13 Determination of Acrylamide in Foodstuffs Using UPLC–MS

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13.1 INTRODUCTION

Acrylamide is one of the latest discovered neurotoxic and carcinogenic substances in food. Upon single exposure, acrylamide is toxic or harmful by all routes of administration [1,2]. Acrylamide has been added to the list of food-borne toxicants since 2002, when the Swedish National Food Administration found relevant amounts of acrylamide in several heat-treated, carbohydrate-rich foods such as potato chips, coffee, and bread [3]. It has been widely used since the last century for various chemical and environmental applications [4]. Some of the common uses of acrylamide are in the paper, dyes, cosmetics, and toiletry industry. Acrylamides have also been used as flocculants for clarifying drinking water, and for waste water treatment. It is produced commercially as an intermediate in the production and synthesis of polyacrylamides [2]. They are also a component of tobacco smoke, which gave the earliest indication that it can be formed by heating of biological material [5]. Acrylamide can also be present in a variety of food cooked at high temperature. For example, the daily mean intake of acrylamide present in some foods and coffee in a Norwegian subpopulation have been estimated to be 0.49 and 0.46 g per kg body weight in males and females, respectively [6]. Acrylamide is formed during frying, roasting, and baking and is not typically found in boiled or microwaved foods. The highest acrylamide levels have been found in fried potato products, bread and bakery wares, and coffee [7]. All the same, a great variability in acrylamide level between different products of each food category as well as between different brands of the same product has been reported. The difference in the concentration of precursors (free asparagine and reducing sugars) in raw materials, difference in food composition, and in process conditions applied can easily explain the observed variability [8]. Moreover, the actual acrylamide content of a food as it is eaten can largely vary according to domestic cooking conditions. Estimates of dietary acrylamide intake have been made for populations in many countries. A great variability between populations has been found according to a population's eating habits and the way the foods are processed and prepared. Dybing et al. [9] reported an average daily intake for adults close to 0.5 mg/kg body wt, with 95th percentile values of about 1 mg/kg body wt. The World Health Organization (WHO) estimates a daily dietary intake of acrylamide in the range of 0.3–2.0 mg/kg body wt for the general population and up to 5.1 mg/kg body wt for the 99th percentile consumers [10].

13.1.1 Chemistry of Acrylamide

Acrylamide (CH_2=CH–CO–NH_2; 2-propenamide; CAS RN79-06-1) is a colorless and odorless white crystalline solid with a molecular weight of 71.08, a melting point of 84.5°C, low vapor pressure of 0.007 mmHg at 25°C, and a boiling point at 136°C of 3.3 kPa/25 mmHg. Acrylamide is soluble in water, acetone, and ethanol; however, it is not soluble in nonpolar solvents. These properties provide a high mobility in soil and groundwater to acrylamide [11–13]. Acrylamide has been used as an industrial chemical since the 1950s and is produced from the hydration of acrylonitrile. Acrylamide is also known as acrylic amide, ethylene carboxamide, vinyl amide, or 2-propenamide. The main use of acrylamide is as a chemical intermediate for

the production of polyacrylamides. Monomeric acrylamide readily participates in radical-initiated polymerization reactions, whose products, polyacrylamides, form the basis of most of its industrial applications [14]. Acrylamide improves the aqueous solubility, adhesion, and cross-linking of polymers that are well known by population due to myriad types of uses in our society. The primary use of polyacrylamide is to strengthen paper, but polyacrylamides are also utilized in the synthesis of dyes; in copolymers for contact lenses; as well as in construction of dam foundations, tunnels, and sewers. Polymers are used as additives for water treatment, enhancers of oil recovery, flocculants, papermaking aids, thickeners, soil conditioning agents, sewage and waste treatment, ore processing, and permanent-press fabrics. Polyacrylamides are also applied in formulations of several types of personal care and grooming products, such as lotions, cosmetics, deodorants, soaps, and shampoos. Further uses are in oil well drilling fluids, for soil stabilization, as dye acceptors, as polymers for promoting adhesion, for increasing the softening point and solvent resistance of resins, as components of photopolymerizable systems, and as cross-linking agents in vinyl polymers. However, other applications of polyacrylamide are in the biomedical, genetic engineering, and research fields, like the separation of proteins by gel electrophoresis [2]. Besides its industrial applications, acrylamide is also present in tobacco smoke, in amounts of 1–2 mg per cigarette [15,16]. Polyacrylamide contains up to 0.1% free acrylamide monomer. Then, low amounts of acrylamide might also migrate from food packaging material into packaged foodstuff [17]. The specific migration limit for acrylamide from materials that come into contact with foodstuffs was defined to not be detectable, with a limit of detection (LOD) of 10 mg/kg [18,19].

13.2 CONTAMINATION OF FOODSTUFFS WITH ACRYLAMIDE

Acrylamide is a heat-induced contaminant naturally formed during home cooking and industrial processing of many foods consumed daily around the world. French fries, potato chips, bread, cookies, and coffee exert the highest contribution to dietary exposure of acrylamide to humans. Furthermore, food safety international bodies and industrial sectors are very active in implementing strategies to minimize its formation during roasting, baking, frying, toasting, and so on. Given the prevalence of acrylamide in the human diet and its toxicological effects, it is a general public health concern to determine the risk of dietary intake of acrylamide. However, associations between dietary acrylamide exposure and increased risk of different cancers are somewhat controversial and do not have a direct extrapolation to the global population. Accordingly, further long-term studies with a general view are ongoing to clarify the risk scenario and to improve the methodology to detect small increases in cancer incidence [20].

13.2.1 Maillard Reaction Acrylamide Production

Recently, after the discovery of the chemical mechanism governing this food-related contaminant, acrylamide production has been described through a series of reactions known as Maillard reaction, between an amino acid, primarily asparagines, and a reducing sugar such as fructose or glucose [21–25] (Figure 13.1). The amino acid

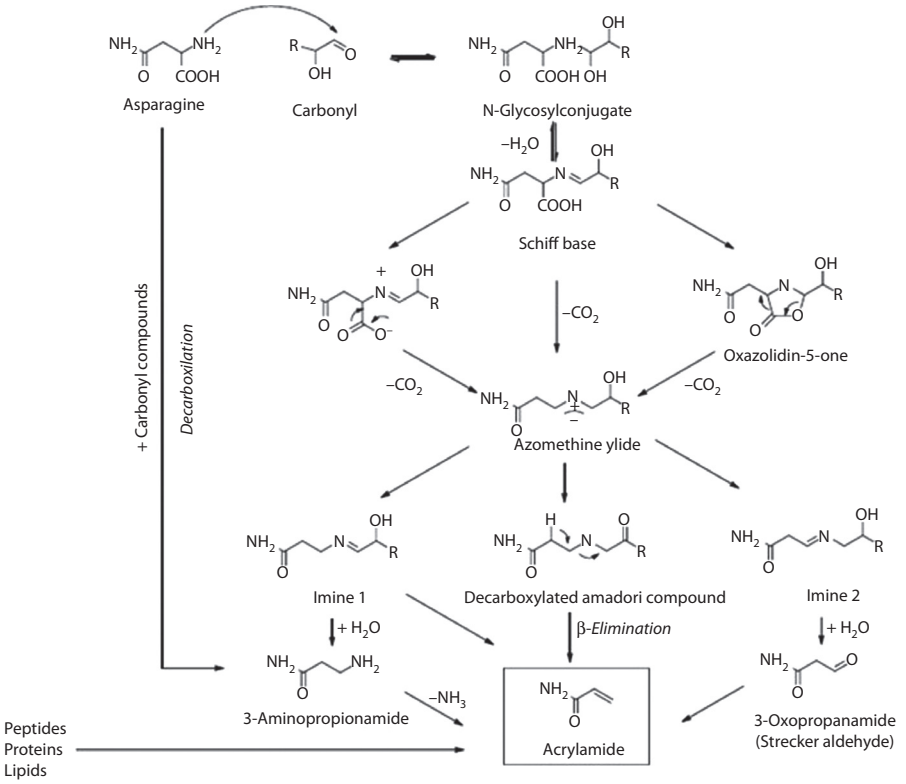

FIGURE 13.1 Reaction scheme for acrylamide formation in the Maillard reaction. (Adapted from Buhlert, J. et al. 2006. *Lett. Org. Chem.* 3:356–357. With permission.)

asparagines was first suspected and later confirmed to be necessary for the formation of acrylamide, as it furnishes the backbone of the acrylamide molecule. Later, additional formation mechanisms, for example, from peptides, proteins, lipids, and biogenic amines, were identified [26–29]. Briefly, acrylamide formation begins at temperatures around 120°C and peaks between 160°C and 180°C [30]. Thus, acrylamide is formed during frying, roasting, and baking and is not typically found in boiled or microwaved food. The highest levels appear in fried and roasted potato products and in cereal products such as breads, crackers, and breakfast cereals. Kinetic studies in model systems and foods clearly demonstrated the strong relationship between processing conditions (time and temperature), moisture and pH, and acrylamide. The formation of acrylamide becomes relevant at temperatures higher than 120°C, and at prolonged heating conditions above 170°C there is a balance between the rates of formation and of loss. Acrylamide losses are due to evaporation, polymerization, or reaction with other food components. Acrylamide is able to react via Michael addition with nucleophilic sites such as amino or thiol groups present in proteins. The almost exclusive formation of acrylamide from asparagines could explain the selective occurrence of acrylamide in certain food products that are rich

in concentration of the dominant free amino acid sparagines such as potatoes and cereals. This is the case of potato processing, wherein asparagines comprise nearly 39% (59–143 mmol/kg) of the total free amino acids, and reducing sugar content can vary up to 5.0% of fresh weight. In the case of cereals, the content of free sparagines in wheat flour ranged from 0.15 to 0.4 g/kg, being up to 1.48 g/kg higher in wheat bran [27]. Shortly after discovering, it was established that the major pathway for acrylamide formation in foods is the Maillard reaction with free sparagines as the key amino precursor [18,19]. Other minor reaction routes for acrylamide formation in foods have been postulated, from acrolein and acrylic acid [28], from wheat gluten [26], and by deamination of 3-aminopropionamide [23]. This rapid identification of the main route of acrylamide formation during thermal processing of food was critical to launch the different mitigation strategies as offered today to industry. The Maillard reaction has also been known for producing other mutagenic compounds such as some dicarbonyl compounds (e.g., acrolein and glyoxal), furans (e.g., furfural and 5-sulfooxylmethylfurfural), heterocyclic amines, pyrroles (e.g., 1-nitro-2-acetylpyrrole), dithianes (e.g., 1,3-dithiane), thiazoles, and thiazolidines (e.g., 2-(1,2,3,4,5-pentahydroxy)-pentylthiazolidine). This background of knowledge from the scientific community was determinant in the rapid identification of the predominant pathway of formation in foodstuffs, and later for searching for ways of mitigation. Any information from the variables affecting the formation of acrylamide in foods could be of great interest, as it may open new opportunities for its mitigation. Because the precursors of acrylamide formation are common sugars and amino acids, it is impossible to eliminate them from our foods to avoid the formation of acrylamide, and alternatives should be studied. Accordingly, the structure-specific health effects of the harmful and beneficial compounds formed from the Maillard reaction can be determined, and the food processing technologies can be optimized toward a more selective formation of the health-beneficial ones. Upon lowering the formation of acrylamide in foodstuffs, the concomitant loss of valuable Maillard components, such as flavor or colored compounds, should also be taken into account [20].

13.3 ACRYLAMIDE TOXICITY

From a chemical point of view, acrylamide is a reactive electrophile, and due to its α-, β-unsaturated structure can react with nucleophiles such as amines, carboxylates, and thiols that are commonly found on biological molecules like DNA [29]. Acrylamide is biotransformed *in vivo* to its epoxide, glycidamide ($C_3H_5NO_2$; CAS RN 5694-00-8), which is genotoxic in a variety of *in vitro* and *in vivo* test systems [31]. Glycidamide is also known as glycidic acid amide, oxirane-2-carboxamide, or 2,3-epoxypropionamide. However, acrylamide is much less reactive than glycidamide toward DNA [32]. This fact, together with the genotoxicity of glycidamide, has led to the assumption that glycidamide is the genotoxic agent and probably also the cancer risk increasing factor in acrylamide exposure [33]. The biotransformation process by which acrylamide is converted into glycidamide is not only plausible in animals, but can be readily demonstrated to occur efficiently in both human and rodent tissues. It was noted in passing by the IARC working group that “acrylamide is not known to occur as a natural product” [34]. Long-term exposure to acrylamide

may cause damage to the nervous system in both humans and animals to a certain extent. Meanwhile, acrylamide is also regarded as a potentially genetic and reproductive toxin with mutagenic and carcinogenic properties in both *in vitro* and *in vivo* studies [35,36]. However, acrylamide has not been known to be produced from the degradation of polyacrylamide gels in biomedical research applications [37]. The major exposure to the population from polyacrylamide comes from cosmetics, which might contain up to 2% of the gel. However, polyacrylamide is not considered to be harmful to humans. Acrylamide monomer, however, is described as having damaging effects in several aspects, which might become apparent after a delay of months or even years. Toxicological studies suggested that acrylamide vapors irritate the eyes and skin and cause paralysis of the cerebrospinal system, and its occupational exposure limit is set to 0.3 mg/m^3 [38,39]. For the general public, a potential source of exposure had only been seen by drinking water that had been treated with polyacrylamide in a refining process [40]. In order to minimize the risk for the general population, a maximum tolerable level of 0.1 mg acrylamide/L water has been established within the European Union [41]. Elevated levels of acrylamide bound to the hemoglobin were found in workers exposed to the chemical grout. Through measurement of reaction products with protein hemoglobin in blood, it was shown that several of the tunnel workers had developed peripheral nerve symptoms similar to those reported for acrylamide poisoning [13,42]. However, unexpected amounts of acrylamide–hemoglobin adducts could be found in the unexposed people, living outside the contaminated area and used as controls. The observation of a regularly occurring high background level (~0.03 nmol/g globin) of adducts from acrylamide to N-terminal valine in hemoglobin in nonsmoking and occupationally unexposed control persons outside the leakage water indicated the existence of another general exposure source [43]. In order to identify the origin of acrylamide adducts in these nonexposed persons, researchers investigated a number of suspected sources, like food. The importance of acrylamide in food was mentioned for the first time by Tareke [44] who showed that rats feeding fried feed led to a large increase in the level of the hemoglobin adduct, which was concluded to be *N*-(2-carbamoyl-methyl)-valine. This finding showed that almost the entire population is exposed to acrylamide on a daily basis and that the major cause for the observed background adducts was the ingestion of heated starchy food. Background hemoglobin adduct levels in adult humans range from 12 to 50 fmol/mg of globin [9]. After the confirmation of the formation of acrylamide in starchy foods, a variety of many other food products containing acrylamide have been identified [45,46]. For some foods, the measured amounts exceeded amply the maximum allowable concentration in drinking water of 0.1 mg acrylamide/L in EU countries [43] as well as the WHO guideline value for the maximum safe level concentration of acrylamide at 0.5 mg/L [47] and, of course, the existing EU regulations on chemical migration from plastic packaging of 10 mg acrylamide/kg [18]. Based on these observations, certain food products were suspected of being a potential source of exposure to acrylamide. These findings attracted worldwide interest, because of toxicological relevance of acrylamide. Thus, the discovery that the compound is found extensively throughout the food supply caused alarm that dietary acrylamide could be an important human cancer risk factor [46]. This finding showed that almost the entire population is exposed to

acrylamide on a daily basis and that the major cause for the observed background adducts was the ingestion of heated starchy food. After rapid confirmation, numerous research activities concerning the extent of exposure, origin of acrylamide in food, health risk to humans, and mitigation of acrylamide in food were initiated. Acrylamide has been shown to be neurotoxic in humans [48] and has been shown to induce tumors in laboratory rats [34,41]. On the basis of tests in animals, a Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that current acrylamide levels in foods may indicate a human health concern and that cancer could be the most important adverse effect of acrylamide [49,50]. As a consequence, a huge number of multidisciplinary studies with a great mobilization of human and economic resources were started around the world. There is some confusion with the denomination "contaminant" to acrylamide, as it is a compound naturally formed in food during cooking. Acrylamide is named as a processing contaminant or neoformed contaminant. Long-term exposure to acrylamide may cause damage to the nervous system in both humans and animals to a certain extent. Meanwhile, acrylamide is also regarded as a potentially genetic and reproductive toxin with mutagenic and carcinogenic properties in both *in vitro* and *in vivo* studies [37,38]. Acrylamide monomer is described as having damaging effects in several aspects, which might become apparent after a delay of months or even years.

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13.3.1 Neurotoxicity

The neurotoxicity of acrylamide in humans is well known from occupational and accidental exposures [51]. For instance, Calleman et al. [52] reported peripheral neuropathy symptoms to highly exposed workers in China. It is characterized by skeletal muscle weakness, numbness of hands and feet, and ataxia. Acrylamide has been shown to be toxic to both the central and the peripheral nervous system [53], although the nerve terminal is now considered to be the primary site of acrylamide action [54,55]. Acrylamide induces nerve terminal degeneration and has effects on the cerebral cortex, thalamus, and hippocampus [56]. In double-blind studies of factory workers, no neurotoxicity was found in workers exposed to less than 3.0 mg/kg/day as determined by biomonitoring [55]. A very recent study demonstrates structural and ultra structural evidence of neurotoxic effects of fried potato chips on rat postnatal development.

13.3.2 Reproductive Toxicity

Acrylamide administered to drinking water of rodents at doses ≥5 mg/kg bw/day resulted in significant decreases in number of live fetuses per litter [57]. At doses of

155 mg/kg bw/day or greater, signs of neurotoxicity and copulatory behavior were noted, as well as effects on sperm motility and morphology. The toxicities in male animals include degeneration of the epithelial cells of the seminiferous tubules, decreased number of sperm, and abnormal sperm, and resulted in decreased fertility rates and retarded development of pups [56]. These toxic effects may be attributed to the interfering effect of acrylamide on the kinesin motor proteins, which also exist in the flagella of sperm, resulting in the reduction in sperm motility and fertilization events [58]. The exposure levels are, however, far above the dietary acrylamide intake in order to pose such a risk. Furthermore, there is no evidence for adverse reproductive or developmental effects from exposure to acrylamide in the general population [59].

13.4 CARCINOGENICITY

Acrylamide is found extensively throughout the food supply, causing alarm that dietary acrylamide could be an important human cancer risk factor [46]. Acrylamide was classified as probably carcinogenic to humans (Group 2A) by the International Agency for Research on Cancer (IARC) [34]. This conclusion was mainly based on positive bioassay results in rodents and supported by the evidence that acrylamide is transformed in mammalian tissues to its more reactive genotoxic metabolite, glycidamide. Several evidences were considered to reach this decision: (i) formation of covalent adducts with DNA in mice and rats, (ii) formation of covalent adducts with hemoglobin in humans and rats, (iii) induction of gene mutations and chromosomal aberrations in germ cells of mice and chromosomal aberrations in germ cells of rats, (iv) induction of chromosomal aberrations in somatic cells of rodents in vivo, (v) induction of gene mutations and chromosomal aberrations in cultured cells in vitro, and (vi) induction of cell transformation in mouse cell lines. Moreover, acrylamide is currently classified as "reasonably anticipated to be a human carcinogen" by the National Toxicology Program [60]. Clear evidence of carcinogenic activity in laboratory animals is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy. Acrylamide has not been found to induce mutations in bacteria, but it induced sex-linked recessive lethal and somatic mutations in Drosophila. Substantial laboratory evidence on experimental rodents shows that acrylamide is carcinogenic, causing tumors at multiple sites such as lungs, skin, brain, mammary gland, thyroid gland, and uterus [61,62]. Acrylamide is clastogenic and mutagenic in mammalian cells [42]. It has been stated that oxidation of acrylamide to glycidamide appeared to be a prerequisite for genotoxicity of acrylamide, due to the higher reactivity of glycidamide to form adducts with DNA [63,64]. Although acrylamide and glycidamide reacted directly with hemoglobin, only glycidamide reacted largely with DNA to produce the N7–guanine adduct and to a much lesser extent the N3–adenine adduct [65]. Hence, acrylamide is not mutagenic in *Salmonella typhimurium* assays; in contrast, glycidamide is mutagenic in this assay. The important role of CYP2E1 in epoxidation of acrylamide to glycidamide and formation of glycidamide–DNA

adducts has been demonstrated by using CYP2E1-null mice, and when such mice were exposed to acrylamide, higher levels of acrylamide adducts were observed compared with wild-type mice [65]. Additionally, DNA adducts can be regarded as biomarkers of a biologically active internal dose of acrylamide. Before use of such DNA adducts, analytical methods with improved sensitivity would be required, but also complete information on the stability over longer periods of time. Acrylamide itself does not show direct reactivity toward DNA. Since 1994, acrylamide has been classified by the IARC as probably carcinogen to humans [34].

13.5 REGULATIONS

For some foods, the measured amounts exceeded amply the maximum allowable concentration in drinking water of 0.1 mg acrylamide/L in EU countries [43], as well as the WHO guideline value for the maximum safe level concentration of acrylamide at 0.5 mg/L [66], and, of course, the existing EU regulation on chemical migration from plastic packaging of 10 mg acrylamide/kg [18]. Based on these observations, certain food products were suspected of being a potential source of exposure to acrylamide. An acrylamide legislative framework is a critical determinant of whether reliable analytical methods can be developed. It stipulates (i) sampling and monitoring plans, (ii) definition of maximum residue limits (MRLs) for tolerated food contaminants and residues and minimum required performance limits (MRPLs) for some of the testing procedures to detect banned substances, and (iii) the performance characteristics of analytical methods [50,67,68]. The development, optimization, and validation of suitable analytical methods are important elements for the determination of acrylamide and to improve the reliability of analysis methods applied to food samples and residue testing. Because of this, a short description of the situation and aims of this legislative set-up is obligatory. An acrylamide legislation is not coordinated throughout the world. However, well-known international bodies, the most representative of which is the Codex Alimentarius Commission established by FAO and WHO, develops science and risk-based food safety standards that are a reference in international trade and a model for countries to use in their legislation. They considered the margin of exposure values to be low and concluded that it may indicate a human health concern [69].

13.6 MITIGATION STRATEGIES

Mitigation steps include replacement of reducing sugars with sucrose and of ammonium bicarbonate with sodium bicarbonate, or changing in process conditions and/or technologies (changing of time–temperature of frying or baking, changing in the type of oven, prolonged fermentation, etc.) [70]. One of the most promising tools to control acrylamide content in heat-treated foods is the addition of the enzyme asparaginase. The enzyme asparaginase (L-asparagine amidohydrolase) is an enzyme able to catalyze the hydrolysis of asparagine in aspartic acid and ammonia, thus lowering the content of precursor asparagine. Asparaginase has been successfully applied at lab scale both to potato [22] and cereal-based products [71] with a percentage of reduction up to 85–90%. It has no effect on product taste and

appearance and is already being used for some products at industrial scale [72]. Some preliminary results achieved at lab scale highlight that asparaginase pretreatment of green beans may represent a viable way to reduce acrylamide concentration in roasted coffee, as well. Up to now, two commercial products, Acrylaway® (asparaginase from *Aspergillus oryzae*) from Novozyme and PreventASe® (asparaginase from *Aspergillus niger*) from Dutch-based multinational life sciences and materials sciences company (DSM), are on the market for food applications. Generally recognized as safe (GRAS) status has been obtained from the US FDA for both types of asparaginases available, and JECFA also endorsed the conclusion that asparaginase does not represent a hazard to human health [73]. Nevertheless, the high cost of the enzyme may represent a serious constraint on its application on a large scale. It should be noted that most of the mitigation measures proposed so far were only tested at laboratory or at pilot scale. Therefore, for those mitigation measures it is not clearly known whether the percentage of reduction in acrylamide claimed at laboratory scale could ever be achievable in food processed at an industrial scale. It has also been emphasized that some mitigation strategies are associated with an increase in other risks or a loss in benefits. For example, prolonging yeast fermentation can efficiently reduce acrylamide concentration in bread, but it is also associated with an increase in the levels of 3-monochloropropandiol (3-MCPD), another neo-formed contaminant [51]. Similarly, replacement of ammonium bicarbonate with sodium bicarbonate as a rising agent for fine bakery products results in an increase of sodium intake [74]. There is a wide consensus that the actions aiming at lowering acrylamide content of foods should be accompanied by a risk–risk or risk–benefit analysis to elucidate all the side effects and their impact on human health. Some options are hardly feasible because of the negative effect they have on HMF content. In addition, some of the mitigation strategies that have been proposed bring about changes in organoleptic properties of foods (excessive browning as a result of glycine addition, generation of off-flavors, insufficient browning as result of changing in time–temperature profile, etc.) that can dramatically affect the final quality and consumers' acceptance [75]. This is a fundamental point for the future, considering that mitigation strategies are not useful if for sensorial reasons consumers do not like the "mitigated" products, giving their preference to the "conventional" ones. In that respect, knowledge of the kinetics of acrylamide accumulation in foods is of utmost importance. Acrylamide starts to form at a temperature >100°C after an initial lag phase, during which no acrylamide forms. Later on, the acrylamide concentration increases exponentially with time to a maximum concentration, after which it can decrease again because of the exhaustion of one of the reactants and/or by the elimination of acrylamide. Acrylamide possesses two functional groups, an amide group and the electron-deficient vinylic double bond, that makes it available for a wide range of reactions, including nucleophilic and Diels–Alder additions and radical reactions. Acrylamide may undergo Michael addition-type reactions to the vinylic double bond with nucleophiles, including amino and thiol groups of amino acids and proteins. On the other hand, the amide group can undergo many reactions, including hydrolysis, dehydration, alcoholysis, and condensation with aldehydes [16]. Many kinetic models have been proposed to describe acrylamide formation and elimination and to predict its final concentration

in model systems and foods. Single-response models based on overall empirical reaction kinetics have been extensively used. Acrylamide formation has thus been modeled as a first-order [76] or second-order reaction [77] according to reaction conditions and reactant concentrations. Acrylamide elimination has usually been modeled as first-order kinetics [78]. Totally empirical models such as those proposed by Corradini and Peleg [79] and Kolek et al. [80] have also been proposed and proven to satisfactorily describe acrylamide concentration in model systems [81] and potato chips [82]. Such empirical models are not based on an underlying chemical mechanism and extrapolation is thus not possible outside the region of variables for which the function has been derived. On the other hand, multiresponse models using acrylamide data supplemented with data on reaction precursors, intermediates, and end products including mechanistic insights in the chemistry involved have also been proposed [83]. With such model systems, not only acrylamide formation but also that of other relevant Maillard-related compounds can be modeled and the estimation of kinetic parameters is much more precise.

13.7 ANALYTICAL METHODS FOR ACRYLAMIDE DETERMINATION IN FOOD

13.7.1 Sample Pretreatment Methods

13.7.1.1 Liquid–Liquid Extraction

A promising approach is to extract the analyte into a polar organic solvent, such as ethyl acetate. Sanders et al. (2002) [84] have employed ethyl acetate to extract acrylamide from the aqueous phase (removing interfering constituents such as salt, sugars, starches, amino acids, etc.). The ethyl acetate extract can then be concentrated and analyzed by either liquid chromatography mass spectrometry (LC–MS) or GC–MS. In most cases, the LOD is significantly lowered, even approaching 10 μg/kg [22]. Similarly, an ethyl acetate extraction step can also be included after the SPE clean-up, providing a significant improvement in sensitivity, especially for more difficult matrices, such as cocoa powder and coffee.

13.7.1.2 Solid-Phase Extraction

The whole extraction and clean-up procedures generally summarized from many peer-reviewed papers before sample injection of GC–MS or LC–MS/MS analysis are shown in Figure 13.2. Water at room temperature has been used to extract acrylamide from many kinds of sample matrices in most analytic methods published to date, because acrylamide is a good hydrophilic small molecule [46,47]. Besides water as an extractant, methanol can also be used to extract acrylamide for the convenience of rotatory evaporation and concentration [85]. Young et al. [86] suggested that acrylamide could be extracted from sample matrices by using NaCl aqueous solution with a relatively high level in order, so that the emulsification process during sample pretreatment was obviously inhibited and the high recovery of analytes was demonstrated. Moreover, one of the laboratories that took part in the proficiency test about acrylamide used a mixture of water and acetone as extractant [87]. In addition, a research group of the National Institute of Health Sciences of Japan chose

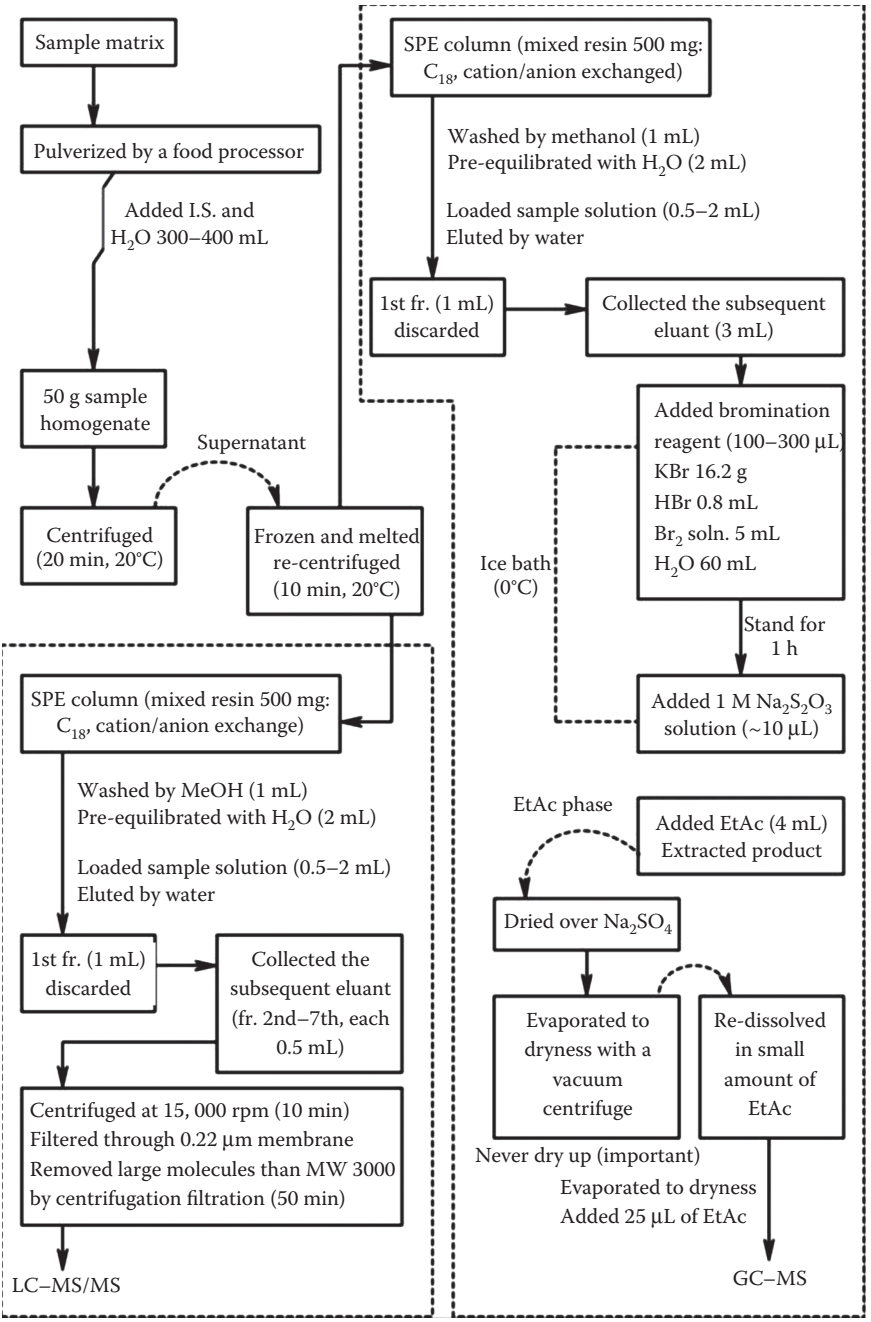

FIGURE 13.2 The whole extraction and clean-up pretreatment of acrylamide before GC–MS or LC–MS/MS. I.S., internal standard; fr., fraction; EtAc, ethyl acetate; MW, molecular weight. (Adapted from Zhang, Y., Zhang, G., and Zhang, Y. 2005. *J. Chromatogr. A* 1075:1–21. With permission.)

this solvent composition for acrylamide extraction [88]. Heating or ultrasonicating during the extraction step may as well be avoided because this may generate large amounts of slight particles that can saturate the SPE cartridges used in further clean-up steps and reduce the efficiency of clean-up and the operating life of SPE cartridges. However, water that had been previously heated to 80°C has been used with no extracting problems.

13.7.2 Chromatographic Techniques

The first method pertaining to the analysis of acrylamide in different cooked and processed foods was reported in May 2002 and is based on the use of isotope dilution LC–MS [89]. Since then, several analytical methods dealing with the analysis of acrylamide in cooked foods have been published in peer-reviewed journals or presented at international scientific meetings [90]. These methods are based mainly on MS as the determinative technique, coupled with a chromatographic step either by LC [64] or GC, the latter either after derivatization of the analyte [91], or, in a few cases, analysis of the compound directly [92]. The Working Group on Analytical Methods that convened during the recent meeting [93] of the European Workshop on Analytical Methods for the Determination of Acrylamide in Food [94], concluded that the majority of laboratories involved in acrylamide analysis use either GC–MS or LC–MS methods. The advantage of the LC–MS-based methods is that acrylamide can be analyzed without prior derivatization (e.g., bromination), which considerably simplifies and expedites the analysis.

13.7.2.1 Methods Based on Gas Chromatography–Mass Spectrometry Techniques

Assays employing GC–MS techniques are either based on bromination of the analyte or direct analysis without derivatization. The latter approach is less laborious and, in both reported cases, employs liquid–liquid extraction of the analyte. In the method reported by Biedermann et al. [94], the determinative step is either positive ion chemical ionization in the selected ion monitoring (SIM) mode or electron impact ionization, achieving an LOD of around 50 and <10 μg/kg, respectively, for potato products. Better sensitivity (level of quantitation, LOQ = 5 μg/kg) can be achieved in the tandem (MS/MS) mode using a high-resolution mass spectrometer. Although more tedious, the bromination of acrylamide to 2,3-dibromopropionamide has multiple advantages, which include (a) improved selectivity, (b) increased volatility, (c) removal of potentially interfering co-extractives, and (d) better sensitivity. Usually the ions *m/z* 150/152 [CH_2 CHBr $CONH_2$]+ and *m/z* 106/108 [CH_2 CHBr]+ are monitored in the SIM mode. Some analysts, however, choose to convert the rather labile di-bromo derivative into 2-bromopropenamide by treatment with triethylamine. This additional step avoids the risk of dehydrobromination in the injector or the ion source of the MS and has no impact on the selectivity or sensitivity of the method. In this case, the ions *m/z* 149/151 [CH_2 CBr $CONH_2$]+ and *m/z* 106/108 are chosen in the SIM mode. Therefore, GC–MS after bromination is probably the best choice for the analysis of acrylamide in foods necessitating a detection level at or <10 μg/kg. A typical flow chart illustrating the individual steps in sample preparation and

extraction for GC–MS analysis is shown in Pittet et al. [95]. A further advantage of this technique is that a relatively simple benchtop, GC–MS, can be employed for acrylamide analysis. Application of GC–MS/MS or coupling to a high-resolution MS would even further lower the detection limit for certain foods, approaching the range of 1–2 µg/kg.

13.7.2.2 Methods Based on Liquid Chromatography–Mass Spectrometry

The first LC–MS method for acrylamide in cooked foods was developed in early 2002 by Rosén and Hellenas [91] to verify the initial results procured in Sweden by GC–MS. The method essentially entailed extraction of the analyte with water, centrifugation, solid phase extraction over a Multimode (Isolute®) cartridge, filtering over a 0.22 µm syringe filter, and, subsequently, over a centrifuge spin filter (cut-off 3 kDa). Due to the low molecular weight of acrylamide (71 g/mol) and also its low mass fragment ions, confirmation of the analyte can be achieved with a three-stage mass spectrometer (monitoring of more than one characteristic mass transition). However, acrylamide is a very polar molecule with poor retention on conventional LC reversed-phase sorbents [64], and despite the use of a tandem mass spectrometer, more effort must, in most cases, be placed on the clean-up steps to avoid interference from co-extractives. Of the LC–MS methods communicated at different expert meetings, workshops, or published in the scientific literature, most are making use of SPE during the clean-up step. Acrylamide is difficult to bind actively to any of the conventional sorbents, but the major advantage of SPE is the retention of interfering matrix constituents. Therefore, enrichment or concentration of the analyte remains a challenge, and relatively low absolute recoveries have been recorded in the range of 62–74% in breakfast cereals and crackers, respectively [96]. Since the initial Swedish announcement, food industry laboratories have worked intensively on the development of LC–MS-based methods to determine acrylamide in processed and cooked foods [97]. Similar to the experiences of private and official food control laboratories, problems have been encountered in the analysis of difficult matrices due to interfering compounds in the characteristic acrylamide transitions (either for the internal standard or the analyte). A promising approach is to extract the analyte into a polar organic solvent, such as ethyl acetate. Sanders et al. [87] have employed ethyl acetate to extract acrylamide from the aqueous phase (removing interfering constituents such as salt, sugars, starches, amino acids, etc.). The ethyl acetate extract can then be concentrated and analyzed by either LC–MS or GC–MS. In most cases, the LOD is significantly lowered, even approaching 10 µg/kg [22]. Similarly, an ethyl acetate extraction step can also be included after the SPE clean-up, providing a significant improvement in sensitivity, especially for more difficult matrices, such as cocoa powder and coffee. Continuous progress is being made in optimizing LC–MS methods and reducing the quantification limits. Recently, Jezussek and Schieberle [98,99] have reported a promising method by derivatizing acrylamide with 2-mercaptobenzoic acid to the thioether and measuring the resulting adduct with a single-stage mass spectrometer. This method achieves an improvement in sensitivity of approximately 100-fold versus the nonderivatized analyte. Such approaches may potentially form the basis for the development of even more simple LC-UV methods for the determination of acrylamide in foods.

13.7.2.3 Online Methods-Proton Transfer Reaction MS

Proton transfer reaction mass spectrometry (PTR-MS) has been shown to be a suitable method for rapid and online measurements of volatile compounds of headspace samples [100] It combines a soft, sensitive, and efficient mode of chemical ionization, with a quadrupole mass filter. The headspace gas is continuously introduced into the drift tube, which contains a buffer gas and a controlled ion density of H_3O+. Volatile organic compounds (VOCs) that have proton affinities larger than water are ionized in the drift tube by proton transfer from H_3O+, that is, $VOC + H_3O + \rightarrow [VOC + H] + +H_2O$. The protonated VOCs are extracted from the drift tube by a small electric field and mass analyzed in the quadrupole mass spectrometer. The four key features of PTR-MS can be summarized as follows: (a) it is fast, and time-dependent variations of headspace profiles can be monitored with a time resolution of about 0.1 s; (b) the volatiles are not subjected to work-up or thermal stress and little fragmentation is induced by the ionization step, hence, mass spectral profiles closely reflect genuine headspace distributions; (c) mass spectral intensities can be transformed into absolute headspace concentrations; (d) it is not invasive. All these features make PTR-MS particularly suited to investigate fast dynamic processes, such as formation of aroma and volatile contaminants in Maillard reactions. The applicability of the PTR-MS approach for monitoring online the formation of acrylamide was evaluated in real food systems using thermally treated potatoes as an example [101]. The mass trace at *m/z* 72 indicated the presence of acrylamide in the headspace obtained by heating a potato at 170°C. The mass at *m/z* 72 was found to be homogeneous, without interference with other volatile compounds, using an offline coupling method. Retention index and EI mass spectrum were identical with those of the acrylamide reference compound, and only one peak with the mass at *m/z* 72 was detected by PTR-MS. The EI spectrum of the compound eluting 49.6 m was conclusively identified by the Wiley EI database as acrylamide. The formation of acrylamide on heating dried potato slices at 170°C for 70 m showed a rapid initial increase, followed by a broad maximum after 6–10 m of reaction time, and, subsequently, with a slow decline of the curve. However, the amounts of acrylamide in the headspace were very low compared to the Maillard model systems [101], which is most likely due to the lower concentration of the precursors (reducing sugars and asparagine), but also to the high polarity and low volatility of acrylamide. According to literature data [102], fresh potato contains about 1000 mg/kg of free asparagine. Taking into account the high water content of ca. 80% [103], an estimated 2.5 mg (19 μmol) of asparagine was available in 0.5 g dried potatoes for generating acrylamide. Despite the low precursor amounts in the experiment with potatoes, these data show that PTR-MS is sufficiently sensitive to monitor the formation of acrylamide under food processing conditions.

In summary, mainly two methods of analysis (LC–MS or GCMS) are used by laboratories worldwide, and based on the early indications of proficiency tests, it is difficult to say that one is more reliable than the other. Limits of quantification range from 30 to 50 μg/kg for LC–MS down to 10–30 μg/kg for GC–MS. However, it is quite clear that for the analysis of acrylamide at <30 μg/kg level, GC–MS after bromination is the best approach. This approach has the advantage of adequate

sensitivity with multiple ion confirmation. A further advantage of this technique is that a relatively simple benchtop GC–MS can be employed for acrylamide analysis. Application of GC–MS/MS or coupling to a high-resolution MS would even further lower the detection limit of certain foods, approaching the range of 1–2 μg/kg [104].

13.7.2.4 LC–Time-of-Flight–MS/MS

Liquid chromatography–time-of-flight (TOF)–mass spectrometry has also been established as a valuable technique for the routine control of the wholesomeness of food. In this sense, TOF techniques can record an accurate full-scan spectrum throughout the acquisition range and have resulted as an excellent tool for the unequivocal target and nontarget identification and confirmation of food contaminants [105,106].

13.7.2.5 LC–Quadrupole Linear Ion Trap (QqLIT)–MS/MS

Recently introduced tandem mass spectrometers, having both features, such as quadrupole linear ion trap (QqLIT, LTQ or Q-trap), quadrupole time-of-flight (QqTOF), LTQ-Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS), and LTQ-Orbitrap, and so on, have allowed for the development of several new methods for acrylamide detection [107,108].

13.7.2.6 Capillary Zone Electrophoresis

A CZE method has been developed for the determination of acrylamide after derivatization with 2-mercaptobenzoic acid in foodstuffs products. The previously established derivatization procedure was improved by reducing several steps, allowing direct injection of AA derivative into the CZE system without the need to remove reagent excess. With this method, a LOD of 0.07 μg/mL was obtained, which involves a 10-fold enhancement when compared with that obtained by MEEKC. Good linearity ($r^2 > 0.999$) and run-to-run and day-today precisions (RSD lower than 5.8 and 11.2%, respectively) were achieved. The addition of a derivatization step did not negatively affect the precision of the established methodology. The results obtained show that the method can be used for quantitative purposes in foodstuffs products. The application of CZE for the determination of acrylamide in french fries, breakfast cereals, and biscuits, using external calibration and standard addition methods, has been demonstrated. As a consequence, external calibration can be successfully selected as a good strategy for the determination of AA in a large variety of samples. This less time-consuming method becomes especially appropriate for a routine screening of foodstuffs with a high risk of AA contamination due to their thermal processing [109].

13.8 UPLC–MS ANALYSIS OF ACRYLAMIDE IN FOOD SAMPLES

In today's global marketplace, the safety and quality of food products are of growing concern for consumers and governments, and analytical information, including surveillance data for both recognized and newly identified contaminants, is also essential. However, information about their occurrence in food is still (very) limited [110]. Against this background, LC–MS, traditionally an important part of the medical

TABLE 13.1
Common Acrylamide Contaminants Present in a Variety of Food Determined by LC–MS

Technique	Matrix	Extraction Method	LOQ (ng/g)	Comments	Ref.
HPLC–MS	Food stuffs (potato chips and biscuit)	Acetic acid after Carrez 1 and Carrez 2 solutions	5.0	Confirmatory and quantitative. Study the formation of acrylamide during cooking	[116]
LC–MS	Food stuffs (beef, chicken, biscuits, etc.)	Hexane and filtration through 0.45_m syringe filter	–	Confirmatory and quantitative. Study the formation of acrylamide during cooking	[116]
LC–QqQ–MS/MS	Roasted chestnuts and chestnut-based foods	Water and cleaned with multimode ENV + ® SPE and eluted with methanol	4–9	Confirmatory, quantitative, and study formation of acrylamide during roasting	[117]
LC–Qq-IT–MS/MS or LC–QqQ–MS/MS	Spanish products, potato chips, pastry products, sweet fritters (“churros”), Spanish omelet	Homogenized with water, clean up with Strata-XC SPE and a ENV+ SPE and elution with MeOH:H_2O (60:40)	2–6	Confirmatory and quantitative	[118]
LC–QqQ–MS/MS	Potato, coffee, cereals	Homogenized with water, clean up with ENV+ and elution with methanol	0.5	Confirmatory and quantitative	[119]
LC–QqQ–MS/MS	Chinese traditional carbohydrate-rich foods	Ethyl acetate clean up with SPE with Bond Elut Accutat mixed mode SPE column consisting of a strong cation and strong anion exchanges into one bed and analyte elution with methanol	4	Confirmatory, quantitative $^{13}C_3$-labeled acrylamide internal standard solution	[120]
LC–QqQ–MS/MS	Processes food (rice, bread, corn chips, potato chips)	C18 SPE and analyte elution with water	2	Confirmatory and quantitative	[121]
LC–APCI–MS	Potato and cereal-based foods	0.01 mM acetic acid in a vortex mixer and clean up with Oasis MCX SPE	–	Confirmatory and quantitative	[122]

laboratory, found a growing market from a new application—food safety testing [111]. LC–MS is particularly suited for the analysis of acrylamide in food samples, since it provides a large amount of information about a complex mixture, enabling the screening, confirmation, and quantitation of hundreds of components with one analysis [112,113]. These instruments are used to test other food safety issues, such as food authenticity and labeling accuracy [114,115]. Table 13.1 illustrates examples regarding major acrylamide contaminants in food determined by LC–MS. Triple quadrupole (QqQ) MS has been the cornerstone technique for screening and confirmation of food contaminants and residues [123]. The majority of current liquid chromatography–tandem mass spectrometry (LC–MS/MS)-based contaminants and residue analysis relies on the high sensitivity and selectivity of the selected reaction monitoring (SRM) mode of QqQ–MS/MS [124,125]. Modern instruments produce high signal-to-noise (S/N) ratios even when relying on short SRM dwell times and can be properly combined with ultra-performance liquid chromatography (UPLC). Conventional LC with C18 columns plays a dominant role for the determination of foodstuff, whereas UPLC along with sub-2 μm particle C18 columns reduces run time and improves sensitivity. Comparative analyses of acrylamide in potato chip extracts, which were analyzed in two alternative LC–MS/MS systems, documented the potential of UPLC to replace "classic" LC separation strategy [126]. The data generated in optimized systems employing either Acquity UPLC (Waters) or Alliance LC (Waters) hyphenated with Quattro Premier (Waters) MS detector

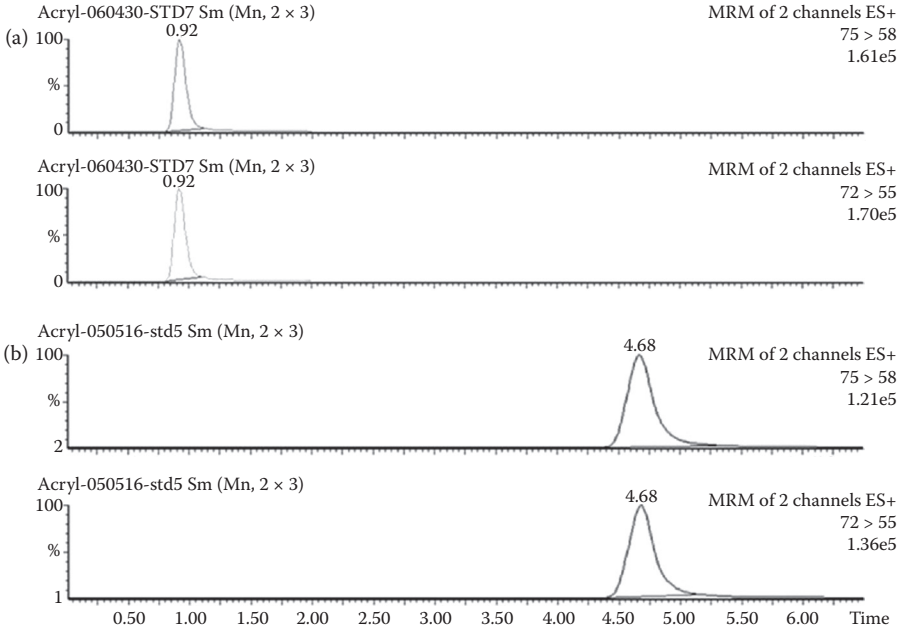

FIGURE 13.3 Comparison study on the determination of acrylamide by (a) UPLC–MS/MS and (b) HPLC–MS/MS. (Adapted from Zhang, Y. et al. 2007. *J. Chromatogr. A* 1142:194–198. With permission.)

(tandem quadrupole), showed that (i) the number of theoretical plates was for most analytes higher in a system employing LC, and with lower variability compared to UPLC, (ii) the values of height equivalent to the theoretical plate obtained in UPLC were mostly higher, however, their variability was also rather high, (iii) the analysis time in the system employing UPLC was reduced by more than 50% with similar analytical output, and (iv) UPLC provided significantly improved S/N followed by decreased LOQs for the majority of compounds [127]. Chromatogram illustrating LC–MS analysis of acrylamide in potato chips is shown in Figure 13.3. The reduced analysis time consequently resulted in significantly lower consumption of organic solvents [128].

13.9 CONCLUSION

This chapter emphasized number of recent techniques for the analysis of acrylamide in food. Despite presence of several analytical methods for acrylamide determination, these techniques still require further development. After review of this chapter, one can conclude that UPLC/MS/MS represents a powerful technique with high-resolution separation of acrylamide, short run time, and high specificity that excludes the matrix interferences of the extract.

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