. MAE was performed at 60°C using a closed-vessel system under pressure (ETHOS T MW digestion/extraction system; Milestone, Italy) with methanol (30 ml) for 2 min (P = 750 W). The overall result obtained from this research suggested that all varieties of *Labisia pumila* Benth. are sources of bioactive compounds that are endowed with interesting antioxidant activities. Thus, the presence of phytochemicals and other bioactive compounds present in this plant may serve as a new potential source of medicines in the future (Table 7.4).

### *7.2.10 Flavonoids and Hydroxycoumarin Glycosides*

In their work, Zhou et al. [45] investigated the potential application of polyethylene glycol (PEG) as a possible alternative green solvent to be used in the MAE of flavone and coumarin compounds from medicinal plants, and PEGs of various molecular weights and at several different concentrations were used to extract the flavone and coumarin compounds. PEG-MAE, organic solvent-MAE, and conventional heating reflux extraction (HRE) were evaluated for their capacity to extract nevadensin from

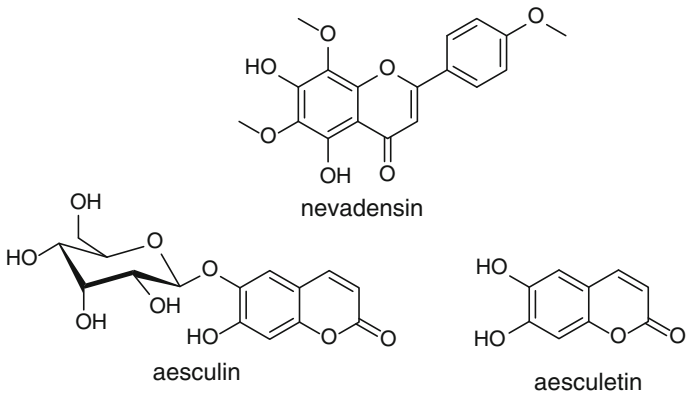



**Fig. 7.10** Chemical structure of nevadensin, aesculin, and aesculetin

*Lysionotus pauciflorus* (a traditional medicinal herb for the treatment of lymph node tuberculosis, coughs with achypnea, and rheumatic pains) as well as extracting aesculin and aesculetin from *Cortex fraxini* (which inhibit the growth of dysentery bacillus and have been shown to possess expectorant, antitussive, and antiasthmatic activity) (Fig. 7.10).

Compared with ionic liquids, PEGs have other advantages, such as their greatly reduced cost, the fact that they are completely nonhalogenated, and their generally well known low toxicity.

The proposed method was able to provide higher extraction yields and a considerable reduction in extraction time and solvent consumption when compared to conventional extraction procedures. Furthermore, PEG solutions are suitably MW absorptive, which is a great advantage in MAE procedures. The PEG-MAE kinetic mechanism indicated that about 10 min was sufficient to obtain high target compound extraction yields and, according to the scanning electron microscopy (SEM) results, this enhanced extraction was mainly the result of the destruction of sample microstructures in PEG-MAE process. The proposed technique is a green, simple, rapid, and effective extraction method for the separation of flavone and coumarin compounds from the Chinese herb that, with the development of green sample preparation techniques, shows great promise in the extraction of useful substances from natural sources.

### *7.2.11 Coumarins*

Bergenin (Fig. 7.11) is an isocoumarin isolated from various medicinal plants that provides a wide range of biological benefits such as antihepatotoxic [46] antiulcer [47], antidiabetic [48], antiarrhythmic [49], antiinflammatory [50], antiarthritic, and antitussive properties [51]; in addition, it shows mild activity against human immunodeficiency virus (HIV) [52].

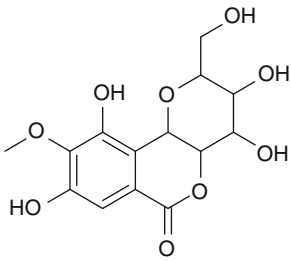



**Fig. 7.11** Chemical structure of bergenin

Deng et al. [53] developed a simple method for the rapid extraction, separation, and purification of bergenin from *Ardisia crenata* s. and *Rodgersia sambucifolia* h. using MAE coupled with high-speed counter-current chromatography (HSCCC). The MAE conditions were optimized, and a 2.0-g sample was extracted using 60% (v/v) aqueous methanol as the extraction solvent with a liquid-to-solid ratio of 10:1 (ml/g). The extraction was carried out at 60°C for 15 min. The crude extract was separated and purified directly by HSCCC using an ethyl acetate/*n*-butanol/water (3:2:5, v/v/v) solvent system. In less than 3.5 h, 18.6 and 25.0 mg bergenin, respectively, were obtained from a 160-mg crude extract of *A. creanta* or *R. sambucifolia* in a one-step separation. The purity of bergenin was more than 99% as determined by HPLC. The results indicate that MAE coupled with high-speed counter-current chromatography is a highly suitable technique for the extraction, separation, and purification of bergenin from *A. creanta* and *R. sambucifolia*.

### *7.2.12 Pectin*

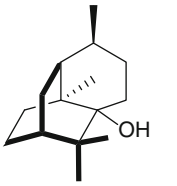
Besides its well-known use in the food industry, pectin is also used in phytotherapy to reduce heart disease and gallstones.

Bagherian et al. [54] found that MAE can lead to a considerable increase in the yield and quality of extracted pectin. For instance, the extraction of pectin from fruit materials pretreated in an electromagnetic field of super-high frequency led to a higher pectin yield and higher degree of esterification (DE) values for gel strength compared with the non-pretreated samples. Furthermore, the 2-min MW heating period was enough to extract the same amount of pectin as obtained from the 90-min conventional extraction period.

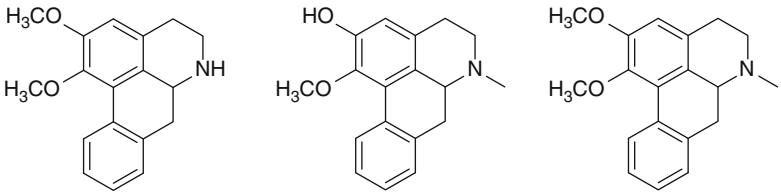
### *7.2.13 Patchouli Alcohol*

Fan et al. [55] developed a MW radiation-accelerated ionic liquid pre-treatment (MRAILP) to enhance the extraction of patchouli alcohol (Fig. 7.12) from *Pogostemon cablin*. Under the optimized conditions, the extraction yield of patchouli

**Fig. 7.12** Chemical structure of patchouli alcohol




patchouli alcohol




*N*-nornuciferine *O*-nornuciferine nuciferine

**Fig. 7.13** Chemical structure of *N*-nornuciferine, *O*-nornuciferine, and nuciferine

alcohol using MRAILP was 1.94%, which was 166% higher than MAE in absence of ionic liquid pre-treatment. The recovery was in the range of 95.71–103.7% with a relative standard deviation less than 3.0%. It is a novel alternative extraction method for the rapid extraction and quantity determination of patchouli alcohol from *Pogostemon cablin*.

### *7.2.14 Alkaloids*

***N-nornuciferine, O-nornuciferine, and nuciferine*** (Fig. 7.13). Nuciferine has a pharmacological profile of action that is associated with the dopamine-receptor blockade; that is, it induces catalepsy, inhibits spontaneous motor activity, and conditions avoidance response, amphetamine toxicity, and stereotypy.

Maa et al. [56] developed an efficient IL-MAE method for the extraction and quantification of the three alkaloids *N*-nornuciferine, *O*-nornuciferine, and nuciferine from lotus leaves. Optimal MAE conditions were studied and noted. Compared with regular MAE and conventional HRE methods, the approach provided higher extraction efficiency (0.9–43.7% enhancement) and a greatly reduced extraction time (from 2 h to 2 min). This observation demonstrates that the aqueous IL solutions are excellent extractants and that IL-MAE is a simple, rapid, and effective extraction method. Moreover, considering the unique properties of ILs, the proposed IL-MAE method has wide-ranging potential as an environmentally friendly sample preparation technique.

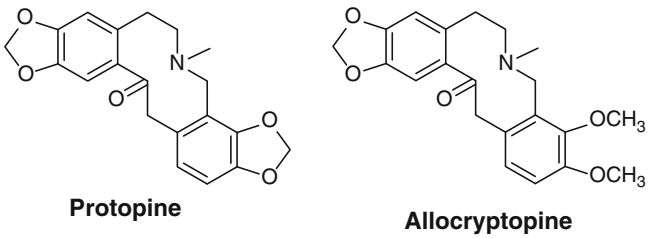



**Fig. 7.14** Chemical structure of protopine and allocryptopine

### *7.2.15 Protopine (PRO) and Allocryptopine (ALL)*

In recent years, PRO and ALL (Fig. 7.14) have been the focus of commercial interest because of their pharmacological properties that include antithrombotic, antiinflammatory [57], antibacterial [58], and antihelminthic activity [59]. They also function as inhibitors of phosphodiesterase enzymes [60]. Therefore, commercial production of PRO and ALL from *Macleaya cordata* is of importance and the plant resources should be utilized efficiently.

Zhong et al. [61] investigated the extraction of protopine and allocryptopine from the stems of *Macleaya cordata* (Willd) R. Br. with MAE using less solvent, less extracta sicca (ES), and in a shorter time. The three variables ethanol concentration (20–80%, v/v), extraction temperature (30–70°C), and solvent/solid ratio (10:1:30:1, ml/g) were investigated in this study. The results showed that the optimal parameters of MAE were an ethanol concentration of 45.2% (v/v), extraction temperature of 54.71°C, and a solvent/solid ratio of 20.4:1 (ml/g). Under these conditions, the extraction yields of protopine and allocryptopine were 89.4% and 102.0%, respectively, and the dry product yield was 12.5%.

### *7.2.16 Oxymatrine Possesses Important Biological Properties Including Anticancer Activity and the Inhibition of Hepatitis B Virus Replication [62]*

Xia et al. [63] studied the MAE of oxymatrine from *Sophora flavescens* and found that MW irradiation was a powerful tool that efficiently improved the extraction of oxymatrine. The effects of several experimental parameters on the extraction yields of oxymatrine were evaluated, and the optimal extraction conditions were determined as 60% ethanol, a liquid-to-material ratio of 20:1, and a procedure that lasted 10 min at 50°C under 500 W MW irradiation. Under these optimal conditions, the yield of oxymatrine was 14.37 mg/g. The crude extract could be used either as a component of certain complex traditional medicines or further isolated and purified to provide oxymatrine.

**Table 7.5** Biomarker compounds

| | Concentration of biomarker compounds (mg 100 $g^{-1}$) ± SD | | | |
|---|---|---|---|---|
| MAE at 100°C | Catechin | Caffeic acid | Epicatechin | Rhynchophylline |
| CYY | 15.8 ± 1.28 | 6.4 ± 0.08 | 20.2 ± 1.28 | 117.6 ± 5.59 |
| YSF | 36.5 ± 1.6 | 17.8 ± 0.52 | 44.0 ± 1.22 | 59.9 ± 2.58 |
| SH | 98.3 ± 1.18 | 11.9 ± 0.24 | 81.5 ± 1.99 | 109.2 ± 1.84 |

Three samples of *Unicaria sinensis* (CYY, YSF, and SH) from different sources
Optimized extraction conditions: water as extraction solvent, extraction time 20 min
MAE at 100°C; concentration of biomarker compounds (mg 100 $g^{-1}$) ± SD
Determination of metabolites in *Uncaria sinensis* by HPLC and GC–MS after green solvent microwave-assisted extraction
*Source*: Reprinted from Tigrine-Kordjani et al. [65], copyright 2011, with permission from Elsevier

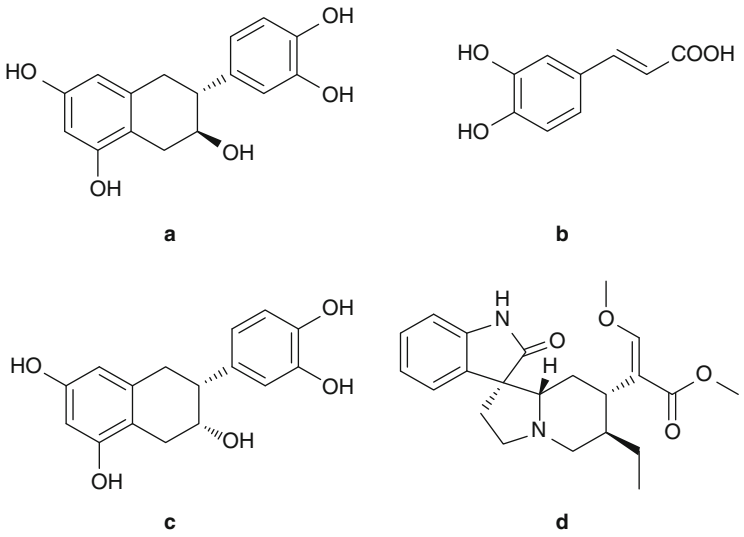



**Fig. 7.15** Chemical structure of catechin (**a**), caffeic acid (**b**), epicathechin (**c**), and rhynchophylline (**d**) in *Uncaria sinensis*

### *7.2.17 Alkaloids and Flavonoids*

Tan et al. [64] extracted the major bioactive secondary metabolites of *Uncaria sinensis* using the MAE method with ultrapure water as the extraction solvent. The optimal extraction conditions for this green solvent MAE method were found to be a temperature of 100°C for 20 min (Table 7.5). *Uncaria sinensis* has long been used as an important traditional Chinese medicine (TCM) herb for the treatment of fevers and various nervous disorders and seems to have three classes of bioactive compounds. These bioactive secondary metabolites are caffeic acid (CA, an organic acid), rhynchophylline (RH, an alkaloid), and catechin (CT, a flavonoid), and epicatechin (epiCT, another flavonoid) (Fig. 7.15). It has been shown that flavonoids

such as epicatechin and catechin are active components that protect the cultured cerebellar granule cells against glutamate-induced neuronal death through the inhibition of $Ca^{2+}$ influx [65]. Other reports have brought to light the anti-dementia effects of phenolic compounds (e.g., caffeic acid) and indole alkaloids (e.g., rhynchophylline and isorhynchophylline), all of which are present in *U. sinensis*.

### *7.2.18 Terpenes*

Tigrine-Kordjani et al. [66] observed a large difference in the chemical composition of the oils of *Zygophyllum album* L. that were obtained with MW-accelerated distillation (MAD) [67] and hydrodistillation (HD). α-Terpineol and carvone (oxygenated monoterpenes) were the major species in the MAD oil, and β-damascenone was the most common component in the HD oil.

This result was explained by the fact that MAD interferes with polarization effects that cannot be easily separated from the physical and chemical properties of isolated molecules.

One of the main advantages of MW extraction is the reduction in heating time, which prevents the degradation of thermolabile components and provides more valuable essential oils. An extraction time of 30 min with MAD provides yields (0.002%) comparable to those obtained after 3 h using HD, which is one of the reference methods in essential oil extraction.

The presence of antioxidants such as thymoquinone and 2,6-di-(*t*-butyl)-*p*-benzoquinone in the essential oils of *Zygophyllum album* L., obtained using either MAD or HD, shows that this plant may have interesting antioxidant properties.

It was also reported that different species of *Zygophyllum* are used against diabetes and as a drug to fight rheumatism, gout, asthma, and hypertension.

In some developing countries, plants, in the form of crude extracts, infusions, and plasters, are commonly used in the treatment of a number of pathologies and as diuretic, local anesthetic, and antihistaminic medicines.

### *7.2.19 Triterpenoid Saponins*

#### 7.2.19.1 Asiaticoside

Shen et al. [68] provided an efficient and reliable MAE method for the quantitative recovery of triterpene saponins from *Centella asiatica*. The substances obtained include pentacyclic triterpenes, mainly asiatic acid, asiaticoside, madecassoside, and madecossic acid, which, it has been claimed, possess various physiological properties (Fig. 7.16). Reports have revealed that triterpene saponins from *C. asiatica* have been used for the treatment of psoriasis, ulceration, eczema, and in wound

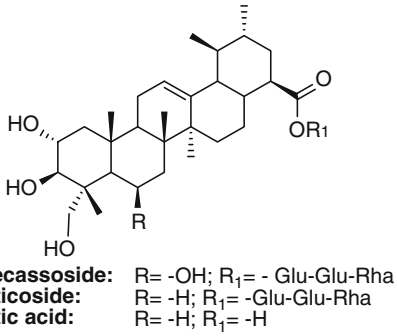



**Fig. 7.16** Chemical structure of structure of madecassoside, asiaticoside, and asiatic acid

**Table 7.6** Comparison of MAE and conventional extraction methods under the optimal conditions for astragalosides (AG) in *Radix astragali*

| | AG I | | AG II | | AG III | | AG IV | |
|---|---|---|---|---|---|---|---|---|
| Method | Yield (mg/g) | RSD (%) | Yield (mg/g) | RSD (%) | Yield (mg/g) | RSD (%) | Yield (mg/g) | RSD (%) |
| MAE | 0.788 | 1.13 | 0.351 | 1.22 | 0.206 | 1.86 | 0.278 | 2.17 |
| SE | 0.770 | 1.68 | 0.347 | 2.27 | 0.193 | 1.73 | 0.242 | 1.95 |
| HRE | 0.761 | 2.19 | 0.352 | 1.94 | 0.203 | 2.01 | 0.257 | 2.32 |
| UE | 0.549 | 1.96 | 0.302 | 2.10 | 0.190 | 2.42 | 0.225 | 1.66 |
| ME | 0.411 | 1.77 | 0.299 | 1.46 | 0.166 | 1.82 | 0.203 | 2.11 |

*SE* Soxhlet extraction, *HRE* heat reflux extraction, *UE* ultrasonic extraction, *ME* maceration extraction

Optimization of the microwave-assisted extraction process for four main astragalosides in *Radix astragali*

*Source*: Reprinted from Zhang et al. [72], copyright 2010, with permission from Elsevier

healing [69]. They also have the benefits of memory improvement and possess antiinflammatory, anticancer, antioxidation, anxiolytic, and other properties [70]. MAE extraction times were much shorter and yields were higher than in Soxhlet extraction. The results showed that the compounds could be entirely extracted in between 20 and 30 min in 90% methanol in a ETHOS 1 (Milestone). Again this finding suggests that MAE is a good alternative to conventional extraction techniques.

### *7.2.20 Astragalosides*

Yan et al. [71] employed an efficient MAE technique to extract astragalosides I–IV from *Radix astragali* (Table 7.6), which are considered by many to be major bioactive constituents with superior pharmacological properties and to possess antioxidant [72], antitumor [73], hepatoprotective, antidiabetic, antimicrobial, antiviral, and

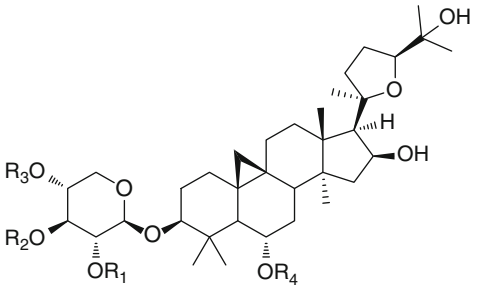



**Fig. 7.17** Chemical structure of four astragalosides in *Radix astragali* (Glu: glucose)

| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| **Astragaloside I** | Ac | Ac | H | Glu |
| **Astragaloside II** | Ac | H | H | Glu |
| **Astragaloside III** | Glu | H | H | H |
| **Astragaloside IV** | H | H | H | Glu |

immunological properties [74]. Their chemical structures are shown in Fig. 7.17. Astragalosides were quantified by liquid chromatography-electrospray ionization mass spectrometry (LC–ESI/MS). The MAE procedure was optimized, validated, and compared with other conventional extraction techniques. MAE gave the best result because it gave the highest extraction efficiency in the shortest extraction time. Optimal MAE conditions were 80% ethanol as solvent, solid/liquid ratio of 1:25 (g/ml), temperature of 70°C, irradiation power of 700 W, and three extraction cycles, each 5 min.

To study the alteration in structure that may occur during the selected extraction techniques and to understand the extraction mechanism, the plant samples were examined using SEM. The different extraction techniques produced notable physical changes in *Radix astragali*. Figure 7.18 shows micrographs of raw material (RM) samples extracted by MAE, Soxhlet extraction (SE), HRE, ultrasonic extraction (UE), and microwatt extraction (ME). The disruption of the sample surface was discernibly higher in MAE than in other methods. The cell structure was affected by MW treatment as a result of sudden temperature rise and internal pressure increase. During this rupture process, the chemical substances within the cell are rapidly released into the surrounding extraction solvents. A further reason for the high efficiency of the technique is the fact that MW energy penetrated both the sample and solvent. In UE, the mechanical cavitation effect of ultrasound enabled the solvent to better penetrate the cellular materials and improve the release of chemical substances into the solvent. Hence, the sample surface was notably disrupted after UE. In HRE and SE, the solvent transfers into the sample and extracts the compounds via permeation and solubilization at higher temperatures. Hence, little disruption of the sample microstructure occurs and only a few slight ruptures take place on the surface of the sample. In these processes, a larger amount of solvent, a longer extraction time, and higher extraction temperature were needed. In MW, the surface of the sample was not considerably different from that of the raw materials, and only a few slight creases were observed. Hence, the astragaloside yields observed were the lowest.

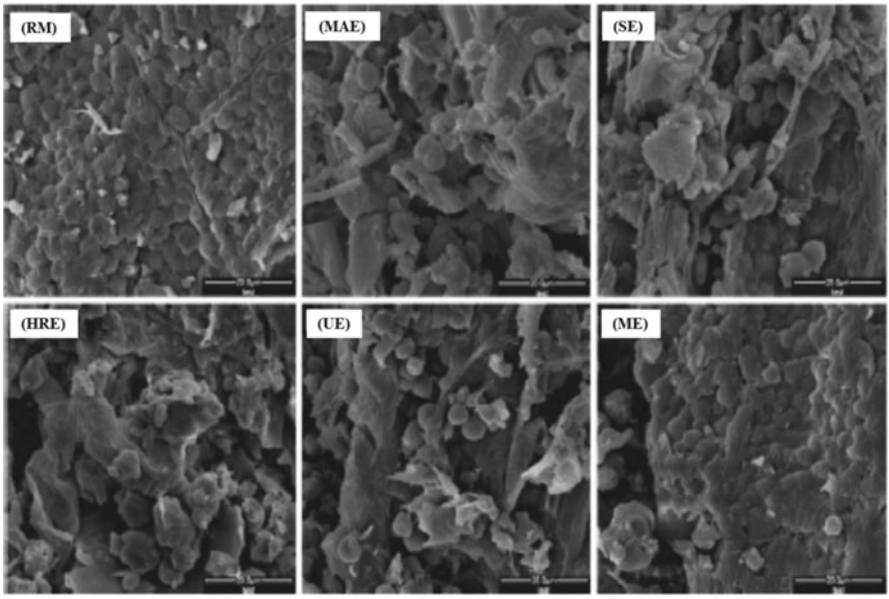



**Fig. 7.18** Scanning electron microscopy (SEM) images (20.0 μm, 15.0 kV) after MAE, SE, HRE, UE, and ME. Optimization of the MAE process for four main astragalosides in *Radix astragali*. (Reprinted from Zhang et al. [72], copyright (2010), with permission from Elsevier)

### *7.2.21 Mineral: Selenium (Se)*

The importance of Se to human health has been a source of great interest in recent years. Se deficiency can cause the induction of some pathological conditions such as viral infections, cardiovascular disease, and even cancer, which may be caused by the loss of immunological competence. Recent evidence indicates that the consumption of Se in excess of the Recommended Dietary Allowance (RDA) may provide substantial cancer prevention benefits in humans [75]. Kolachi et al. [76] studied the applicability of MAE to the extraction of Se from medicinal plant (MP) samples. A simple and rapid MAE of Se from medicinal plants was carried out and studied using varied nitric and hydrochloric acid concentrations, sample masses, heating times, MW energy settings, and plant particle sizes. Electrothermal atomic absorption spectrometry was used to determine Se content (Table 7.7).

### *7.2.22 Lichenes Metabolites*

Bonny et al. [77] tested *Aspicilia radiosa*, *Diploicia canescens*, and *Ochrolechia parella* for the extraction of norstictic acid (NA), diploicin (DP), and variolaric acid

**Table 7.7** Variable range used in the factorial design for extraction of selenium (Se) samples

| Conditions | Low (−) | High (+) | Unit |
|---|---|---|---|
| HCl, %[a] | 5.0 | 20 | ml |
| $HNO_3$, %[a] | 5 | 20 | ml |
| MW power | 40 | 80 | W |
| Treatment time | 0.5 | 2 | min |
| Sample weight | 100 | 200 | mg |
| Particle size | 65 | 125 | μm |

[a]10.0 ml of both extractant acids was used at different concentration levels
*Source*: Reproduced in part from Bonny et al. [77]

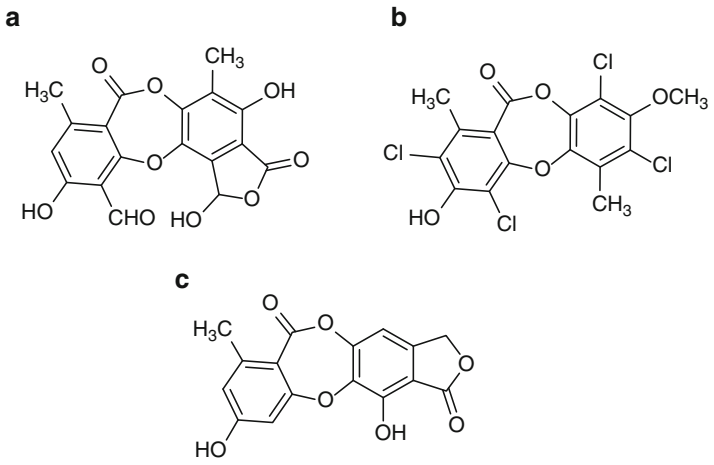



**Fig. 7.19** Chemical structure of (**a**) norstictic acid, (**b**) diploicin, and (**c**) variolaric acid

(VA) (Fig. 7.19). These compounds are the major metabolites found in these lichens and have shown significant biological activity; for example, NA [78] and VA [79] display antioxidant properties and DP shows significant cytotoxicity against human and cell lines.

MAE showed a drastic reduction in extraction time (7 min vs. 3 h) and solvent consumption (15 ml vs. 30 ml) compared to the reflux method. The total NA yield was 90% for both methods. The optimal conditions were applied to other crustose lichens *Aspicilia radiosa*, *Diploicia canescens*, and *Ochrolechia parella* for the extraction of NA, diploicine (DP), and variolaric acid (VA), which showed 83%, 90%, and 95% recovery, respectively.

## 7.3 Conclusions

Several naturally derived food substances have now moved from the territory of traditional and folklore medicine to rigorous studies aimed at identifying natural preventive therapies for diseases [80]. It should also be mentioned that phytochemical

and antioxidant characteristics of some bioactive substances can also be modified by chemical treatments, which may have either positive or negative impacts in their properties [81]. Therefore, reliable and safe extraction procedures should be improved and assessed. Because of the growing interest in the extraction of bioactive compounds and nutraceuticals from plants and herbs and the search for sustainable extraction techniques, MAE is fast realizing its promise as a technique that can respond to the rigorous demands this field necessitates. The studies reported in this chapter demonstrate the many advantages that make MAE an excellent substitute to traditional methods such as Soxhlet and solid–liquid extractions, as well as other environmentally benign technologies [82].

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# Chapter 8
# From Laboratory to Industry: Scale-Up, Quality, and Safety Consideration for Microwave-Assisted Extraction

**Ying Li, Marilena Radoiu, Anne-Sylvie Fabiano-Tixier, and Farid Chemat**

## 8.1 Introduction

The past century has clearly demonstrated the impact of technology on society. As industrial processing continues the drive toward higher performance and lower costs, demanding new processes become a necessity. Balanced with the performance and quality requirements for current and future processing materials are the health, safety, and environmental concerns surrounding these processes. The shape and direction of further technological development also must consider that many of these process steps are critically dependant on energy transfer into the reaction to deliver the highest levels of process performance and end-product reliability.

Microwave heating, which is based on "green chemistry" principles, has been studied as an innovative technique of heating dielectric materials, that is, materials that have no or little electrical conductivity; in most cases these materials are also poorly conductive thermally. Conventional heating techniques, based on heat transfer phenomena, fail for heating dielectrics. MW techniques dissipate heat within the material by the electromagnetic phenomenon of energy transfer.

When discussing the performance of the microwave technique in comparison with other dielectric heating techniques (i.e., radiofrequency), two main advantages should be highlighted: high energy transfer as a consequence of higher frequency (915 or 2,450 MHz vs. 13.56 or 27.12 MHz) and reduced breakdown risk because of the

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lesser electric field. It is well known that energy is often one of the most significant uncontrolled variables in an experiment. Microwaves have the potential to change that, partly by the fundamental way the energy is delivered to the molecules of interest and partly by the electronic controls in the apparatus, facilitating the setting and documentation of time, energy level, final temperature, and so forth.

Although microwave technique can bring about benefits, the quality of the whole procedures and the safety of the operators are equally significant but rarely a concern in European regulations. Therefore, quality management tools in the industry are becoming increasingly important to ensure a certain product in proper working conditions. Various methods exist to identify the occurrence of possible risks. The concepts of HACCP (hazard analysis and critical control points) and HAZOP (hazard and operability analysis) are used to ensure safety and to optimize the efficiency and management of hazards related to manufacturing, people, and the environment. As mentioned in the current edition of the *Codex Alimentarius*, one of the major requirements for a food safety system is that it must be based on the principles of hazard analysis and the study of critical control points. Therefore, with the aim of providing safe and wholesome assurance, it is meaningful to establish a hazard analysis system to monitor throughout the processing operation before implementing the innovative techniques. The design of a microwave-assisted processing operation should be based on the concepts of HACCP and HAZOP. It also should be noted that this design is a preliminary step toward approval by regulatory agencies such as the European Community. The HACCP concept defines the following critical control points (CCPs) in a microwave processing facility: (1) the raw material receiving area, (2) treatment chamber, and (3) packaging line. Knowledge of processing parameters and conditions, such as microwave power, material and product temperature, and cleanliness, are fundamental in the inactivation of microorganisms and enzymes. Product characteristics such as conductivity, pH, and composition must be considered in designing the process. In addition, treatment materials, chamber geometry, electrical components, and connections are key engineering aspects at the design and construction phases of microwave systems to avoid safety hazards with respect to both equipment operation and product integrity [1].

Microwave-assisted extraction (MAE) is broadly used not only in extraction of high value-added compounds such as aromas, antioxidants, and pigments, but also in food products, for instance, enriched vegetable oils and fruit juices. Nonetheless, potential hazards in terms of the safety of processing procedures and operators safety emerge during MAE operation. Therefore, developing HACCP and HAZOP hazard analyses is vital to establish a set of specifications by regulatory agencies, which can be taken into account in the design, construction, and startup of MAE, so that all possible hazards during processing can be controlled to a minimum level. These guidelines stem from a reflection process on the application of HACCP and HAZOP principles in MAE processes.

## 8.2 The Problems of Scaling Up

Generally, microwave heating (dielectric heating) utilizes relative permittivity measurements via network analyzers as the indicator of the heat that can be generated in a sample when subjected to microwaves. To scale up microwave technology for unit

processes, quantitative characterization has to be successfully carried out for (a) electric field profile in the microwave reactor and (b) the resulting changes occurring in the substrate. However, these are complex tasks, difficult to achieve in an industrial microwave apparatus because of their poor reproducibility and thus giving rise to poor control over process quality.

Summarizing from the current literature, the complexity in scaling up microwave processes arises from the following:

- Theoretical modeling of an empty microwave cavity has little predictive capability for events in a partially loaded cavity because of the enormous perturbation of electric field by the presence of a load
- The dielectric constant of a given compound varies spatially depending on the chemical composition and bulk density of the load as well as temperature variation during the process
- Low penetration depth of microwaves giving rise to competing heat transfer mechanisms; uniform heating is rarely achievable in conventional microwave systems, often giving rise to both unprocessed and severely overheated spots
- Various mixing operations applied in mainstream thermal technologies are inapplicable in microwave systems because of material selection issues imposed by the microwave environment

In addition to microwave complexity, there are other 'more conventional' aspects to be considered when designing industrial chemical equipment for microwave-assisted processing, such as safety, corrosion, uptime, and maintenance intervals.

Sairem has already produced a number of microwave-assisted industrial chemical installations such as Laurydone synthesis (BioEurope, France) and nitroglycerin removal from the waste acid resulted from nitrocellulose fabrication (PB Clermont, Belgium). Based on the results obtained in these early installations, during the past 5 years or so, Sairem has been working on the equipment attempting to overcome all the scale-up problems in a simple and reproducible design toward better process quality control at the industrial scale:

a. The design of a microwave reactor that overcomes the nonhomogeneity profile of the electric field and the nonuniform heating of materials caused by the shallow penetration of microwaves, with a high level of control especially of the temperature inside the reaction mixture to avoid overheating [nonhomogeneity may lead to hot spots and degradation of products and the reactor, usually made of polytetrafluoroethylene (PTFE)]
b. The design of a microwave reactor that is easily adaptable to different chemistries: batch, flow, elevated temperature and pressure, safe to use, and with a high degree of flexibility
c. The possibility to run reactions automatically in a continuous and repetitive manner
d. The design of experimental methods to follow the process after different periods of microwave exposure by characterizing the products and making a comparative study with the existent published data

The comparative study is not a trivial challenge as most of the results published up to now are based on experiments carried out in very small batch or flow reactors,

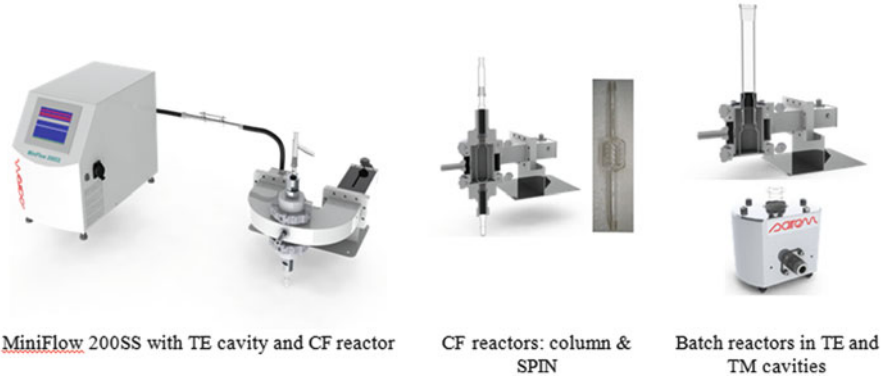


**Fig. 8.1** MiniFlow 200SS and its possible configurations

without any appropriate method for measuring the real energy absorbed by the process, but based only on the temperature measured at one or multiple points inside the reactor. Sairem proposes equipment that allows the scale-up of the process starting from basic research and process parameters control at a very small scale up to many hundreds of liters.

### *8.2.1 MiniFlow 200SS*

The MiniFlow 200SS is an easy-to-use microwave-assisted reactor, engineered as a system specifically designed for the laboratory use. The equipment has the flexibility to meet all laboratory requirements; the system can be easily configured to perform different reactions in continuous flow or in batch (Fig. 8.1), with or without cooling of the reactor. The microwave energy is provided via a built-in solid-state microwave generator with adjustable power from 0 to 200 W with 1-W power increments and controlled variable frequency from 2,425 to 2,475 MHz with 0.1-MHz increments.

Among the advantages of using the MiniFlow 200SS as a first step of a process development are the following:

- Compact size and flexible configuration with microwave energy transmitted via coaxial cable
- Stable operation from microwave power levels as low as 0.5 W and power adjustable in 1-W steps
- Very good frequency spectrum even at low power; the frequency spectrum of magnetron-based generators has poor stability below 100–150 W
- The continuous control and monitoring of the forward power, reflected power, and temperature are achieved via an integrated PLC/digital display; temperature is measured using a built-in fiberoptic thermometer.

**Fig. 8.2** LABOTRON X & S: example of multi-treatment installation with Labotron X6000 and Labotron X2000 in batch configuration. Installed microwave power 6 kW + 2 kW, 2,450 MHz

### *8.2.2 LABOTRON for Extraction and Synthesis*

The LABOTRON X & S are equipment for microwave-assisted synthesis and extraction, designed to overcome all the scale-up problems and to carry out quick, reproducible, and safe chemistry and the extraction of delicate biological or vegetal-specific molecules.

There are two important features of this equipment: WO 2009/122101 and WO 2009/122102 (Figs. 8.2, 8.3, 8.4):

a. The microwave energy is transmitted to the reaction via an internal transmission line (INTLI). This method allows for the microwave energy to be transmitted directly into the reaction using high-density electromagnetic fields adapted to each reaction mixture and, as such, the availability of very high densities of activation energy. The new modality for microwave transmission also makes it easy to make the microwave reactors of metals (e.g., stainless steel, hastelloy), which will help with pressure containment but also faster thermal transfer
b. The U-shaped waveguide that allows the mounting of several types of reactors, standard or custom made, and for a smaller footprint of the equipment

The LABOTRON can be used for a wide variety of substances and has adjustable microwave power output with reflected power reading and automatic tuning as well as temperature control. The LABOTRON offers a high level of flexibility and control providing optimized microwave energy efficiency and enhanced safety:


**Fig. 8.3** LABOTRON extraction: batch reactors mounted on the U-guide

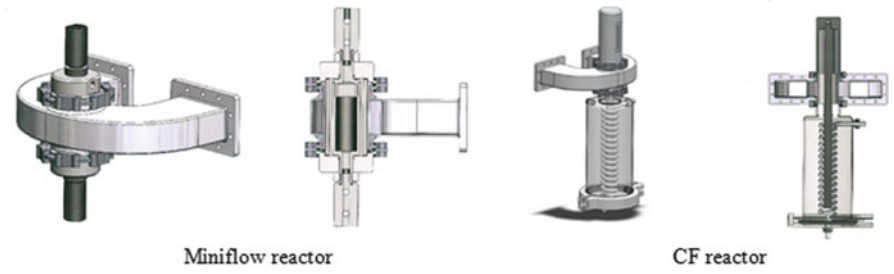


**Fig. 8.4** LABOTRON synthesis: continuous flow reactors mounted on the U-guide

- The Labotron can equally function with a batch reactor (volume, 1.5–17 l) or continuous flow reactor (from a few ml/min to several l/h); recognition of the reactor type is done via the Labotron's PLC
- Adjustable power from a few watts to 6 kW
- Optimized geometry of the INTLI to achieve high power densities inside the reactor
- Direct reading of forward and reflected power values and calculation of the energy absorbed by the irradiated sample
- Automatic impedance matching for minimal levels of reflected power
- Efficient external cooling via a cooling jacket
- PLC-based controller and touch screen user interface; all system functions and status, including recipe changes, alarms, and chemical level, are accessible from the touch screen display
- Quick connections for increased flexibility and rapid cleaning and maintenance
- On-line reagents addition and product removal and sampling
- Based on a mobile platform for quick laboratory installation and positioning

In collaboration with École Nationale de Chimie in Montpellier, SAIREM has installed a platform unique in Europe for experimentation at a semi-industrial scale (Figs. 8.5, 8.6). The main parameters of the pilot-scale installation are as follows:

- Installed maximum microwave power of 30 kW, 915 MHz
- Fast control of forward and reflected power
- High attainable microwave power densities
- Integration between microwave generator and reactor ensures internal compatibility and control of all system components
- Continuous flow reactor up to 5 l/min
- Batch reactor with variable speed mechanical stirring, with maximum volume of 100 l adapted particularly for vegetal-type extraction in aqueous phase or solvents
- Possible recirculation of the extracted product back into the reaction mixture
- Process could be carried out under an inert atmosphere ($N_2$, Ar, $CO_2$, etc.)
- Possibility of on-line filtration or distillation of the products
- Reactor external cooling via a cooling jacket with automated temperature control
- In situ temperature measurement
- Range of interlocks for safe and reliable operation

Process intensification based on microwave technology is actively pursued to achieve a better position at the industrial scale. Increase of reaction rates and improved selectivity, combined with the possibility of repetitive procedures, demonstrate the advantageous application of these enabling techniques. To make microwave heating feasible on a larger scale, this technique should perform better than operation with conventional heating and, most importantly, any aspects such as productivity, selectivity, safety, and investment costs in equipment have to be compensated by the added value of improved reaction conditions. An important role of the

**Fig. 8.5** Microwave-assisted batch reactor, 100 l. Installed microwave power 30 kW, 915 MHz. (Pilot installation at Ecole Nationale Superieure de Montpellier, France)

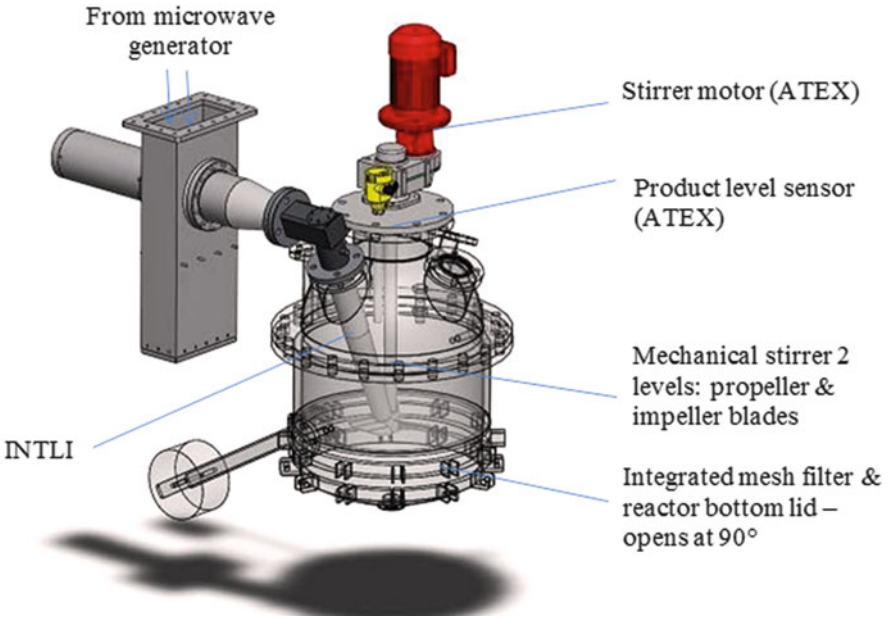


**Fig. 8.6** Schematic of batch reactor 100 l: stainless steel with jacket for water cooling

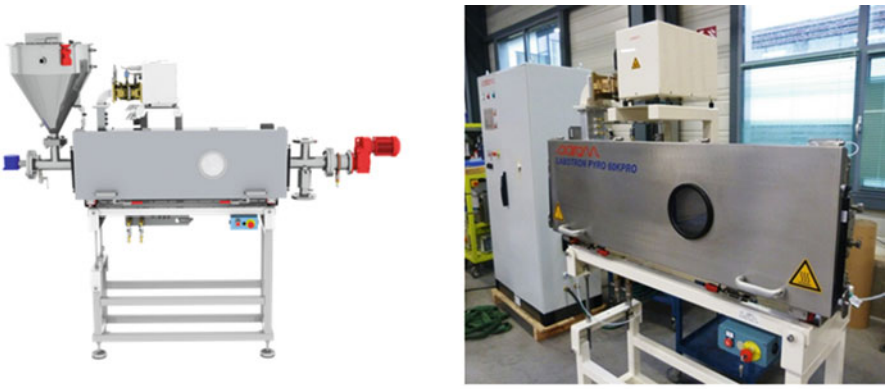


**Fig. 8.7** Continuous flow reactor. Microwave installed power, 6 kW, 2.45 GHz

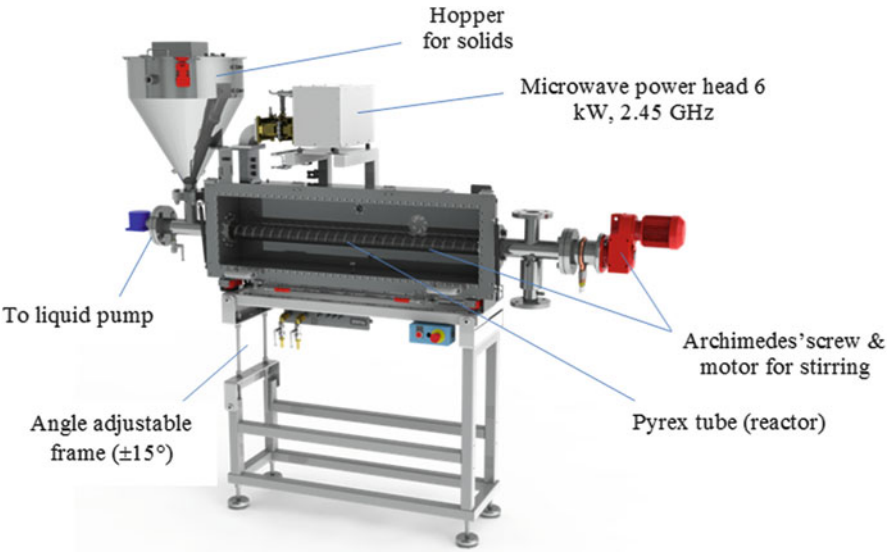


**Fig. 8.8** Continuous flow reactor for microwave-assisted extraction (MAE) (installation optimized for liquid–solid or solid processes). Installed microwave power 6 kW, 2.45 GHz)

scaling-up is microwave-assisted continuous flow processing (Figs. 8.7, 8.8). The main advantage of continuous flow processing is the ease with which reactions can be scaled through the operation of multiple systems in parallel (numbering-up, scaling-out) or related strategies, thereby readily achieving production-scale quantities. The typically short reaction times, on the order of a few minutes or even seconds, experienced in microwave chemistry protocols form an ideal basis for continuous flow processing, in which short residence times within the flow device are essential to achieve efficient throughput.

## 8.3 Definitions and Principles of the HACCP and HAZOP Concepts

The hazard analysis critical control point (HACCP) and the hazard and operability (HAZOP) concepts are considered as total quality and safety management tools in the design of a MAE operation. The HACCP concept was originally developed as a microbiological safety system that was applied in the production of the foods destined to be used in space in the early days of the USA manned space program. It was advanced in the 1960s by the Pillsbury Company working alongside NASA and the United States Army laboratories at Natick and was used as a "zero defect" program aiming at guaranteeing production of safe foods that would be consumed by astronauts in zero gravity [2]. To the late 20th century, HACCP has been unceasingly developed from the first three principles to the current five initial steps and seven principles. Now HACCP is used as a systematic approach for ensuring food safety in the modern world, and it plays an important role in preventing the occurrence of hazardous substances in food processing operations, including manufacturing, storage, and marketing.

The HAZOP study that was initially presented in 1963 was based on so-called critical examination techniques and was followed by the first guide in 1977 and first main textbook by Kletz. In 2001, the first HAZOP standard IEC 61882 came out and more and more countries or communities moved to HAZOP studies so that the HAZOP method was developed as a structured and systematic examination of a planned or existing process or operation which was usually applied in the chemistry or food chemistry industries [3–5].

### *8.3.1 Specific Vocabularies and Principles in a HACCP System*

To better understand the idea of the HACCP concept, it is important to understand and differentiate its specific vocabularies, which can give a different meaning or context. The common terms are defined as follows [6]:

- *Hazard: Any biological, chemical, or physical factor that can lead to an unacceptable risk for consumer safety or product quality.*
- *Biological hazards: Any pathogenic bacteria, yeast, mold, virus, or some parasites that may be p*resent in raw materials or occur during the processing operation as the result of food contamination.
- *Chemical hazards*: Excessive toxic residues, pesticides, heavy metals, antibiotics, detergents, etc. This hazard occurs in food processing operations naturally (e.g., mycotoxins), intentionally (e.g., preservatives, nutritional additives, color additives), or unintentionally (e.g., agriculture chemicals, toxic elements).
- *Physical hazards*: Physical objects that can get into food during the production processing are classified as ferrous (metal particles, stones, jewelry) and nonferrous (plastic, glass, wood).

- *Critical Control Point (CCP)*: Any place, personnel, operation, or protocol where inadequate control would result in the appearance of food dangers.
- *Preventive action*: All the techniques, methods, and actions that would allow eliminating the danger or reducing it to an acceptable level.
- *Corrective action*: Procedure to follow when the monitoring indicates that a CCP is not monitored.
- *Critical limit*: Criterion or parameters that must be respected to ensure that the monitoring is effective; that is, a criterion that separates acceptability from unacceptability.
- *Deviation*: Non-respect of a critical limit.
- *Verification*: Methods, procedures, and controls used to determine if the hazard control plan is effective and reaches the objectives fixed.
- *Validation*: Methods, procedures, and controls used to assess the effectiveness of the entire HACCP plan.

The HACCP system is not dependent on the quality analysis of the final product for guarantee of food safety but on the process control of food processing. In this way, we can prevent known hazards or reduce them to an acceptable level. This system does not exist independently. It must be established on the basis of food safety programs such as GMP (Good Manufacturing Practice), SOP (Standard Operating Procedure), and SSOP (Sanitation Standard Operating Procedure). As the result, the HACCP system, which is based on many work standards, is considered as the most effective control system of food-borne diseases by more and more countries and societies. It is a more complete quality assurance system.

Generally, setting up an HACCP system is based on the following seven principles [7]:

1. Conduct a hazard analysis and consider any preventive measures to control the identified hazard in the process.
2. Determine CCPs that are required to control the identified hazard.
3. Establish critical limits for each identified CCP.
4. Establish a monitoring system for each CCP.
5. Establish corrective actions to be taken when monitoring indicates a deviation from an established critical limit.
6. Establish documentation and effective record-keeping procedures.
7. Establish verification and validation procedures to ensure that the HACCP system is functioning properly.

### *8.3.2 Specific Terms and Principles in the HAZOP System*

In HAZOP methodology, some useful terms need to be known before executing a HAZOP analysis process.

- *Hazard*: Any operation that has a potential to cause a catastrophic result of explosive, flammable, or toxic chemicals or any action that could possibly harmful to personnel, property, or environment.

- *Operability*: Any operation in the HAZOP process that would stop the steps which could possibly lead to a violation of safety, health, or environmental regulations or negatively impact profitability.
- *Node*: A specific equipment or location in the process in which the design intent is evaluated.
- *Design/process intent*: A description of how the process is expected to work at the node.
- *Deviation*: A way in which the process parameters may depart from their design intent.
- *Parameter*: The relevant parameters are made according to the process conditions.
- *Guideword*: A short word that creates an image of a deviation of the design intent. The most commonly used set of guidewords are no, less, more, as well as, reverse, and other than, which could apply to all the parameters to identify unexpected and yet identifiable deviations from the design intent.
- *Cause*: The reason why the deviation occurs and may result in the worst possible consequence. It is worth mentioning that several causes may be identified for one deviation.
- *Consequence*: The result of deviation, in case it occurs. It may comprise both process hazards and operability problems. Several consequences may correspond to one cause and vice versa; one consequence also can have several causes.
- *Safeguard*: Facilities that assist to reduce the occurrence frequency of the deviation or to alleviate its consequences.

The HAZOP method was initially developed to analyze chemical process systems in the chemistry industry and petroleum industry. This analysis has been designed to control the identifiable hazards that may be harmful to personnel, equipment, processing operations, or environment. The HAZOP analysis has now been developed as a structured and systematic technique for system examination and risk management that can help to optimize the processes for achievement of higher efficiency and productivity.

As in the HACCP analysis, a multidisciplinary team is also created to carry out the HAZOP plan. This critical analysis encourages the imagination of the HAZOP team to discover the causes and possible deviations and to identify other inconspicuous operation steps in which a hazardous condition may arise. To develop and implement a HAZOP analysis, the following eight principles should be taken into account [8]:

1. Definition of system, including concept and surroundings of the studied system.
2. Identify the potential relevant hazardous parameters associated with the studied system.
3. Combination of each parameter to appropriate guidewords to constitute deviations.
4. Study the causes and consequences of each relevant credible deviation.
5. Establish corrective actions to prevent the hazardous condition and efficiency problem.
6. Implement corrective actions for modification of procedures used in the operation.
7. Establish monitoring procedures to verify the effectiveness of the corrective actions.

8. Establish personnel training programs.
9. Establish effective record-keeping systems.
10. Establish control procedures for verification of the effectiveness of the corrective actions.
11. Update the process scheme with corrective actions.

Although the principles of the HACCP and HAZOP concept are almost the same, they are used for different objectives. The HACCP method focuses on the production and processing of safe foods in analyzing health hazards concerning processing, distribution, and consumption of food, whereas the HAZOP is used to identify hazardous working conditions in a specific step of processing operations in terms of the equipment design, process requirements, personnel protection, and equipment safeguards. Both methods can be applied as complementary systems in the design, construction, and troubleshooting of a MAE facility.

## 8.4 Application of HACCP to Microwave-Assisted Extraction (MAE)

All the unitary operations of a MAE process are summarized as several steps, where key operations are receiving and storage of the raw materials, preparation and mixing, microwave processing and packaging, and finished product storage. According to the HACCP's seven principles, the first step in developing an HACCP system for the MAE operation is assessment of the hazards involved.

### *8.4.1 Hazard Assessment*

For each step of this process, evaluation and classification of hazards are done. In this way, each hazard is identified with a corresponding grade according to its significance and its detectability. The significance of hazards is based on their risk, severity, frequency, and possibility of occurrence. Furthermore, preventive measures with the aim of eliminating or reducing hazard occurrence are set up with procedures.

In general, microwaves will not destroy nutrients in comparison with conventional heating methods. However, uneven distribution of the electromagnetic field in an oven and heterogeneity of the material matrix will cause some severe problems in microwave processing. When microwave frequency is set, heating is controlled by microwave power and treatment time, which may lead to inadequate heating or superheating that cause nutrient loss or degradation of materials. Nevertheless, these physical burns induced by superheating are not relevant to food safety, and thus they are considered but not as a hazard in a HACCP plan.

Microbial hazards are the main concern throughout the MAE operation. Raw materials contain pathogens and spoilage microbes which, under poor storage conditions, will be able to spoil the raw materials or ingredient and may increase the

risk to consumers. In addition, the attention of cleanliness and machinery leakage may prevent cross-contamination problems. For instance, cross-contamination could happen in a microwave treating step when surfaces of processing equipment are not well cleaned, disinfected, or rinsed. Furthermore, the different temperature inside the treating materials caused by uneven microwave treatment will lead to microbiological proliferation and survival. Finally, the packaging line should be properly sterilized to avoid recontamination, as well as storage conditions and handling of processed products and hygiene of the relevant personnel.

Chemical hazards involved in MAE operation are apparition and migration of chemical compounds. Unsafe chemical components may appear during microwave treatment such as acrylamide, heterocyclic amines, or acrolein. Moreover, oil oxidation should also be noticed because accelerated rancidity reduces the quality of the sanitation environment. Further, some chemicals can migrate from packaging into food under microwave treatment, such as the carcinogenic compound DEHA (bis-(2-ethylhexyl) adipate) found in polyvinyl chloride (PVC) films [9]. However, the amount of this substance, is not significant with the exception of certain rare conditions.

To reduce the aforementioned hazards to an acceptable level, a good knowledge of microwave processing is a prerequisite. The electric field distribution depends on its geometry and its dielectric properties, and no methods have existed to control the electromagnetic field until now [10]. The microwave power and treatment time need to be adapted to the product and optimized before large-scale processing. The cross-contamination can be prevented with references of SSOP and GMP, which ensures a disposed environment in food industry areas. Furthermore, selection of appropriate materials can also reduce hazards.

### *8.4.2 Critical Control Point*

The second step in development of an HACCP system consists of establishing CCPs, setting critical limits for each CCP, describing monitoring procedures, and determining corrective actions when CCPs deviate from the set critical limits. Table 8.1 suggests some CCPs with regard to different affecting parameters and actions. The HACCP team uses gathered information in the first step to identify whether the steps are CCPs in the process. A decision tree presented in Fig. 8.9 is useful for CCP decisions [6].

The CCPs in a MAE operation should be selected to ensure the safety of MAE products: they focus on the four key operations already mentioned. The main factors of considering and monitoring a CCP are handling of materials in each step including raw materials, intermediate products, and final products, processing conditions, and cleanliness of equipment, utensils, or containers used during processing. Processing conditions include various parameters such as properties of products, microwave power, and treatment time in the microwave chamber. The purpose of selection of variables is to eliminate all potentially hazardous microorganisms that may not be inactivated to the acceptable level if the appropriate handling was not

**Table 8.1** Possible critical control points (CCP) limits and associated corrective actions in a microwave-assisted extraction operation/system

| Critical control point | Danger | Target | Deviation | Corrective actions |
|---|---|---|---|---|
| | Microbiological and chemical spoilage | Good raw material quality | Detected | Reject |
| Raw material receiving and storage | Foreign materials | Absence | Detected | Reject |
| | Storage time | Correct, above the consumption date limit | Exceeded | Reject |
| | Storage temperature | Adequate, according to purveyor recommendations | Does not conform to the requirements | Adjust temperature<br>Laboratory controls |
| | Temperature inside treating materials | Adequate | Does not conform to the requirements | Adjust electromagnetic field<br>Reprocess the product or reject |
| Microwave processing operation | Microwave frequency | Adequate | Does not conform to the requirements | Adjust frequency<br>Laboratory controls<br>Reprocess the product |
| | Microwave power | Adequate | Does not conform to the requirements | Adjust the power<br>Laboratory controls<br>Reprocess the product |
| | Treatment time | Adequate | Does not conform to the requirements | Adjust the time<br>Laboratory controls<br>Reprocess the product |
| | Machinery leakage | Absence | Detected | Reject |
| Packaging operation and storage | Package seal | Without leaks, no cross-contaminations, and correct seal | Does not conform to the requirements | Reject or reprocess the product<br>Adjust the packaging machine |
| | Storage time | Above specifications | Exceeded | Reject |
| | Storage temperature | Adequate, above specifications | Exceeded | Adjust temperature |
| | Spoilage | Absence | Detected | Laboratory controls or reject<br>Reject |

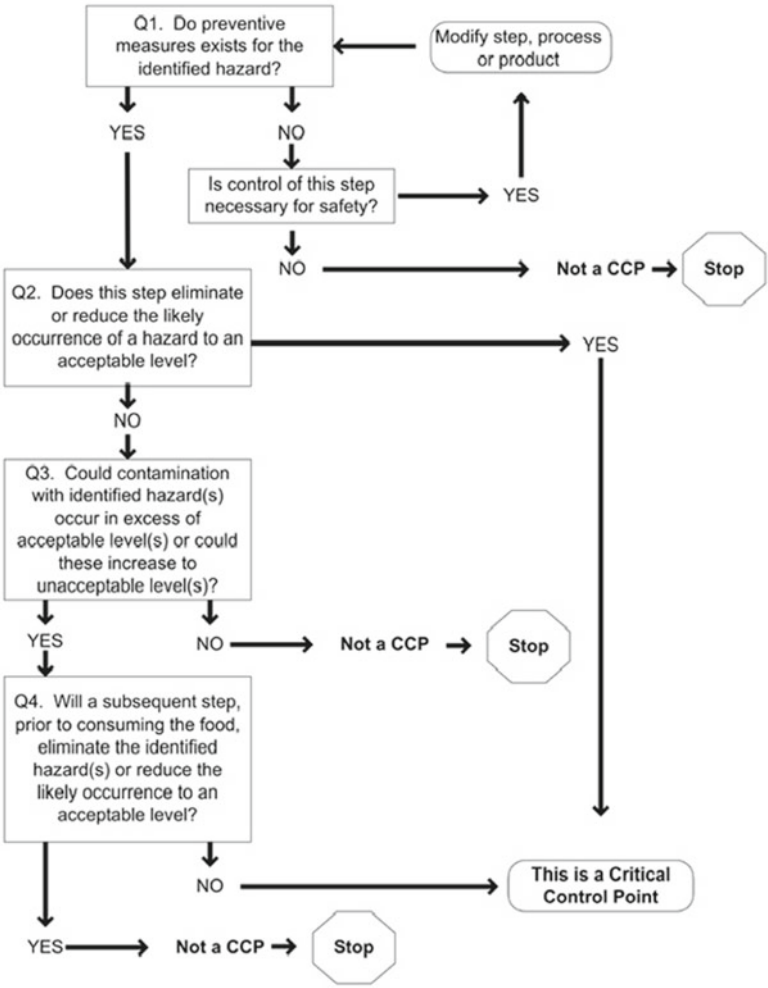


**Fig. 8.9** A decision tree for identification of critical control points (CCPs)

performed. It is also worth mentioning that a uniform microwave heating treatment may require the design and modification of microwave equipment, which is discussed in the later HAZOP analysis.

### *8.4.3 Record Keeping*

Record keeping as the third main step of developing a HACCP system is extremely essential. It should cover all daily documented records related to all the processing steps, from the raw materials to finished product distribution, as well as documentation

**Table 8.2** Records for a microwave-assisted processing operation

| Checkpoints | Yes | No | By | Time |
|---|---|---|---|---|
| Product | | | | |
| Raw materials | | | | |
| Item | | | | |
| Description | | | | |
| ID number | | | | |
| Amount | | | | |
| Equipment and utensils | | | | |
| Treatment chamber | | | | |
| Integrity and leakproofness | | | | |
| Sterile utensils | | | | |
| With detergent | | | | |
| Rinsed with sterile water | | | | |
| Processing system | | | | |
| Leakproofness | | | | |
| Clean with detergent | | | | |
| Rinsed with sterile water | | | | |
| Aseptic package | | | | |
| Number of bags | | | | |
| UV light exposed | | | | |
| With $H_2O_2$ (%) | | | | |
| Rinsed with sterile water | | | | |
| Workbench | | | | |
| UV light exposed | | | | |
| With detergent | | | | |
| Rinsed with sterile water | | | | |
| Dry and clean | | | | |
| Assembling | | | | |
| Safeguards in place | | | | |
| Setup frequency for processing | | | | |
| Process parameters | | | | |
| Microwave power | | | | |
| Treatment time | | | | |
| Temperature in treatment chamber | | | | |

for each HACCP steps. Table 8.2 is an example of a simplified MAE operation checklist, converting all the records into a single document.

## 8.5 Application of HAZOP to Microwave-Assisted Extraction

The HAZOP analysis is usually used in the chemistry industry and apparatus maintenance but us rarely applied in food industry. Now the HAZOP program, as a complementary system, is being attempted to be used in the food industry with an

integration of HACCP analysis. In this way, this collective brainstorming effort can make a thorough review of the process with deliberate opinions on the unsafe conditions and practice that may occur during the MAE operation. It takes all the risks into account, not only those associated with food safety but also those regarding potential hazards that may be harmful to the operators and environment.

In an MAE operation, nothing is more important than safety of personnel, especially the safety of operators. Although the processing parameters of MAE can be controlled, the emergency switch must be accessible in case of failure of the process. Appropriate warning signs must set up and relevant information on the operation and maintenance procedures must be included in standard operating procedures. In HAZOP procedures, the function of each worker must be defined in terms of "who," "what," "how," "when," and "where." Operators should be properly trained and instructed in the use of microwaves. Adequate protection such as radiation protection and wearing gloves should be required during the MAE operation. In addition, implementation of the concept of HAZOP on microwave treatment can solve some important potential unsafe problems that would not have been identified yet.

As a HACCP system enumerates all the hazards of safety of product and process, the HAZOP system attaches importance to the safety of operators and equipment. Apart from traditional safety considerations, there are some other safety concerns unique to microwave systems such as direct effects of microwave energy, performance characteristics, and processed materials. For operators, microwave radiation exposure is the main concern in MAE operation. Microwave exposure is unlike the surface phenomenon of sunlight; its energy can penetrate the skin into subcutaneous tissue and thus raise the temperature level of tissue and blood. Different frequencies of microwave energy penetrate to different depths and result in different amounts of energy being absorbed [11]. For this reason, it is difficult to establish a single standard for exposure to microwave energy. Furthermore, the exposure value for different organs of a professional is defined by the International Commission on Non-Ionizing Radiation Protection (INCIRP) [12]. Hence it is important to prevent the equipment from microwave leakage, and appropriate notices must set up to regulate the operation. Microwave energy is absorbed not only by polar solutions to produce heat and to accelerate reactions, but also by some sample molecules, container materials, and surfaces of apparatus that may not be heated during a reaction. The choice of microwave materials should be rigorous. The type of materials used must adapt to the microwave processing. In addition, wearing gloves can avoid scald and appropriate caution should be maintained to eliminate this kind of thoughtless accident.

For equipment, safe usage of laboratory microwave instrumentation is necessary and also responsible for the safety of operator. With regard to some ineffective equipment or subassembly, modification and replacement is necessary. An intact vessel is then noted to be about to perish when it has been stressed for a long time or has not received the proper maintenance, or for other reasons. The vessel may have cracks or chemical degradation. In this sense, it is advisable to permanently remove it from use. Otherwise, longtime heating may lead the vessel to a catastrophic failure, in which a blast may occur (Fig. 8.10), and the vessel will break into several scattered pieces of debris (Fig. 8.11).

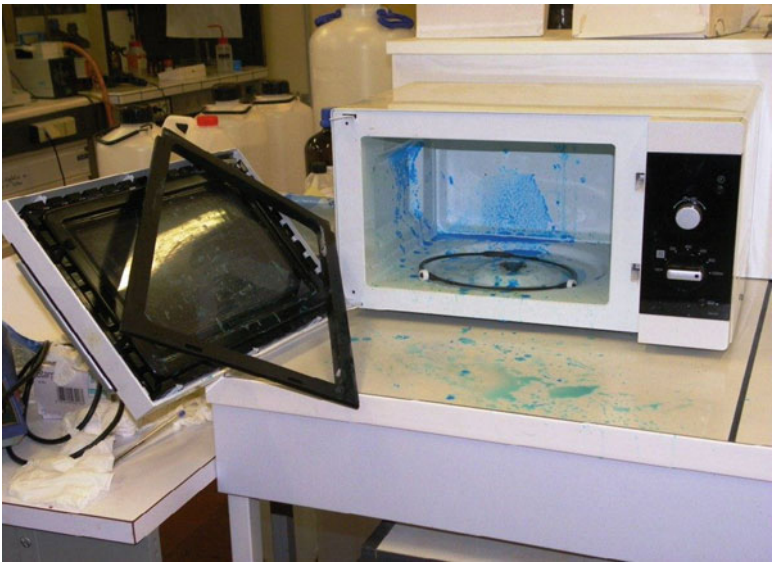

**Fig. 8.10** Microwave oven after explosion in laboratory

**Fig. 8.11** Debris of catastrophic failure of a lined digestion vessel

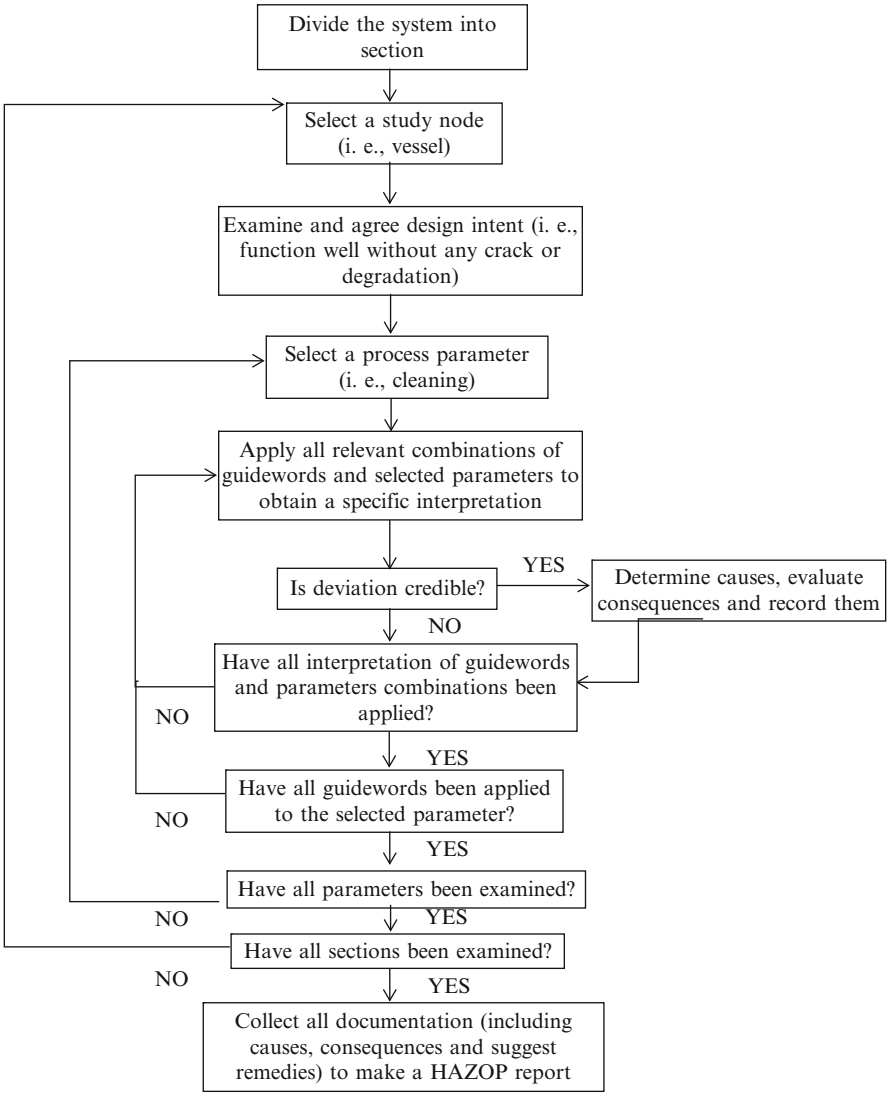


**Fig. 8.12** Flow chart of a hazards and operability (HAZOP) procedure representing the MAE operation

Based on the HAZOP principles, a HAZOP analysis process corresponding to MAE operation is graphically illustrated in Fig. 8.12. All HAZOP procedures are clearly shown in this flowchart; microwave reactor and vessel are selected as study section and node, respectively. Microwave leakage is examined as a process parameter to which a process HAZOP work sheet corresponds (Table 8.3). It is noticeable that all sections, nodes, and parameters that pose potential hazards should be considered to help to develop an all-inclusive analysis system.

**Table 8.3** A hazards and operability (HAZOP) worksheet template used in microwave-assisted extraction (MAE) operation [13]

| Study title: | | | | | | Page: | | | |
|---|---|---|---|---|---|---|---|---|---|
| Drawing no.: | | | Rev. no.: | | | Date: | | | |
| HAZOP team: | | | | | | Meeting date | | | |
| Section considered: | | | | | | | | | |
| Design intent: | | | Materials:<br>Source: | | Activity:<br>Destination: | | | | |
| No. | Guideword | Element | Deviation | Possible causes | Consequences | Safeguards | Comments | Actions required | Actions allocated to |
| 1 | More | Microwave leakage | More microwave leakage during MAE operation | Aging of equipment, carelessness of operators | Materials not effectively processed, instrument wear, and unsafe for personnel | Operators check the leakproofness before process cycle | 2 watts per kilogram for head and trunk, 4 watts per kilogram for limbs | Personnel training, warning sign | Technician Trainer |
| 2 | Other than | Microwave leakage | Other leakage during MAE operation | Incorrect operation or usage of some improper raw materials or chemical reagents | Erosion of equipment, ineffective processing, harmful to personnel | Check the chemicals used in protocol, read instruction book, or training before operation | Assumes operators can reliably choose the raw materials and chemical reagents used in process | More knowledge to ensure correct materials selection, technical training | Scientist Trainer |

## 8.6 Conclusions

Microwave heating is considered as proven technology. The past decades have widened its scope of applications by the progress of power electronics and numerical modeling. Homogeneous and internal heating over the whole of the volume is always mentioned as the main characteristic and advantage of dielectric heating; as well, selective heating and reduced floor space of the installation must be also attributed to this technology.

Further benefits are as follows:

- Shorter production time, possibly as much as 75–80%
- Shorter time needed for preparing the process (filling and emptying)
- Savings of electrical energy in comparison with traditional methods, frequently in the range of 25–50%
- Lower production cost from decreased waste and production losses
- Reduction of the footprint by as much as 50–90% in comparison with conventional processing
- Shorter setup and commissioning time
- Easy maintenance during a shorter time

It is highlighted that there are no exhaust gases to evacuate, and there is no flame, which gives a relatively safer operation so that insurance premiums are lower. There is no noise, and the production room is more comfortable because there is not much heat transferred to the surroundings.

It is also worth mentioning that the HACCP concept allows us to determine biological, chemical, or physical hazards concerning food safety, while the HAZOP concept is used to identify necessary actions to ensure the safety of personnel, equipment, and environment. Although there have some efforts in recent years on integration of the two concepts in the design and construction of food processing industries, application of the two methods in a complementary way remains limited but still has potential space for microwave-related technology.

Although microwave-assisted extraction is increasingly applied in the food and chemistry industries, it also needs to be implemented correctly to guarantee the safety of product and operators, as well as the environment. Microwave treatment time, uneven temperature inside products, and unsafe chemical applications are critical points to be taken into account in HACCP. Microwave materials, overradiation, and hidden dangers of equipment are essential parameters to consider in the HAZOP approach for the safety of personnel and equipment.

Finally, we must emphasize the importance of these two approaches to the systematic examination throughout the whole process that defines a specification to ensure a safe process for implementing microwave processing in the plants. Nowadays, safety has become a priority in both food and chemical industries all over the world. In such an environment, it is necessary to take into account all aspects of microwave technology and the constraints of usage in the industrial world before implementing a microwave-assisted extraction.

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