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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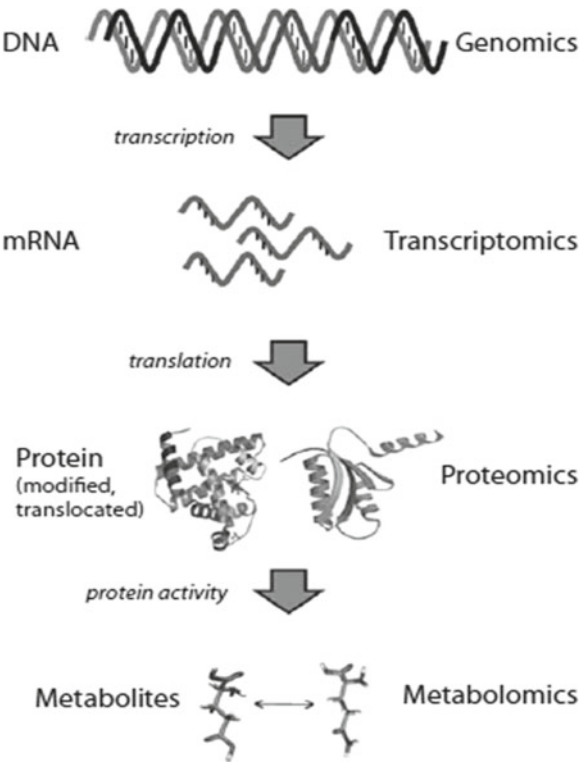

Fig. 6.1 The 'omics cascade.' This cascade depicts the pathway from the gene to metabolism. The *grey arrows* indicate the biochemical processes, which communicate the genetic information to the next level of molecules (Reproduced with permission of Springer Verlag. From Michlmayr and Oehler [3])

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,

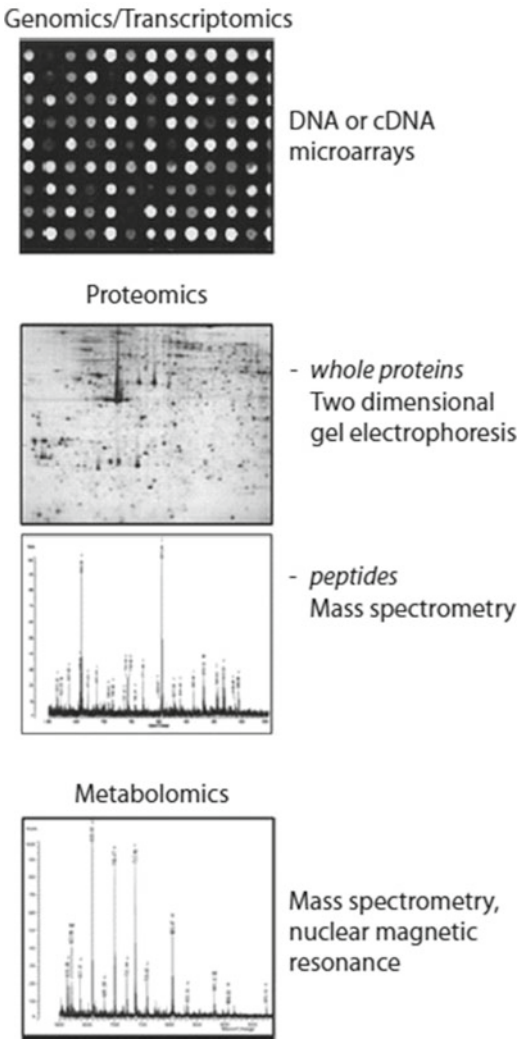

Fig. 6.2 Technologies in omics analysis. For molecular profiling at different levels of gene expression different techniques are required. (Reproduced with permission of Springer Verlag. From Michlmayr and Oehler [3])

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,

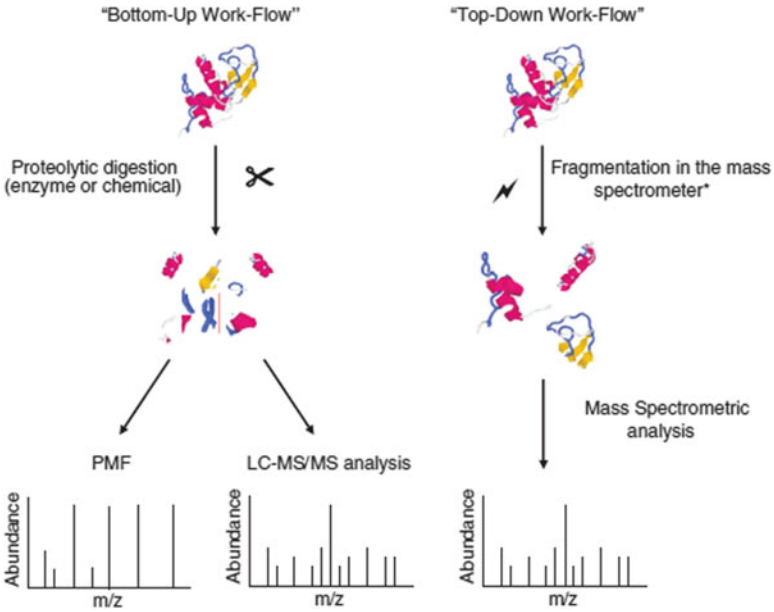

Fig. 6.3 An overview of *bottom-up* and *top-down* proteomic work flows for the characterization of proteins. (Reproduced by permission of The Royal Society of Chemistry. From Lill [8])

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

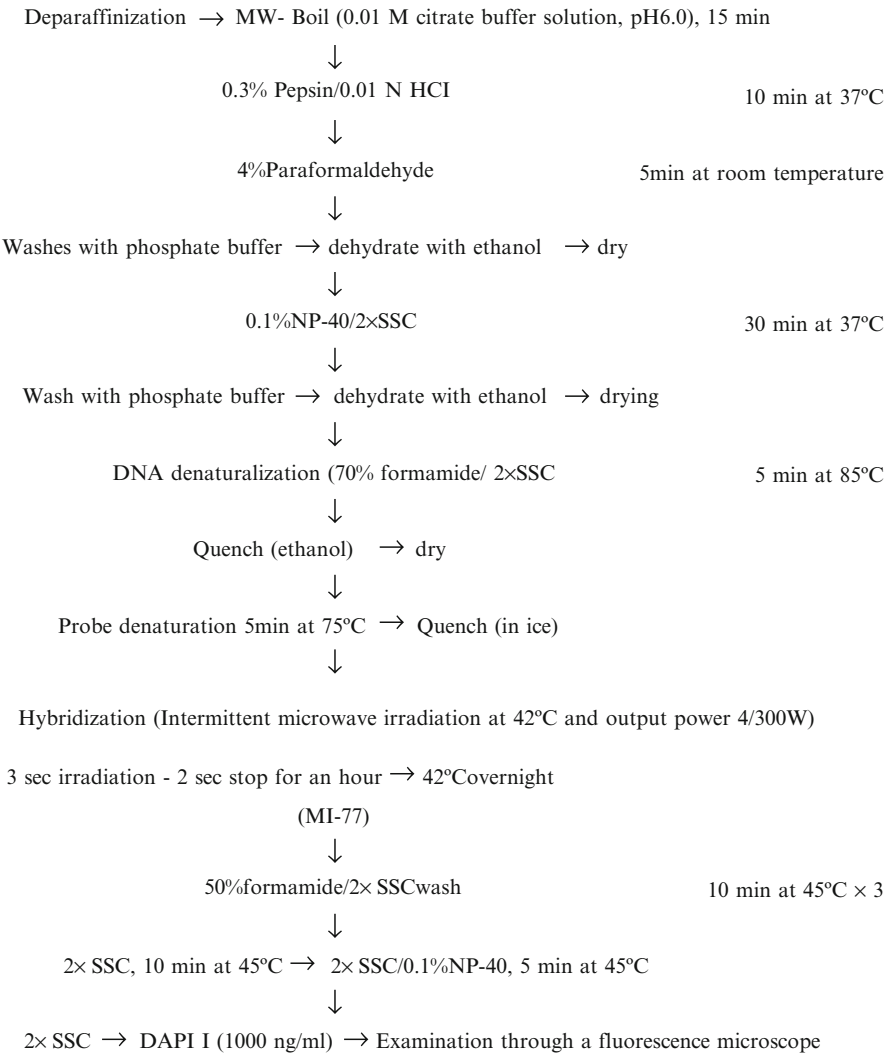

Fig. 6.4 Flowchart of the microwave-fluorescence in situ hybridization (MW-FISH) protocol. (Reproduced with permission of Oxford University Press. From Sugimura [81])

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

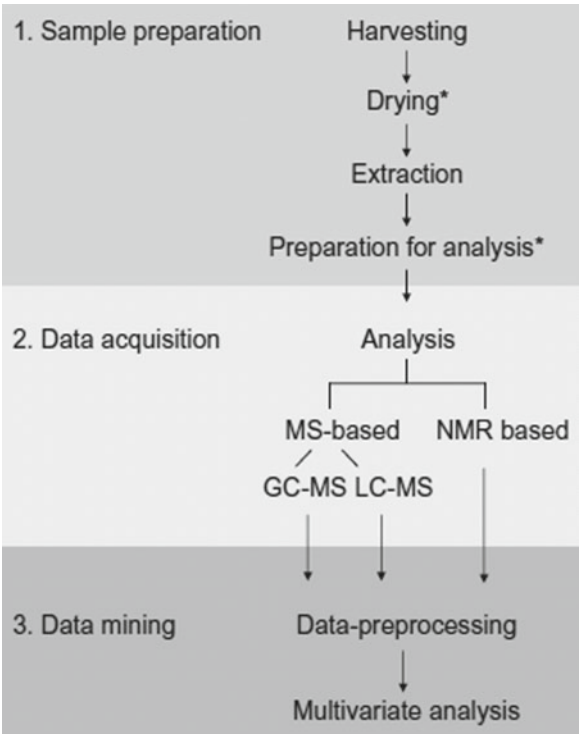

Fig. 6.5 Flowchart of the metabolomic study in plants. Sample preparation steps can be changed depending on the analytical methods; however, in general many steps are common. (*This step can be omitted in some analyses.) (Reproduced with permission of Wiley Interscience. From Kyong Kim and Verpoorte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

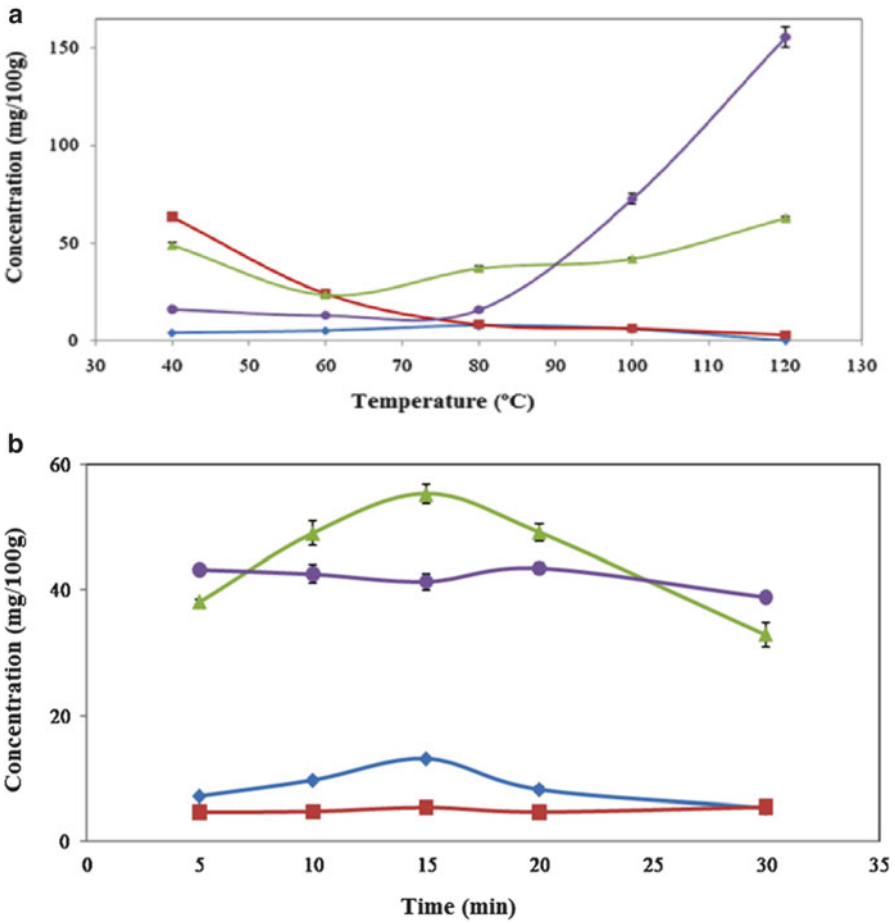

Fig. 6.6 (**a**) Effect of different extraction temperatures on the recovery of biomarker compounds from *Uncaria sinensis* by microwave-assisted extraction (MAE) at 20 min ($n=3$): caffeic acid and rhynchophylline (**a**) and epicatechin and catechin (**b**). The decrease of catechin at 40°C and 80°C 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 Δ^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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