CHAPTER

1

INTRODUCTION: BASIC PRINCIPLES OF ASSAYS TO BE COVERED, SAMPLE HANDLING, AND SAMPLE PROCESSING

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1.1 INTRODUCTION

1.1.1 Current Situation and Challenges of Food Safety and Regulations

Food can never be entirely safe. In recent years, food safety concern has grown significantly following a number of highly publicized incidents worldwide. These incidents include bovine spongiform encephalopathy in beef and benzene in carbonated drinks in the United Kingdom, dioxins in pork and milk products in Belgium, pesticides in contaminated foods in Japan, tainted coca-cola in Belgium and France, melamine in milk products in China, salmonella in peanuts and pistachios in the U.S. [1], and phthalates in drinks and foods in Taiwan [2]. Governments all over the world have taken many measures to increase food safety, resulting in a marked increase in the number of regulated compounds.

The European Union (EU) made a considerable effort to centralize food regulatory powers. The European Food Safety Authority (EFSA) and the national competent authorities are networks for food safety. The European Commission has designated food safety as a top priority, and published a white paper on food safety [3]. Legislative documents, such as 657/2002/EC, which sets out performance criteria for veterinary drug residue methods, are published as European Commission Decisions [4].

The Japanese government implemented a "positive list" to regulate the use of pesticides, veterinary drugs, and other chemicals in 2006, which replaced the old "negative list" regulations [5]. Over 700 compounds have to be monitored and reported. A certified safety report is now a requirement for both importing and exporting countries. The new regulations are listed as addendums to the positive list. In Japan, strengthening regulations for industrial use of perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA), additives, and residual pharmaceutical and personal care products (PPCPs) in the environment is progressing, which in turn creates a demand for instrumentation that provides reliable trace determination.

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In the United States, federal laws are the primary source of food safety regulations, for example, related codes under CFR Title 7, 9, 21, and 40. The law enforcement network comprises state government agencies and federal government agencies, including the U.S. Department of Agriculture (USDA), Food and Drug Administration (FDA), Centers for Disease Control and Prevention (CDC), and National Oceanic and Atmospheric Administration (NOAA). The Food Safety Modernization Act (H.R. 2751) is a federal statute signed into law by President Barack Obama on January 4, 2011. The law grants FDA authority to order recalls of contaminated food, increase inspections of domestic food facilities, and enhance detection of food-borne illness outbreaks.

As a result of regulation change and globalization, most nations around the world have now increased regulations on food safety for their domestic and export markets. International coordination and standardization are mainly conducted by the Codex Alimentarius Commission (CAC). The CAC is an intergovernmental body established in 1961 by the Food and Agriculture Organization of the United Nations (FAO), and joined by the World Health Organization (WHO) in 1962 to implement the Joint FAO/WHO Food Standards Program. There are 185 member countries and one organization member (EC) in the Codex now. The Codex standards are recommendations for voluntary application by members. However, in many cases, these standards are the basis for national legislation. The Codex covers processed, semiprocessed, and raw foods. The Codex also has general standards covering (but not limited to) food hygiene, food additives, food labeling, and pesticide residues [6].

1.1.2 Residues and Matrices of Food Analysis and High-Throughput Analysis

From the examples listed above, it is simply impossible to test every single item for every imaginable food-borne pathogen, including bacteria, viruses, and parasites; food allergens such as milk, eggs, shellfish, and soybean; naturally occurring toxins and mycotoxins; residues of pesticides and veterinary drugs; environmental contaminants; processing and packaging contaminants; spoilage markers [7]; food authenticity; and labeling accuracy [8].

Fortunately, modern analytical techniques, especially mass spectrometry-based techniques, such as gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS), can help speed up the processes. In the past decade, LC–MS, including tandem LC–MS techniques, or LC–MS/MS, has been applied in pesticide residue analysis and other food safety issues. The use of LC–MS has increased exponentially in recent years [9]. For example, an LC–MS/MS method using a scheduled selected reaction monitoring (sSRM) algorithm was developed and applied to analyze 242 multiclass pesticides for fruits and vegetables [10]. The high selectivity of LC–MS can effectively reduce interference from matrices, which significantly simplifies the process of sample preparation.

In addition, other high-throughput methods, including bioactivity-based methods, have also been widely applied today and will continue to be applied at least for the

foreseeable future, although false-positive results were found in a high number of cases for these methods [11]. A striking example is the rapid microbiological assays used routinely by dairies to screen milk inexpensively and rapidly for residues of antimicrobial drugs. In the United Kingdom alone, dairy companies run millions of such assays per year, with a test duration of only minutes from sampling to result. These tests are widely used internationally by dairies for completeness.

1.1.3 Food Safety Classifications

Food safety analysis can be broadly classified and grouped based on the residues or analytes and food matrices, accepting that there will be some degree of crossover between groups. Based on the analytes, it can be classified to pesticide residues, drug residues, mycotoxins and environment pollutants, and other industrial chemicals. Based on food matrices, the most accepted classification of groups consists of high-moisture foods, low-moisture foods, and fatty foods. Examples of such matrices are fruits and vegetables, dry grains (wheat, rice, bean, etc.), and tissues, including fish and meat.

Food safety analysis methods can be further divided into two categories: screening methods and confirmation methods. The regulatory agencies and international standard organizations have clear guidelines for screening methods and confirmation methods. The requirements are slightly different for both, depending on the residues to be analyzed, matrix, risk factor, and techniques available. A screening method is qualitative or semiquantitative in nature, comprises establishment of those residues likely to be present based on an interpretation of the raw data, and tries to avoid false negatives as much as possible. A false negative rate of 5% is accepted for both the EU and the US FDA [12,13]. A confirmation method can provide unequivocal confirmation of the identity of the residue and may also confirm the quantity present on residues found in screening. Therefore, an analyst has to use appropriate guidelines to develop a new method based on the regulation, residue category, and matrices and to provide expert advice on the findings to those commissioning the analysis.

1.1.4 "High Throughput" Definition

The "high throughput" concept has become popular in the pharmaceutical industry after combinatorial chemistry was introduced for drug discovery [14], such as in "high-throughput screening" and "high-throughput drug analysis." However, "high-throughput analysis for food safety" has only recently drawn more attention, especially after China's melamine milk crisis and Taiwan's phthalates scandal.

Although there is no numeric definition of "high-throughput screening" in the pharmaceutical industry, the standardized sample plate of 96-, 384-, or even 1536-well plates can indicate how quickly many analyses can be completed. Compared with single digits of targets in drug screening, food analysis often involves multiclass compounds ranging from a few dozens to a few hundred targets. All these kinds of

GC–MS or LC–MS methods can be considered as high-throughput analyses because one way to calculate sample throughput is to use the following equation [15]:

$$\text{sample throughput} = \frac{\text{screening capacity} \times \text{number of samples}}{\text{total analysis time}} \tag{1.1}$$

where screening capacity or analysis capacity = number of target analytes that can be screened or analyzed by the method; total analysis time = time for sample preparation + instrument data acquisition + data analysis (data process) + documentation. Given this definition, analyses using GC–MS and LC–MS as already discussed can qualify as "high throughput" because their screening capacities can be, in some instances, quite high. High screening capacities eliminate the need for many analyses on the same sample that simply screen for just one or two analytes at a time. Practically, as long as the sample throughput of a new method is significantly higher than that obtained using the current prevailing method, the new method should be considered as a high-throughput method.

1.1.5 Scope of the Book

Food safety analysis usually involves the simultaneous measurement of multiple analytes from a complex matrix. Separation of the analytes from matrices is often crucial for mass spectrometry-based analyses. Although separations can be achieved electrophoretically on one- and two-dimensional gels, by capillary electrophoresis and by GC and LC, both LC and GC are still the most applied separation methods due to their good reproducibility, recovery, sensitivity, dynamic range, and quantifiability [8,16].

GC–MS has been widely used for food safety analysis for a long time. However, the use of LC–MS for food safety analysis is among the fastest developing fields in science and industry [17]. Currently, both LC–MS and GC–MS are widely used for every food safety issue, as already mentioned. There are many modern approaches in LC–MS- and GC–MS-based methods that enable the reduction of "analytical" time and increase the sample throughput.

The book is divided into eight chapters: Chapters 1–3 discuss technology background, statistical background, industrial standards, and governments' regulations. Chapters 4–8 discuss specific fields of method development, applications of new technologies, and practice of analytical work to compile industrial standards and government regulations. The topics include pesticide residues analysis, veterinary drug residue analysis, mycotoxins analysis, and industrial chemical analysis. The discussions will show not only the current dynamic interaction between technology development and laboratory practice but also the trends of food safety analysis. Advanced sample preparation techniques and future perspectives will be discussed in the following sections, with an emphasis on an evaluation of or improvements in the throughput of the methods.

1.2 ADVANCED SAMPLE PREPARATION TECHNIQUES

Food safety analysis is a difficult task because of the complexity of food matrices and the low concentrations at which target compounds are usually present. Thus, despite the advances in the development of highly efficient analytical instrumentation for their final determination, sample pretreatment remains a bottleneck and an important part of obtaining accurate quantitative results. A past survey has shown that an average chromatography separation accounts for about 15% of the total analysis time, sample preparation for about 60%, and data analysis and reporting for 25% [18,19]. However, some new technologies and automation have significantly accelerated the sample preparation process.

Sample preparation can involve a number of steps, including collection, drying, grinding, filtration, centrifugation, precipitation, dilution, and various forms of extraction. The most conventional sample preparation methods are protein precipitation (PPT), liquid–liquid extraction (LLE), and solid-phase extraction (SPE). In addition to these traditional methods, many advanced approaches have been proposed for pretreatment and/or extraction of food samples. These approaches include salting out LLE (SALLE) such as QuEChERS (quick, easy, cheap, effective, rugged, and safe) and SweEt (Swedish extraction technique), supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), matrix solid-phase dispersion (MSPD), solid-phase microextraction (SPME), stir bar sorptive extraction (SBSE), turbulent flow chromatography (TFC), and others [8,20–23]. To avoid overlap with other chapters, only automation of weighing and preparing standard solutions, QuEChERS, SWEET, TFC, PLE, automated 96- and 384-well formatted sample preparation, headspace, SPME, MEPS, and liquid extraction surface analysis (LESA™) are discussed in the following sections.

1.2.1 Automation of Weighing and Preparing Standard Solutions

The first step of an analysis is to weigh standards for calibration solutions. With an automatic dosing balance, a tablet, paste, or powder sample can be easily weighed into a volume flask. Combined with liquid dosing, a specified target concentration can be obtained by adding the exact amount of solvent automatically.

Many routine sample preparations, such as calibration curve generation, sample dilution, aliquoting, reconstitution, internal standard addition, or sample derivatization are often time consuming. The technology development of liquid handlers has provided full automation or semiautomation solutions. Basically, there are two approaches: one is the multiple pipette liquid handler; another is the multifunction autosampler. For example, a sample preparation workbench was applied to determine eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in marine oils found in today's supplement market [24]. The workbench was programmed to methylate the analytes (derivatization) for each analytical run, to avoid sample exposure to oxygen in a closed system, and to transfer the top layer of sample to a final GC vial for injection. The workbench not only gave results comparable to three widely applied

methods (AOAC 991.39, AOCS Ce 1i-07, and the GOED voluntary monograph for EPA and DHA) but also reduced analysts' time and solvent consumption.

1.2.2 QuEChERS

Anastassiades et al. developed an analytical methodology combining the extraction/isolation of pesticides from food matrices with extract cleanup [25]. The traditional method was LLE followed by salting out of water and cartridge cleaning up. Their new method used dispersive SPE sorbent (d-SPE) together with salting out in a centrifugation tube, which simplifies the whole procedure and reduces solvent consumption and dilution error. They coined the acronym QuEChERS for it. Since its inception, QuEChERS has been gaining significant popularity and has achieved official method status from international organizations (AOAC Official Method 2007.01 and European Standard Method EN 15662) for pesticide analysis.

Besides pesticide residue analysis in food samples, QuEChERS has also been used for the analysis of other industrial chemicals or environmental pollutants such as polycyclic aromatic hydrocarbons in fish, veterinary drugs in animal tissue and milk [26], and hormone esters in muscle tissues. QuEChERS and its variations have also been used for the determination of xenobiotics, mycotoxins, veterinary drugs, environmental or industrial contaminants, and nutraceutical products [27].

1.2.3 Swedish Extraction Technique (SweEt) and Other Fast Sample Preparation Methods

The SweEt method [28] was developed by the Swedish National Food Agency. It is a LLE technique that uses ethyl acetate to differentiate the polar impurities from less polar residues of pesticides or other chemicals. Based on the SweEt method, food samples are classified into four categories: fruit and vegetable, cereals, animal origin A, and animal origin B with high fat. For fruit, vegetable, cereals, or animal origin A matrices, the sample cleanup is filtration–centrifugation or centrifugation–filtration prior to injection for GC–MS/MS or liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) analysis. For animal origin B matrix, an additional gel permeation chromatography (GPC) cleanup step to remove the coextracted fat from the extracts and solvent exchange step is needed prior to GC–MS or LC–MS injection. The method can cover multiresidues or single group of residue(s). The method uses smaller volumes of solvent and provides extracts that are compatible with GC or LC injection methods. It eliminates complicated cleanup steps (except animal origin B samples with high fat) and introduces very low concentrations of matrix components such as proteins and sugar. The method has been used to determine pesticides in fruits, vegetables, cereals, and products of animal origin [28].

QuEChERS and SweEt are general methods for multipesticide residue screening. Based on the same principles of LLE and SPE, many other methods were recently developed for other analytes such as special groups of pesticide residues or veterinary drugs.

A set of methods was developed to analyze pesticide residues that could not be covered in large groups of multiresidue analysis [29]. An example is the analysis of polar pesticides such as paraquat and mepiquat. In the method, stable isotopically labeled internal standards were added to samples before extraction. For dry samples, water was added to the sample first and then methanol with 1% formic acid was used to extract the samples. After centrifugation and filtration, the extracted solutions were injected into LC–MS/MS for quantification. For the analysis of paraquat and diquat, H_2O:MeOH (1:1) with. 0.05 M HCl was used as the extraction solution.

An efficient acetonitrile extraction method followed by using a C-18 SPE cartridge for cleaning up the extracted solution was developed and fully validated to detect tetracycline and seven other groups of veterinary drug residues in eggs by LC–MS/MS [30]. The method can detect 1–2 ng/g of 40 drugs from eight different classes.

1.2.4 Turbulent Flow Chromatography

TFC was introduced in the late 1990s as a technique for the direct injection of biological fluids into a small-diameter column packed with 30 μm spherical porous particles [31]. A high flow rate mobile phase runs through the column to form a turbulent flow. Then, the eluents are directed to an analytical column or waste controlled by a switch valve. The first column (turbo flow column) runs SPE, which can be reversed phase, hydrophilic interaction liquid chromatography (HILIC), size exclusion, or some other modes. The second column runs regular HPLC separation. Today, TFC has been developed as an automated online high-throughput sample preparation technique that makes use of high flow rates in 0.5 or 1.0 mm internal diameter columns packed with particles of size 30–60 μm. These large particle columns allow much higher flow rate with lower backpressure. The smaller analytes diffuse more extensively than larger molecules (e.g., proteins, lipids, and sugars from the matrix) into the pores of the sorbent. The larger molecules do not diffuse into the particle pores because of high flow rate and are washed to waste. The trapped analytes are desorbed from the TFC column by back-flushing it with an organic solvent and the eluate can be transferred with a switching valve onto the analytical LC–MS/MS system for further separation and detection.

Compared with traditional SPE, TFC reduces the time required for off-line sample preparation from hours to minutes because it uses reusable extraction columns in a closed system. It also allows automatic removal of proteins and larger molecules in complex mixtures by combining turbulence, diffusion, and chemistry. TFC technology also allows a broad selection of stationary phases for different matrices. For example, melamine and eight veterinary drugs, belonging to seven different classes, were detected by TFC–LC–MS/MS in milk [31,32].

1.2.5 Pressurized Liquid Extraction

PLE is a rapid extraction of solid/semisolid matrices using organic solvents or water by applying high temperatures (up to 200 °C) and high pressures (up to 1500 psi) to keep solvents in a liquid state above their atmospheric boiling points to increase

solvation power and change extraction kinetics. Raised temperature can also disrupt the strong solute–matrix interactions. The process reduces solvent consumption and operating time so as to increase the extraction efficiency. The automated PLE system can automatically load up to 24 samples in one batch. The sample cell is of different sizes, such as 1, 5, 10, 22, 34, 66, and 100 ml. Azamethiphos, avermectins, carbamates, and benzoylurea pesticides as well as chemotherapeutic agents in seaweeds were determined using PLE and separation of analytes by LC–MS/MS [33]. The applications of PLE in the analysis of food samples have been comprehensively summarized by Mustafa and Turner [34].

1.2.6 Automated 96- and 384-Well Formatted Sample Preparation as well as Automated SPE Workstations

Although automated 96- and 384-well extractions (e.g., LLE and SPE) have been widely used for bioanalysis [35], they have not yet been widely applied for food safety analysis. The possible reasons are mainly attributed to the high cost of automated extraction equipment and more varieties and relatively larger sample size of food samples. Some new automated SPE workstations can handle a much wider range of sample sizes (1–6 ml/40 ml). Therefore, they can overcome some of the limitations. For example, an autosampler-compatible cartridge (Strata-X, 3 ml/200 mg, SPE cartridge) was applied in an automated SPE workstation to detect acrylamide in brewed coffee by LC–MS/MS [36]. We predict that the application of automated extraction systems will draw more and more attention for food safety analysis in the near future.

1.2.7 Solid-Phase Microextraction

SPME was introduced in the early 1990s as a simple and effective adsorption/absorption (based on the solid/liquid coating) and desorption technique. Instead of using a syringe to pick up and inject sample into a chromatography instrument, SPME uses a piece of bonded-phase capillary tube or metal/polymer fiber to load (adsorption) and introduce (desorption) sample into instrument. The device with a bonded-phase capillary tube is called in-tube SPME and the device with a bonded-phase fiber is called fiber SPME.

The capillary tube for in-tube SPME is like a short GC column. When the sample solution goes through the tube, the bonded phase is enriched in analytes through absorption/adsorption. After the solvent is dried by a gas flow, the sample becomes a film adsorbed on the surface of the tube, and is then desorbed with heat and introduced into the instrument. The fiber SPME uses the same steps of absorption/adsorption–desorption as does in-tube SPME. The difference is that the fiber can be immersed into a solution, which is called liquid immersion SPME, or be held above solutions or solid particles/powders to adsorb the vapor from such samples, which is called headspace SPME [37].

Because different surface coatings (bonded phases) have different selectivities to different compounds, choosing an appropriate SPME fiber or tube can differentiate these compounds from a sample matrix. Therefore, SPME can combine sampling,

isolation, and enrichment in one step. SPME can be connected easily to a GC and LC system using available interfaces. Thus, SPME can reduce the time required for sample preparation and eliminate the use of large volumes of extraction solvents.

Besides the properties of surface coating, analytes, and sample matrix, the concentration of analytes is also an important factor for optimization with both tube and fiber SPME. The headspace sampling SPME is a little more complicated because of the heterogeneous phases in sample vials at the adsorption step: one factor is the distribution coefficient of the analyte in two phases (gas–solid or gas–liquid); another factor is the volume ratio of the two phases. These factors are affected by temperature, sample volume, and sample matrix. Since the introduction of SPME, it has become a practical, low-cost alternative for sample preparation for GC–MS. New surface coating materials extended SPME from small molecule to large molecule analysis, from food sample to blood or tissue samples, and from in-lab sample preparation to on-site sample preparation. Besides the application of SPME to GC–MS, SPME has been applied to analyze mycotoxins (ochratoxins A and B) in nuts and grain samples and insecticides in honey by LC–MS [38,39]. It is believed that SPME will become a practical alternative for sample preparation for LC–MS in the future.

1.2.8 Microextraction by Packed Sorbent

Microextraction by packed sorbent (MEPS) is a new development in the field of sample preparation and sample handling. It entails the miniaturization of conventional SPE packed-bed devices from milliliter bed volumes to microliter volumes. MEPS can be connected online to GC or LC without any modifications. In MEPS, ~1 mg of the solid packing material is packed inside a syringe (100–250 µl) as a plug or between the barrel and the needle as a cartridge. Sample preparation occurs on the packed bed. The bed can be coated to provide selective and suitable sampling conditions. The combination of MEPS and LC–MS is a good tool for screening and determining drugs and metabolites in blood, plasma, and urine samples [40].

MEPS has also been applied to food and beverage analysis, including the analysis of bioflavonoids from red wine, diterpene glycosides from tea extract, pesticides and PCB in fats, aflatoxin B_2 and M_2 metabolite trace analysis in milk, mycotoxin trace analysis in cereals, fatty acid methyl esters (long chain) in fermentation medium, omega-6 fatty acid in malt lipid, pigment anthocyanidins in wine, atrazine in cereals, sulfonamide trace analysis in meat, penicillin in dairy products, and cork taints in wine [41].

1.2.9 Liquid Extraction Surface Analysis

LESA was developed at Oak Ridge National Laboratory [42] to bring the benefits of nano-ESI/MS to surface analysis and to automate surface sampling for faster and more effective analyses. This approach mainly involves three steps. In step 1, a robot aliquots a sample of extraction and sprays solvent into a pipette tip. In step 2, the solvent in the pipette tip is dispensed/aspirated onto the sample surface (e.g., an apple skin) to perform extraction of any chemicals on the surface of the apple. The pipette

tip diameter is 800 μm, which produces a surface area wetted with extraction solvent. In step 3, the pipette tips and the sample extract are robotically positioned at the inlet of the ESI chip for nano-ESI-MS analysis [43]. It has been applied to analyze pesticides on apples.

1.2.10 Headspace GC

Headspace analysis has been used for more than 30 years [44] and is still one of the most important sample preparation techniques for gas chromatography [45]. It is based on the principles of gas extraction, that is, on the partition of an analyte in a heterogeneous liquid–vapor system. A good example is that headspace gas chromatography–mass spectrometry (HS-GC–MS) has been successfully applied to rapidly detect benzene, toluene, ethylbenzene, *o*-, *m*-, and *p*-xylenes, and styrene in olives and olive oil [46].

1.2.11 Summary

Using these advanced extraction techniques and their automated analogs, as already discussed, coupled with LC–MS and GC–MS techniques, more analytes per unit time can be analyzed from an increasing range of matrices, thereby increasing throughput in food analyses.

1.3 FUTURE PERSPECTIVES

In addition to GC–MS and LC–MS techniques, other techniques such as near-infrared (NIR), nuclear magnetic resonance (NMR), and capillary electrophoresis have also been developed for high-throughput food safety analysis. A handheld unit based on NIR spectroscopy and chemometrics has been developed for the rapid (<5 min) detection and quantification of economic adulterants in foods, specifically melamine in skimmed milk powder, for potential field use [47]. A new NMR procedure has been developed for routine nontargeted and targeted analyses of foods [48]. Capillary electrophoresis combined with inductively coupled plasma mass spectrometry (CE-ICP-MS) has been developed as an analytical tool for the characterization of nanomaterials in dietary supplements. These nanoparticles are difficult to separate with other techniques such as asymmetric field flow fractionation and size exclusion chromatography, due to their smaller particle sizes (typically less than 20 nm) [49].

Compared with bioanalysis, high-throughput analysis for food safety using mass spectrometry-based techniques (LC–MS and GC–MS) is not popular and gets less attention. However, we predict that throughput for food safety analysis will be significantly improved with the use and development of automated sample preparation technologies, ultrahigh-performance liquid chromatography (UHPLC) and high-resolution MS.

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