

Challenges and trends in the determination of selected chemical contaminants and allergens in food

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Abstract This article covers challenges and trends in the determination of some major food chemical contaminants and allergens, which—among others—are being monitored by Health Canada's Food Directorate and for which background levels in food and human exposure are being analyzed and calculated. Eleven different contaminants/contaminant groups and allergens have been selected for detailed discussion in this paper. They occur in foods as a result of: use as a food additive or ingredient; processing-induced reactions; food packaging migration; deliberate adulteration; and/or presence as a chemical contaminant or natural toxin in the environment. Examples include acrylamide as a food-processing-induced contaminant, bisphenol A as a food packaging-derived chemical, melamine and related compounds as food adulter-

ants and persistent organic pollutants, and perchlorate as an environmental contaminant. Ochratoxin A, fumonisins, and paralytic shellfish poisoning toxins are examples of naturally occurring toxins whereas sulfites, peanuts, and milk exemplify common allergenic food additives/ingredients. To deal with the increasing number of sample matrices and analytes of interest, two analytical approaches have become increasingly prevalent. The first has been the development of rapid screening methods for a variety of analytes based on immunochemical techniques, utilizing ELISA or surface plasmon resonance technology. The second is the development of highly sophisticated multi-analyte methods based on liquid chromatography coupled with multiple-stage mass spectrometry for identification and simultaneous quantification of a wide range of contaminants, often with much less requirement for tedious cleanup procedures. Whereas rapid screening methods enable testing of large numbers of samples, the multi analyte mass spectrometric methods enable full quantification with confirmation of the analytes of interest. Both approaches are useful when gathering surveillance data to determine occurrence and background levels of both recognized and newly identified contaminants in foods in order to estimate human daily intake for health risk assessment.

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Introduction

Food safety has become of increasing concern for consumers, governments, and producers as a result of the global

marketplace where foods are produced and distributed throughout the world and also because of an increase in public awareness of health and quality. Several worldwide incidents related to chemical contaminants in food have also attracted much media attention. Trace levels of chemical contaminants in foods can originate from natural sources (for example mycotoxins [1, 2] and phycotoxins [3, 4]), environmental contamination (for example PCB [5], dioxin-like compounds [6], pesticide residues [7], and perchlorate [8]), migration of chemicals from packaging materials (for example phthalates and bisphenol-A) [9], veterinary drug residues [10], by-products from food processing (for example acrylamide [11, 12]), and other intentional and unintentional adulteration (for example melamine [13] in milk products and ethyl carbamate in wine [14]). Furthermore, instances of emerging contaminants, for example perfluorinated organic compounds [15] entering the food supply are also increasing. The occurrence of allergens in food is another important topic in food safety, resulting in the need to verify that the food labelling information on the product is accurate and enables consumers to make informed choices about their diet.

In Canada, Health Canada's Food Directorate is the federal health authority responsible for establishing policies, setting standards, and providing advice and information on the safety and nutritional value of foods available for sale. An important part of the Food Directorate's mission is to ensure that human exposure to chemical contaminants and residues in food, whether from natural or man-made sources is kept to a minimum and that it is not detrimental to Canadians' health. To support its food safety standard-setting activities and to provide information about the background levels of these chemicals and of possible hidden or undeclared allergens in foods, it is essential for Health Canada's Food Directorate to analyse the food samples using sensitive, fast, and accurate analytical methods. This requirement, in conjunction with the increasing number of sample matrices and analytes of interest, has led to the development of both rapid screening methods for a variety of analytes, mostly based on immunochemical techniques, and of highly sophisticated multi-analyte methods based on liquid chromatography coupled with multiple-stage mass spectrometry, to enable identification and simultaneous quantification of a wide range of contaminants. The Food Research Division of the Bureau of Chemical Safety within Health Canada's Food Directorate maintains an active research program studying the distribution, sources, and potential effects of food processing on chemicals in food, conducts major national surveys, for example the Canadian Total Diet Study, in support of science-based risk management approaches, when warranted, and engages in a range of research activity related to the determination and characterization of chemical contaminants and allergens in food.

This article covers challenges and trends in the determination of selected food allergens and chemical contaminants, which—among others—are being monitored by Health Canada's Food Directorate and for which background levels in food and the human exposure to these chemicals are being analyzed and calculated.

Eleven different chemical contaminants/contaminant groups and allergens have been selected for detailed discussion in this paper. They are all introduced into foods at some point in the food supply continuum and are listed below:

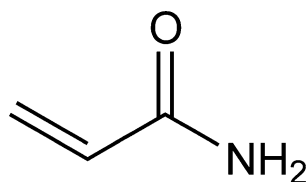
- acrylamide, as an example of a food-processing-induced contaminant
- bisphenol A, as an example of a food packaging-derived chemical
- melamine and related compounds, as examples of food adulteration
- persistent organic pollutants and perchlorate, as examples of environmental contaminants in food
- ochratoxin A and fumonisins, as examples of mycotoxins, i.e. natural toxins produced by fungi
- paralytic shellfish poisoning toxins, cylindrospermopsin, and β -methylamino-L-alanine, as examples of phycotoxins, i.e. natural toxins produced by algae
- sulfites, as examples of allergenic food additives
- peanuts, milk, and other allergenic food ingredients which exemplify the need for accurate food labelling

Acrylamide

Acrylamide is currently the most actively investigated chemical among process-induced food toxicants. The discovery of acrylamide in heat-treated foods [16] led to many subsequent investigations related to its formation and presence in foodstuffs [17, 18]. The intensity of this research is because acrylamide is a rodent carcinogen [19], a human neurotoxin [20] and is classified as a probable human carcinogen [21].

Owing to the ubiquity of asparagine and sugars—the most common reactants required for formation of acrylamide—acrylamide was detected in many heat-processed foods at levels ranging from $\text{sub-}\mu\text{g kg}^{-1}$ to over $5000 \mu\text{g kg}^{-1}$. The potential human health risk of acrylamide is still being assessed and, although a maximum allowable concentration in foods has not been promulgated, efforts to minimize its formation are in progress, as also is continuous monitoring of its concentration in the relevant food categories [22].

Acrylamide is a moderately reactive, very polar, α,β -unsaturated amide (Fig. 1). It is relatively stable at room temperature in typical food matrices, except for roast and ground coffee, in which significant degradation has been observed over time during storage [23]. Exposure of food

Fig. 1 Chemical structure of acrylamide

samples to elevated temperatures within a laboratory environment should, however, be avoided and samples should be stored at temperatures $< -15^{\circ}\text{C}$ [24, 25].

A plethora of analytical methods has been developed to measure acrylamide in foods over the past decade. With the exception of a few methods utilizing non-MS detection, most methods employ isotope dilution with $^{13}\text{C}_3$ -acrylamide or $^2\text{H}_3$ -acrylamide with GC-MS and LC-MS or LC-MS-MS detection [26–28]. The use of a $^{13}\text{C}_3$ labelled isotope is beneficial when using bromination/dehydrobromination derivatization procedures [27], and when the deuterium isotope effect [29] could affect the accuracy of the analysis (in cases when only selected column fractions are collected during cleanup).

Owing to the extreme solubility of acrylamide in water ($\sim 200\text{ g}/100\text{ mL}$), water is the solvent most widely used for its initial extraction. The main disadvantage of aqueous extraction is the formation of severe, viscous colloids with starchy foods. These colloids can be completely removed by ultrafiltration with a 5-kDa molecular filter [30]; however, care should be exercised because some batches of filters were found to harbour low-ng levels of acrylamide [31]. Alternative extraction solvents are methanol or *n*-propanol, because formation of colloids is suppressed [26, 32, 33]. When using alcohols, some sample matrices must be pre-swelled with water to afford effective extraction. The cleanup which follows initial extraction varies with the method of detection. For GC-MS with derivatization with bromine, an additional cleanup step of brominated acrylamide on silica or Florisil is often needed, especially when detection limits lower than $10\text{ }\mu\text{g kg}^{-1}$ are required [27, 34]. An alternative matrix solid-phase dispersion/SPE step before bromination has been used for coffee samples [35]. This last study also

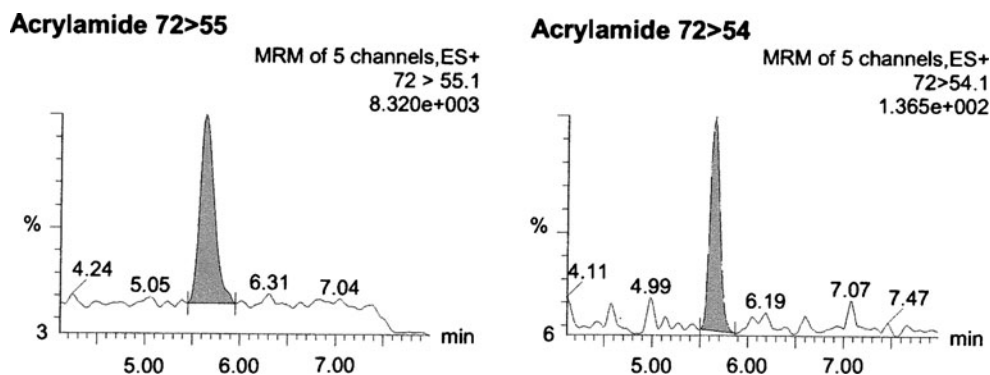
summarized current methods used for analysis of acrylamide in coffee.

For methods employing LC-MS or LC-MS-MS cleanup usually consists of sequential SPE using a variety of cartridges. The combination of Oasis HLB and Bond Elut Accucut has been used most frequently [36]; however, a detailed study of SPE sorbents reported a superior combination consisted of Isolute Multimode and ENV+cartridges [29]. Figure 2 shows the ESI(+)-LC-MS-MS selected reaction monitoring (SRM) chromatogram (also called multiple reaction monitoring (MRM) by some suppliers) at a contamination level of $110\text{ ng acrylamide g}^{-1}$ corn obtained from a regular corn chip sample after extraction and cleanup in accordance with Ref.[30]. As quantifier transition the protonated precursor ion ($\text{M}+\text{H}^+$) $\rightarrow m/z$ 55 was used.

Extraction of coffee and chocolate samples by pressurized fluid extraction using acetonitrile with concomitant in-situ filtration through Florisil afforded extracts that did not require any subsequent cleanup before LC-MS-MS analysis [37].

Chocolate, cocoa, and, especially, coffee, remain as matrices for which new and improved methods are still being developed with the objective of achieving greater robustness. It is often difficult to compare the quality of the data because chromatograms of samples with the concentration of acrylamide close to the detection limit and depicting data from the quantification and confirmation channels are seldom available. However, comparison of published chromatograms indicates that the cleanup methodology for LC-MS-MS described above (pressurized fluid extraction using acetonitrile with concomitant in-situ filtration through Florisil) is superior to the GC-MS method using a solid-phase dispersion/SPE step before bromination.

GC-MS analysis of acrylamide without derivatization might be affected by the formation of artefacts in the heated GC inlet from any co-extracted acrylamide precursors. This may be avoided by selecting extraction solvents such as *n*-propanol or acetonitrile which do not co-extract potential

Fig. 2 Acrylamide in a regular corn chip sample: ESI(+)-LC-MS-MS SRM chromatograms at a contamination level of $110\text{ ng acrylamide g}^{-1}$ corn. The protonated precursor ion ($\text{M}+\text{H}^+$) $\rightarrow m/z$ 55 was used as quantifier transition

precursors [38]. Most GC–MS methods for acrylamide are based on derivatization of the molecule by bromination, subsequent dehydrobromination with triethylamine (to improve the thermal stability of the derivative), and detection in EI mode. The most common bromination reagent is a bromine–water solution with HBr and KBr [28]; however, a safer KBr+KBrO₃ mixture has also been used [39].

Interlaboratory studies have been conducted in which the performance of a few analytical methods were compared [40, 41]. Although GC after derivatization can provide high-quality data down to 1–5 µg kg⁻¹ in some laboratories [27], the broader consensus was that LC–MS–MS is generally the superior method [42]. The advantage of LC–MS–based methods is the determination of acrylamide without prior derivatization and this could be one reason methods which use derivatization of acrylamide before LC–MS determination have not been more widely accepted [43].

The state-of-the-art in the extraction and LC–MS–MS determination of acrylamide has been described by Rosen et al. [29] and this methodology is able to achieve a limit of quantitation (LOQ) of 5 µg kg⁻¹. Further modifications for baby foods afforded an LOQ of 0.5–2 µg kg⁻¹ [31]. This method and other, related, methods use electrospray ionisation [30, 36, 44–47]. On the other hand several other methods use atmospheric pressure ionization [37, 48–50], but it seems that neither mode of ionization is clearly superior to the other in sensitivity or selectivity [51]. In recent studies, an analytical column which contains porous graphitic carbon (Hypercarb) was used as a stationary phase [50]. This phase has a larger capacity factor for acrylamide than any other phase on the market; however, because of its unique sorption characteristics, it is prone to contamination by co-extractives, which might be present in the final analytical extracts [47, 52]. It seems that cocoa and coffee are the most difficult sample matrices for LC–MS based methods. Unfortunately, the exact nature of the co-extractives for such samples is not known, so an empirical approach for cleanup of these matrices is necessary.

While analysis of acrylamide by traditional methods is maturing, development of immunoassays for acrylamide is progressing [53, 54]. So far, these immunoassay-based methods are still lacking ability to detect acrylamide at trace levels in a wide variety of food matrices. Other interesting developments include analysis of acrylamide and other analytes (reducing sugars and asparagine) in one analytical run [48].

Bisphenol A

Bisphenol A (BPA) is the common name for 2,2-(4,4'-dihydroxydiphenyl)propane (Fig. 3). It is used as a

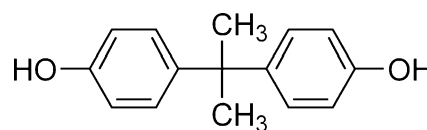


Fig. 3 Chemical structure of bisphenol A

monomer in the production of polycarbonate plastics and epoxy resins, and as an additive for elimination of surplus hydrochloric acid in the production of poly(vinyl chloride) (PVC) organosols. Polycarbonate is used in food-storage containers, for example water bottles and baby bottles, and epoxy resins and PVC organosols are used in the internal coating for food and beverage cans, and also in the internal coating on metal lids for foods in glass jars to protect the food from direct contact with metal. Residues of BPA in polycarbonate plastic containers and coatings can migrate into foods, especially at elevated temperatures; thus, humans are inevitably exposed to BPA, primarily through the diet. Because BPA is an endocrine disruptor that mimics the action of the hormone oestrogen, the specific migration limit for BPA in food or a food simulant has been set at 0.6 µg g⁻¹ in an EC directive in an amending document relating to plastic materials and articles intended to come into contact with foodstuffs [55]. The maximum acceptable dose and tolerable daily intake (TDI) for BPA were established at 50 µg kg⁻¹ body weight day⁻¹ by the United States Environmental Protection Agency [56] and the European Food Safety Authority [57], respectively, and Health Canada established the provisional tolerable daily intake for BPA at 25 µg kg⁻¹ body weight day⁻¹ [58].

Gas chromatography–mass spectrometry (GC–MS) and high-performance liquid chromatography (HPLC) with a variety of detectors are commonly used in analytical methods for the determination of BPA in foods [59–67]. Although method detection limits as low as 1 ng g⁻¹ have been reported [62, 64], methods with detection limits as high as 10 ng g⁻¹ have frequently been used in many of the surveys to determine BPA in foods. This will overestimate human exposure assessment if the detection limit (or half of the detection limit) is used for samples with BPA levels less than the detection limits. Thus, sensitive methods with detection limits as low as possible should be developed and used for determination of BPA in food samples to assess human exposure to BPA more accurately.

BPA was evaluated by the Government of Canada in 2008 and was concluded to be harmful to both human health and the environment at current levels of exposure. The Government of Canada has subsequently taken several regulatory actions, including adding BPA to Schedule 1 (the List of Toxic Substances) of the Canadian Environmental Protection Act, 1999 [68] and prohibiting the import, sale, and advertising of polycarbonate baby bottles containing BPA under the Hazardous Products Act [69]. As part of its

risk-management strategy, the Government of Canada has committed itself to monitoring levels of BPA in foods, particularly those intended for infants [70]. To support this initiative and generate exposure data for BPA in a variety of foods, a sensitive method based on solvent and solid-phase extraction followed by derivatization of BPA with acetic anhydride and GC–MS analysis was developed and validated. This method was used to investigate levels of BPA in canned liquid infant formula [71], powdered infant formula [72], canned soft drinks [73], baby foods in glass jars with metal lids [74], and canned food products [75]. A method based on headspace solid-phase microextraction and GC–MS analysis has also been developed [76] and used to investigate migration of BPA from polycarbonate baby and water bottles under severe conditions [77] and BPA levels in bottled water products [78].

Since BPA in foods is mainly a result of migration from can coatings, BPA is rarely measured in non-canned foods. Thus, the contribution from the non-canned foods to the overall dietary intake of BPA is unknown. There is also a lack of information on the effect of food processing, preparation, and cooking procedures on BPA levels in the final cooked foods. Because polycarbonate tools and containers and containers with epoxy coatings may be used during food preparation for cooking, BPA could be introduced into the final cooked foods, because of migration from polycarbonate and coatings. To address these issues, Health Canada is currently investigating the presence of BPA in the composite food samples from the Canadian total diet study. Health Canada is also investigating the presence of BPA in human milk samples as part of the MIREC study (Maternal–Infant Research on Environmental Chemicals) to update the exposure assessment for infants.

Because of the wide varieties and complex matrices of food samples, interferences are sometime unavoidable during analysis. For example, substantial interference with the quantification ion m/z 213 and the qualification ion m/z 228 from sample matrices were observed during analysis of all beer samples for BPA [79]. Figure 4 shows typical GC–MS ion chromatograms obtained from a beer sample with interferences and from a canned soup sample without interferences. However, if MS was not used as the detection method, false results for BPA would have been reported for all beer samples. Thus, MS detection and subsequent identification by use of ion ratios are essential in the analysis of foods for various contaminants to avoid reporting of false positive results.

Melamine and the related triazines ammeline, ammelide and cyanuric acid

Melamine (MEL) and the related triazines ammeline (AMN), ammelide (AMD), and cyanuric acid (CYA) are

small, polar, nitrogen-rich compounds (Fig. 5). MEL and CYA are used in the production of a variety of materials, including plastics, adhesives, cleansers, and flame retardants; AMN and AMD are not industrially produced but are often found as impurities in MEL and CYA feedstocks.

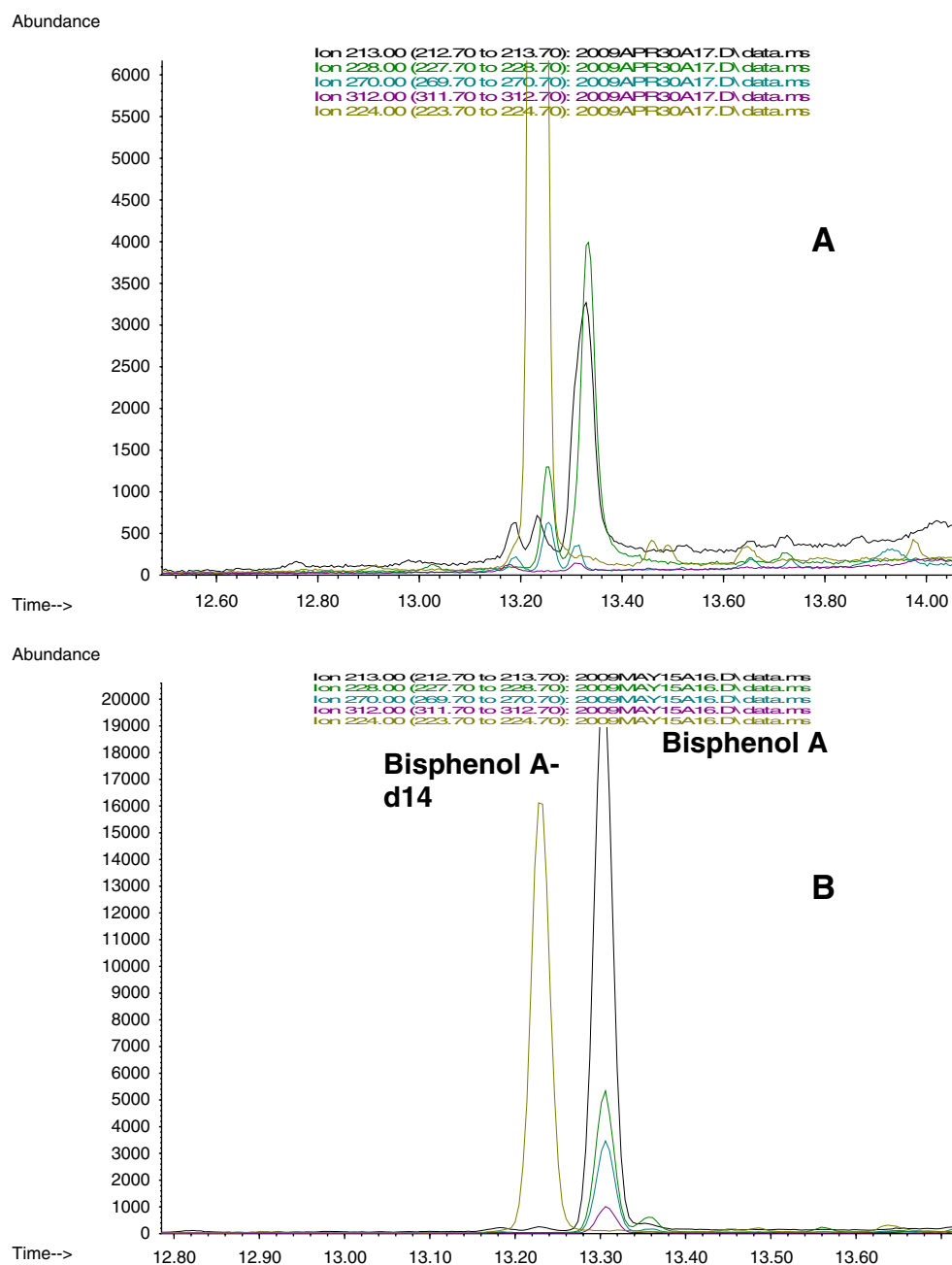
In two separate adulteration incidents, MEL and CYA were added to foods to boost their apparent protein content, thereby increasing their market value. In 2007, vegetable protein containing both MEL and CYA was sold by several Chinese companies. Its use in pet foods resulted in the deaths of a number of pets in Europe and North America, because of the formation of an MEL–CYA complex that precipitates in the kidneys and induces renal failure [80]. In 2008, thousands of Chinese infants became ill after drinking MEL-adulterated milk, with six confirmed deaths [81]. These incidents prompted many countries to examine the extent of contamination in their own food supply, to establish limits for these chemicals in foods, and to recall products which violated these limits.

Methods for the analysis of MEL and related compounds in foods for human consumption have been adapted from those used for animal feed [82]; there are also methods for analysis of the pesticide cyromazine, which degrades to MEL [83] and methods used to evaluate the migration of MEL from melamine–formaldehyde plastic food-contact materials [84]. Typically, homogenized food samples are extracted with a polar solvent, for example water, acetonitrile–water [82, 85] or methanol–water [86]. Aqueous extraction has been performed under acidic [87, 88] and basic [89, 90] conditions, which are thought to dissolve or inhibit the formation of the MEL–CYA complex. However, basic extraction and storage conditions, and other practices intended to prevent MEL–CYA complex formation (for example keeping MEL and CYA extracts separate) do not seem to be necessary unless a threshold concentration is exceeded [80, 91]. Others have reported that basic conditions promote hydrolysis of MEL into AMD and CYA [92].

After extraction, samples are centrifuged and/or filtered, then diluted for analysis. Methods requiring high sensitivity usually incorporate further cleanup by solid phase extraction (SPE). Mixed-mode reversed-phase and/or ion-exchange sorbents have frequently been used: cation-exchange phases are used to isolate MEL [82, 88] whereas CYA is isolated with anion-exchange sorbents [86]. AMN and AMD are partially retained by both anion and cation-exchange sorbents [85]. Graphitic carbon has also been used for sample cleanup [87].

Because MEL and its analogues are highly polar, they are usually chromatographed using normal-phase LC. Some groups have used ion-pair reagents to increase retention on reversed-phase columns [93], but most recent methods use hydrophilic interaction liquid chromatography (HILIC)

Fig. 4 GC–MS ion chromatograms: (A) canned beer sample (interferences with ions m/z 213 and 228), (B) canned soup sample (no interferences). Bisphenol A: m/z 213, 228, 270, 312 (in decreasing order); bisphenol A-d14: m/z 224

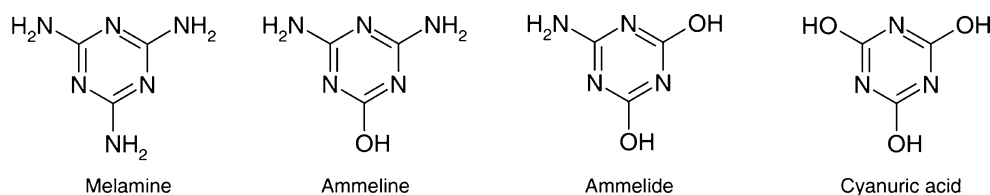


[86, 91]. Gas chromatography (GC) has also been used [94], but derivatization of the analytes is required.

The analyte is usually detected by single-stage or tandem mass spectrometry (MS). UV-based detection methods have also been used [89], but because these are less selective than MS methods, greater care must be taken to cleanup

samples and validate for each sample matrix. For LC–MS (–MS), MEL is analyzed in positive-ionization mode and CYA in negative-ionization mode: AMN and AMD have been analyzed in either mode [85, 90]. If there is sufficient chromatographic separation between compounds analyzed in positive mode and those analyzed in negative mode, the

Fig. 5 Chemical structures of melamine, ammeline, ammelide, and cyanuric acid



MS polarity can be switched during the run and all compounds can be analyzed in a single run. Figure 6 shows ESI(+)-LC-MS-MS chromatograms obtained from the quantification SRM transitions for an infant formula sample fortified at 0.05 mg kg^{-1} (CYA) and 0.01 mg kg^{-1} (MEL, AMN and AMD). CYA was analyzed in negative mode, AMN and AMD in positive mode.

For GC-MS(-MS), derivatized MEL analytes are analyzed in positive electron-impact mode [94]. ^{13}C and ^{15}N -labelled MEL, AMN, AMD and CYA have recently become commercially available for use as internal standards, to correct for the matrix effects that are frequently observed during food analysis [87].

MEL can also be determined using a variety of rapid methods, including enzyme-linked immunosorbent assay [95] and non-chromatographic mass spectrometric methods [96, 97]. Although currently limited to MEL, these techniques require little or no sample preparation, and many are of equivalent sensitivity to LC-MS-MS methods.

Further research is required to resolve conflicting information on the behaviour of some analytes, including conditions required for MEL-CYA complex formation, and transformation and/or hydrolysis of MEL into AMN, AMD, and CYA. These questions have important toxicological and analytical implications.

Although MEL is of low toxicity, the adulteration incidents in China have prompted many countries to re-evaluate the safety of their food supply. The intentional nature, economic motivation, and technical sophistication of the contamination, combined with a global food ingredient network, highlighted significant weaknesses in the food-control system. The World Health Organization has recommended the development and use of rapid, inexpensive, and reliable methods that directly

determine the protein content of food, rather than using nitrogen as a proxy [98]. This approach should be extended to other nutritional assays, particularly for foods for which the result of the assay has a direct result on the commodity price. Such testing would discourage the intentional addition of other novel adulterants, chosen specifically to evade the food control system [99].

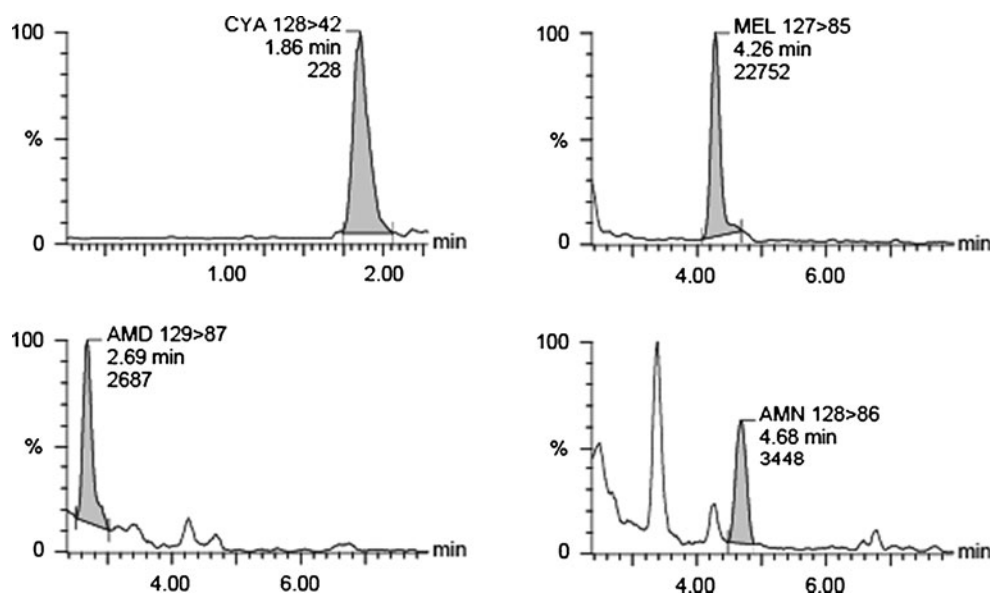
Persistent Organic Pollutants (POPs)

The general category of chemicals classified as persistent organic pollutants (POPs) are halogen-substituted organic compounds that have similar behaviour when released into the environment. POPs are known to persist in the environment, are subject to long-range transport and deposition, and are ubiquitous throughout the environment [100]. These compounds also are lipophilic and accumulate in the lipid tissues of living organisms causing levels to biomagnify through the food chain [101, 102].

Owing to the toxicity of POPs to humans and wildlife, international action was initiated via the Stockholm Convention on POPs to regulate their release into the environment. The compounds listed by the Convention may be identified for elimination of their production, restriction of their production and use, or for reduction of unintentional release. Originally, the Stockholm Convention identified twelve compounds or groups of compounds which included organochlorine insecticides, industrial chemicals, and industrial by-products (Table 1) [103]. Since the original POPs were identified, amendments to the list have occurred.

The toxicological effects associated with exposure to POPs include alteration of endocrine function, effects on

Fig. 6 ESI-LC-MS-MS chromatograms of the quantitation SRM transitions for an infant formula sample fortified at 0.05 mg kg^{-1} (CYA) and 0.01 mg kg^{-1} (MEL, AMN, and AMD). CYA was analyzed in negative mode, AMN and AMD in positive mode



the reproductive and immune systems, on liver function, and on neurobehavioral development and carcinogenesis [104–106]. In some cases, the POPs identified are classes of compounds (e.g., polychlorinated dibenzo-*p*-dioxins/ dibenzofurans (PCDD/Fs), and polychlorinated biphenyls (PCBs)) which have many individual structural analogues within each class. The individual analogues or congeners may cause similar toxic responses, although they have different potency, as in the case of 2,3,7,8 tetrachlorodibenzodioxin (TCDD) which is the most toxic PCDD/F. Because of this different toxicity, toxic equivalency factors (TEFs) relative to TCDD have been developed for the other congeners [107, 108]. The combination of TEF values and concentration data are generally used to establish the toxic equivalent (TEQ) concentration which are used for risk characterization and risk management activity [107, 108].

These lipophilic compounds (see chemical structures in Fig. 7) are present in foods of animal origin and it is generally accepted that diet is the main route of exposure of humans to these compounds. Food safety authorities, therefore, have been required to develop methods for their detection and quantification in foods [109]. POPs are amongst the

chemicals measured in The Canadian Total Diet Study (TDS), a dietary surveillance program for chemical contaminants that has been conducted by the Food Directorate for over 30 years. Results from the TDS, covering five cities over the period 1992 to 2008 are available online. Levels of these environmental contaminants in food are generally low (pg g^{-1} to ng g^{-1} fresh weight) compared with other chemicals in foods that are present because of direct addition, contact with food during processing, or are produced during food processing.

Although concentrations of POPs are not high, their presence in food and dietary supplements can result in court challenges, as has been observed recently in the US [110]. POP levels in fish are frequently higher than observed in other food types [111, 112]. PCDD/F levels in foods are generally low and may be reported on a TEQ basis (pg TEQ g^{-1} sample fresh weight) whereas other POPs (e.g. organochlorine insecticides, PCBs, PBDEs) are generally observed at higher concentrations (ng g^{-1} fresh weight) [112–115].

The determination of POPs generally involves multi-residue methods requiring extensive sample preparation before analysis. The methodology applied to both extrac-

Table 1 Persistent organic pollutants (POPs) as identified by the Stockholm Convention (Stockholm Convention Secretariat, 2010)

Compound (or class)	Type of chemical	Listed
Aldrin	Pesticide	Original POP
Chlordane	Pesticide	Original POP
Dichlorodiphenyltrichloroethane (DDT)	Pesticide	Original POP
Dieldrin	Pesticide	Original POP
Endrin	Pesticide	Original POP
Heptachlor	Pesticide	Original POP
Hexachlorobenzene	Pesticide	Original POP
Mirex	Pesticide	Original POP
Toxaphene	Pesticide	Original POP
Chlordecone	Pesticide	Added in 2009
α -Hexachlorocyclohexane	Pesticide/by-product	Added in 2009
β -Hexachlorocyclohexane	Pesticide/by-product	Added in 2009
Lindane (γ -hexachlorocyclohexane)	Pesticide	Added in 2009
Pentachlorobenzene	Pesticide/industrial chemical/by-product	Added in 2009
Hexachlorobenzene	Industrial chemical/by-product	Original POP
Polychlorinated biphenyls (PCBs) [209 congeners]	Industrial chemical/by-product	Original POP
Hexabromobiphenyl	Industrial chemical	Added in 2009
Hexabromodiphenyl ether	Industrial chemical	Added in 2009
Heptabromodiphenyl ether	Industrial chemical	Added in 2009
Perfluorooctane sulfonic acid and its salts	Industrial chemical	Added in 2009
Perfluorooctane sulfonyl fluoride	Industrial chemical	Added in 2009
Tetrabromodiphenyl ether	Industrial chemical	Added in 2009
Pentabromodiphenyl ether	Industrial chemical	Added in 2009
Polychlorinated dibenzo- <i>p</i> -dioxins and polychlorinated dibenzofurans (PCDD/Fs) [75 congeners]	Industrial chemical	Original POP

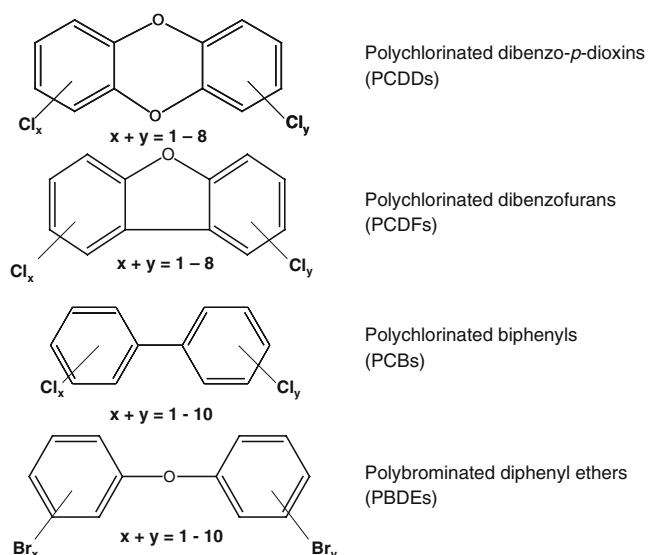


Fig. 7 The structures of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs)

tion and cleanup for removal of co-extracted compounds is critical. Analyte extraction generally involves homogenization with organic solvent [111, 116, 117]. Soxhlet extraction is also used for isolation of POPs from foods [114, 118]. Cleanup techniques applied to POPs may include acid digestion or gel-permeation chromatography to remove large-molecular-weight co-extractives (e.g., lipids), followed by column chromatography using a variety of sorbents including Florisil, silica gel, alumina, and activated carbon [111, 119, 120].

With the numerous steps required for cleanup of sample extracts, the potential for analyte loss is greater than for methods requiring no cleanup. The application of stable isotopes (e.g., ^{13}C) as surrogate standards enables analysts to correct for losses during sample preparation and is routinely used [121–123].

The analytical methods employed for some of the POPs include GC coupled to specific detectors (e.g., electron-capture and flame-photometric) [124, 125], although GC–MS is most frequently applied to the determination of POPs in foods [114, 119, 121, 126]. Because of the great number of POPs of interest and the low detection limits required (for, e.g., PCDD/Fs), their measurement requires the application of high-resolution mass spectrometry rather than low-resolution GC–MS. Examples of GC–MS chromatograms obtained from measurement of PCB, PCDD/F/coplanar PCB, and PBDE in egg yolk extracts is given in Fig. 8. In contrast, the more recently identified POPs (e.g., perfluorinated compounds) are analysed by use of LC–MS [127].

There has recently been increased interest in developing rapid methods for screening of contaminants in food, although quantitative analytical methods are required for

congener-specific determination. The dioxin-responsive chemical-activated luciferase expression assay (DR-CALUX) is used when total dioxin-like compound levels are required [128].

As the number of POPs continues to increase, the requirement for methods of analysis for these compounds will continue to grow. Brominated flame retardants (e.g., hexabromocyclododecane; HBCD) and organochlorine insecticides still in use (e.g., endosulfan) have been of recent interest.

Perchlorate

Perchlorate is an environmental contaminant that arises as a result of anthropogenic processes and from natural sources. Perchlorate can originate from the use of perchlorate salts in military and industrial products, for example solid rocket fuels, munitions, explosives and fireworks, road flares, air bag inflation systems, some fertilizers, and places near potash deposits [129–132]. In the human body, the perchlorate anion can inhibit uptake of iodide, an essential trace element, by the thyroid gland at the sodium iodide symporter (NIS) [133, 134]. Sustained inhibition of iodide uptake can impair thyroid function by reducing the amount of iodide stored in the thyroid and, consequently, that which is available for production of the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4), leading to hypothyroidism [135]. Perchlorate exposure is an especial risk to pregnant women and their foetuses, neonates, and people with iodine deficiency or with thyroid dysfunction.

Perchlorate contamination emerged as a public health concern in the late 1990s. This initiated the conduct of scientific studies and guideline development related to perchlorate in drinking water, plants, food, and other contamination sources in the United States and around the world. A number of recent studies have shown that plant species are able to absorb perchlorate from soil and irrigation water, and elevated levels of perchlorate have been found in food crops, including lettuce [136], fish [137], seaweed [138, 139], and some beverages [140]. Studies from different regions of the United States [141], Chile [142], Japan [143], and China [144] have also reported detectable levels of perchlorate in milk samples. In 2005, the United States Food and Drug Administration (US FDA) launched a study to determine perchlorate levels in total diet study (TDS) samples, and a mean value of $5.8 \mu\text{g L}^{-1}$ perchlorate was reported in 125 dairy milk samples [145]. The recent discovery of perchlorate in infant formula samples by the United States Centers for Disease Control and Prevention (US CDC) gave rise to intense public concern regarding infant exposure to perchlorate [146].

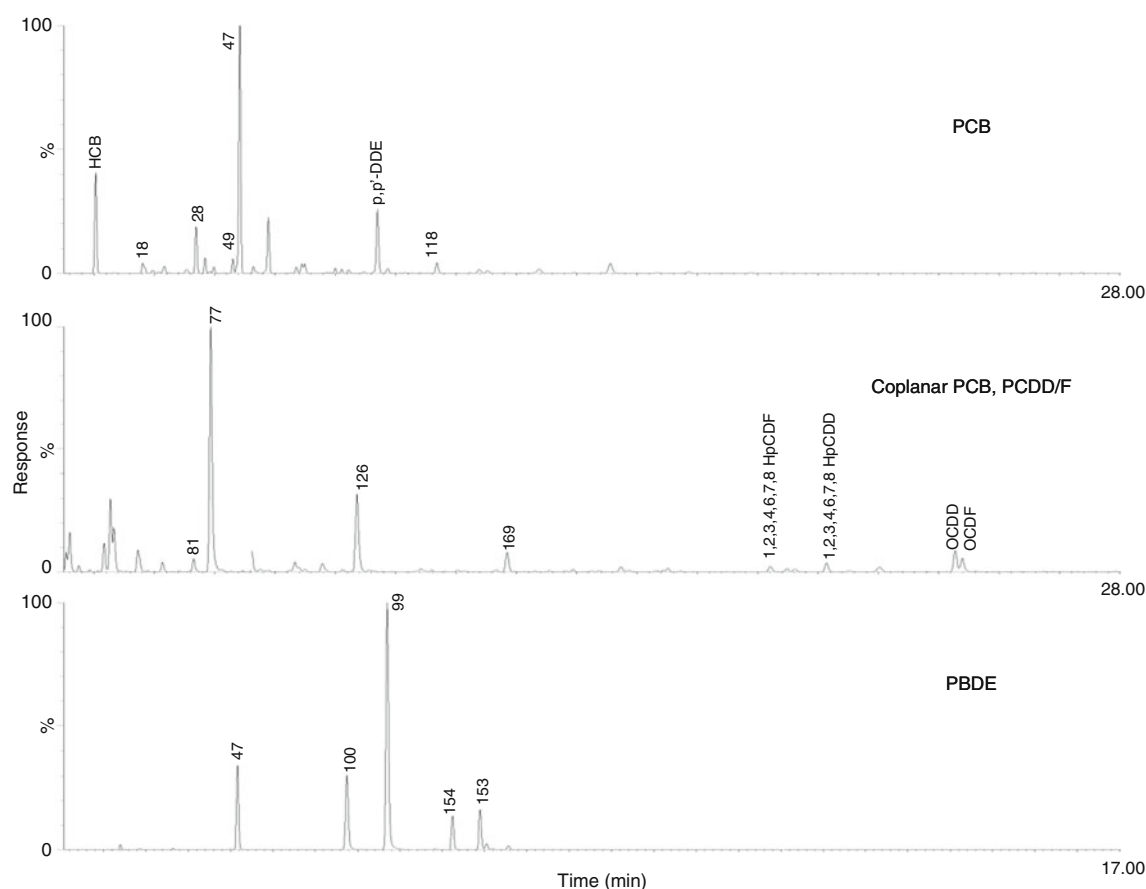


Fig. 8 GC–HRMS chromatograms obtained from measurement of PCB, PCDD/F/coplanar PCB and PBDE in egg yolk extracts

A variety of analytical methods has been developed for determination of perchlorate. Ion chromatography (IC) coupled with conductivity cell detection (CD) is the most common approach [147], and it is the first method approved by the Environmental Protection Agency (EPA) for determination of perchlorate in drinking water [148]. To determine trace levels of perchlorate in complex matrices, IC was coupled with tandem mass spectrometry (MS–MS), taking advantage of the high selectivity and sensitivity of mass spectrometry operated in SRM mode. IC–MS–MS was demonstrated to yield limits of detection (LOD) of 5–25 ng L^{−1} when determining perchlorate in a variety of matrices, including water, urine, amniotic fluid, wine, and food [140, 149–151]. Figure 9 shows a typical SRM mass chromatogram obtained from perchlorate analysis by ID IC–MS–MS.

In Canada, preliminary analysis of ground water and surface water samples revealed non-detectable or very low concentrations of perchlorate [152], indicating that perchlorate contamination of drinking water supplies is not expected to be a significant issue. Nevertheless, exposure to high concentrations of perchlorate from sources such as food has the potential to cause adverse health effects. In 2005, Health Canada began to investigate the occurrence of perchlorate in

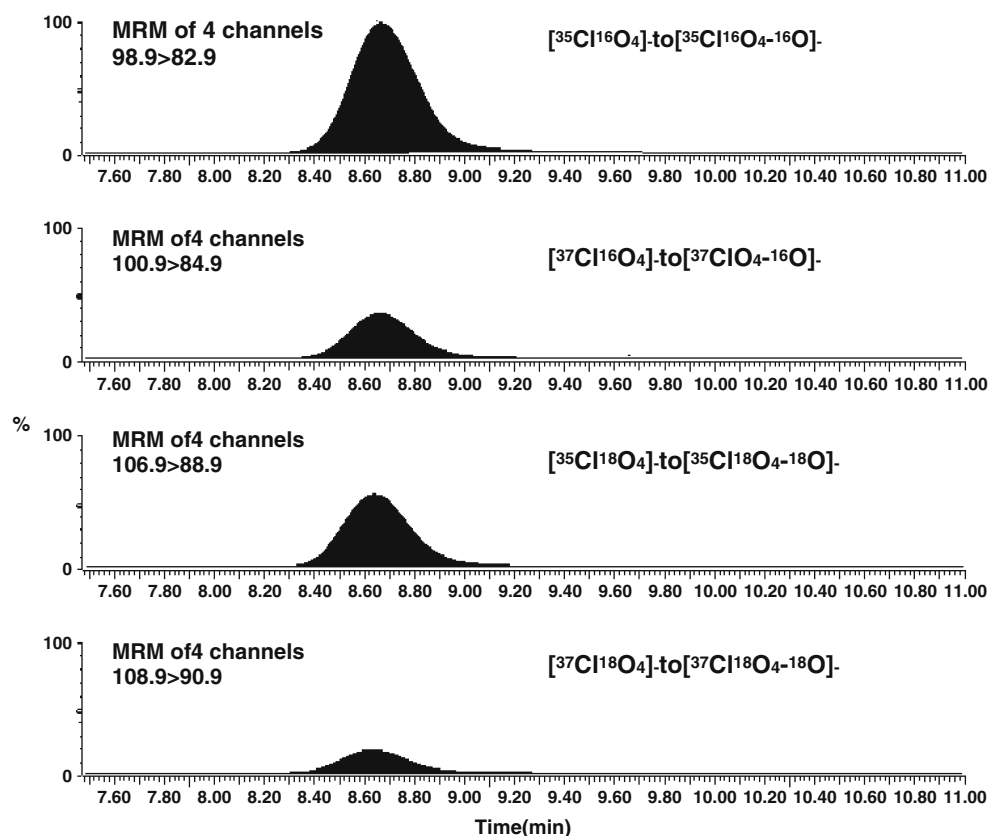
fruits and vegetables, dairy milk, and human milk. Table 2 shows the mean and range values for 150 fruit and vegetable samples collected in Ottawa, Canada [153].

As a continuous study of perchlorate surveillance, an improved ID IC–MS–MS method enabling accurate identification and quantification of perchlorate in infant formula was developed and 39 infant formula samples collected in Ottawa, Canada were analyzed by this method.

Future work is planned to determine the concentration of perchlorate and iodide in Canadian human milk in order to better assess dietary risk. Additionally, direct sampling of foods, for example infant foods, specific to high risk groups, is also planned.

Sulfites

Sulfites are sulfiting reagents that include SO₂, sulfite, sodium and potassium salts of metabisulfite, and bisulfite. They are widely used additives in the food industry and have several technological functions, including as an antioxidant, bleaching agent, flour-treatment agent, and preservative [154]. Sulfites have attracted increasing attention recently because of their allergy-like effect on

Fig. 9 Typical SRM mass chromatograms from perchlorate analysis by ID IC–MS–MS

hypersensitive individuals. The symptoms include respiratory sensitivity, triggering asthma, severe skin irritant, vitamin B and E deficiencies, bronchospasm, wheezing, chest tightness, flushing, hypotension, etc [155].

When sulfites are present at levels above 10 mg L^{-1} but are not indicated on the label, this may pose a potentially serious health risk to sulfite-sensitive consumers. As a result, the international regulations on residual sulfite levels in food in Europe and the US specify sulfites at concentrations $>10 \text{ ppm}$

(expressed as sulfur dioxide equivalent) must be declared on the label and also prohibits the use of sulfites in any product to be served or sold raw to consumers [156]. Although when added as a food additive, the presence of sulfite has long been required to be declared on the ingredient list of most processed foods, Health Canada has recently proposed that when sulfites are present in pre-packaged foods at levels exceeding 10 ppm they must also be declared on the labelling, to increase the level of protection and product choice for

Table 2 Mean and range of perchlorate concentration in fruit and vegetables

Food product	Sample number (<i>n</i>)	Mean $\pm$ SD ($\mu\text{g kg}^{-1}$)	Range ($\mu\text{g kg}^{-1}$)
Apple	24	0.48 ± 0.60	N.D.-1.60
Cantaloupe	18	56.1 ± 151	N.D.-536
Carrot	12	1.31 ± 1.26	N.D.-3.08
Grapes	12	27.7 ± 23.0	1.96-62.1
Honeydew	6	0.30 ± 0.33	N.D.-0.66
Lettuce	18	11.5 ± 14.2	1.27-46.7
Orange	12	0.50 ± 0.82	N.D.-2.89
Potato	24	N.D.	N.D.
Spinach	6	133 ± 24.9	99.4-175
Tomato	18	0.30 ± 0.47	N.D.-1.38

sulfite-sensitive individuals [157]. However, sulfites occurring naturally in foods would be exempt from the label declaration.

A variety of methods are available to quantify sulfites in foods. The most widely used is the optimized Monier–Williams (M–W) method [158]. The method converts free and reproducible amounts of bound oxosulfur(IV) anions in foods to sulfur dioxide, which is distilled and oxidized to sulfuric acid in a hydrogen peroxide trap. The acid is then determined by titration. Alternative methods include enzymatic [159], LC [160], differential pulse polarographic [161], capillary electrophoresis [162], and flow-injection techniques [163, 164]. The discrepancies in sulfite content amongst methods, and the potential for natural sulfur-containing compounds (besides sulfur dioxide) to contribute to overestimation with the M–W assay, bring into question the actual sulfite content of some samples. Therefore, a more accurate method that is able to differentiate between added sulfite and matrix-derived sulfite must be developed, and accurate information on sulfur speciation at low detection limits is becoming an important component of health risk assessment.

Challenges in developing a sensitive and specific sulfite detection method are twofold: first, sensitive monitoring of low-level sulfite is difficult owing to the effect of complex matrix and low signal response of sulfite in previously used techniques. Second, it is difficult to analyze the mixtures of sulfur species, because they are unstable and participate in complex equilibria with other sulfur compounds formed by their auto-redox reactions [165].

Ion chromatography (IC) has been the favoured separation technique for sulfur species, with electrochemical detection and spectrophotometric detection being widely used [166–168]. The inductively coupled plasma mass spectrometer (ICP–MS) is a more robust detector with very high selectivity and sensitivity. Health Canada's Food Research Division has developed an IC–ICP–MS method for determination of sulfur species in order to achieve a substantial reduction of the detection limit of sulfite, and species-specific detection [169]. An ICP–MS has been used as an element-specific detector for sulfide, sulfite, sulfate, and thiosulfate after their separation on a Dionex IonPac AS17 column. The ^{32}S species were detected as SO at m/z 48 with the ICP–MS tuned to high oxide ion production. The stability of sulfite, metabisulfite, and bisulfite in different stabilizers was also studied. Preliminary studies on wine and preserved vegetable samples gave satisfactory results. Figure 10 shows a typical chromatogram obtained from a standard solution containing four sulfur species and spiked and unspiked pickled vegetable samples.

Future studies will focus on extension of the IC–ICP–MS method to a greater variety of food matrices including wine or other alcoholic beverages labelled with “no sulfites added” [170]. In addition, matrix-independent accuracy can

be achieved by use of the isotope dilution (ID) techniques, which was demonstrated by incorporating ID into GC–ICP–MS systems in which detection limits in the range of $0.5\text{--}10\text{ ng g}^{-1}$ sulfur species could be achieved [171]. The use of matrix-independent detection and calibration methods could also possibly simplify quantification of sulfur species and improve the accuracy of results for natural sulfur-containing foods, for example onions, garlic [172], broccoli, and soy products [173].

Mycotoxins: ochratoxin A and fumonisins

Recent advances in the determination of mycotoxins in foods have been well reviewed by Shephard et al. [174], who covered aflatoxins, *Alternaria* toxins, cyclopiazonic acid, fumonisins, ochratoxins, patulin, trichothecenes, and zearalenone. A recent review by Maragos and Busman of methods for analysis of mycotoxins [175] stresses the need for methods with increased throughput. Ochratoxin A and fumonisins are mycotoxins of concern selected below for further discussion.

Ochratoxin A (OTA, L-phenylalaninylcarbonyl-5-chloro-8-hydroxy-3,4-dihydro-3-*R*-methylisocoumarin; Fig. 11) is a mycotoxin formed by some species of *Aspergillus* and *Penicillium* [176, 177]. It is carcinogenic, nephrotoxic, teratogenic, immunotoxic, and hepatotoxic [178] and has been classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans (group 2B) [179]. OTA is found in grain and many other kinds of foodstuffs, notably including wine, beer, spices, dried vine fruits, botanicals, liquorice, coffee, and cocoa.

New developments in analytical methods for OTA have been summarized by Shephard et al. [174]). LC with fluorescence detection (FLD) continues to be important. A simple, sensitive, and reproducible LC–FLD method for determination of OTA in wine was developed by Tafuri et al. [180]. It features direct injection of the wine on to a $5\text{-}\mu\text{m}$ monolithic C_{18} column, with no need for extraction or cleanup of the wine. A new hollow-fibre liquid micro-extraction method for determination of ochratoxin A (and T-2 toxin) in wine and beer has been reported; extraction was followed by UHPLC–MS–MS [181]. LC–MS–MS is now increasingly being used, particularly in multimycotoxin methods, which usually include OTA. Other types of method for OTA include use of biosensors, aptamers, solid-phase microextraction (SPME), immunochromatographic strips, and molecularly imprinted polymer (MIP) solid-phase extraction.

Fumonisins produced by *Fusarium verticillioides* (formerly known as *F. moniliforme*) which caused leucoencephalomalacia in horses were discovered in South Africa in 1988. Fumonisin B₁ (FB₁) is hepatocarcinogenic in rodents and a kidney carcinogen in rats [182, 183]. It is classified as a

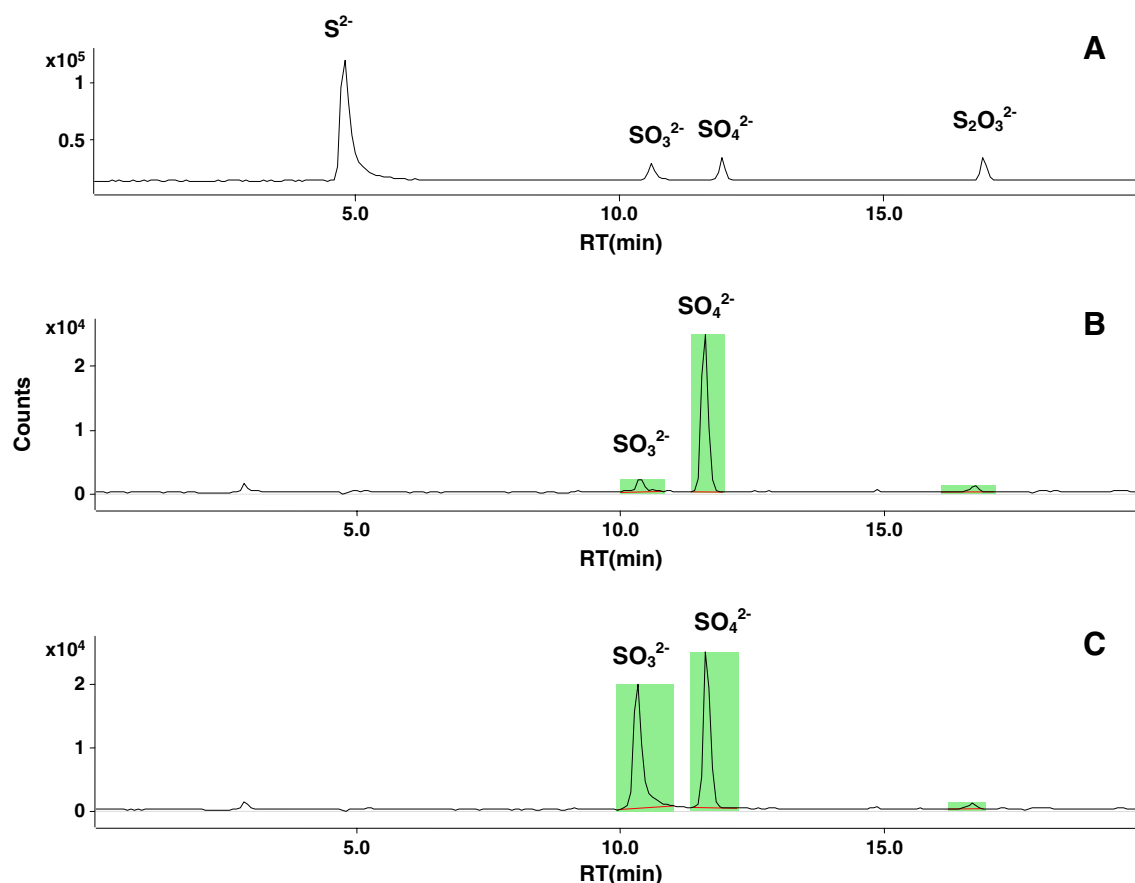


Fig. 10 (A) standard solution containing S^{2-} , SO_3^{2-} , SO_4^{2-} , and $S_2O_3^{2-}$, (B) pickled vegetable sample, (C) pickled vegetable sample spiked with SO_3^{2-}

possible human carcinogen (IARC Group 2B). The structures of FB_1 and two related metabolites are shown in Fig. 12.

Several other *Fusarium* spp., *Alternaria alternata* f. sp. *lycopersici* and, most recently, *Aspergillus niger* [184] are also known producers of fumonisins. FB_1 is the most commonly found fumonisin, mainly in corn (maize) and corn foods, and also in beer, rice, sorghum, triticale, cowpea seeds, beans (navy, mung, adzuki), milk, and asparagus. Now fumonisins have been found in additional grain foods/feed, including barley, wheat, millet, and oats, and in other types of food, including garlic and onion powder, turmeric, tea, coffee beans, medicinal plants, soy products, peanuts, figs, buckwheat, and wine. The widespread occurrence of FB_2 in the last of these [185] will probably prove a topic of interest comparable with that of ochratoxin A in wine. Also, *A. niger* contaminates many foodstuffs, for example cereals, peanuts, coffee beans, and grapes.

Newly discovered fumonisins that have recently been detected by LC–MS–MS in *F. verticillioides* cultures are the FBX series comprising 12 compounds with the tricarballic acid moiety replaced by other carboxylic acids (for example *cis*-aconitic acid, oxalysuccinic acid, and

oxalylfumaric acid), the FD series with fewer carbon atoms in the backbone and several others, including FB_5 and FBK_1 [186]. FB_6 is a new fumonisin found in cultures of *Aspergillus niger*, which is mainly a producer of FB_2 and FB_4 [185]. The sensitivity of LC–MS–MS for fumonisin determination is illustrated by the mean FB_1 contamination level of $0.26 \mu\text{g L}^{-1}$ found in commercial milk samples in Italy [187]. Also, with the wide range of different fumonisins and food matrices of concern, it is apparent that LC–MS–MS is now the procedure of choice for their analysis [174, 188, 189]; fumonisins are frequently included in multimycotoxin determination by this procedure.

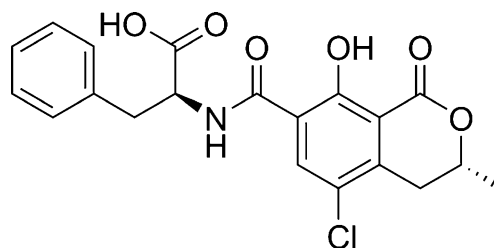
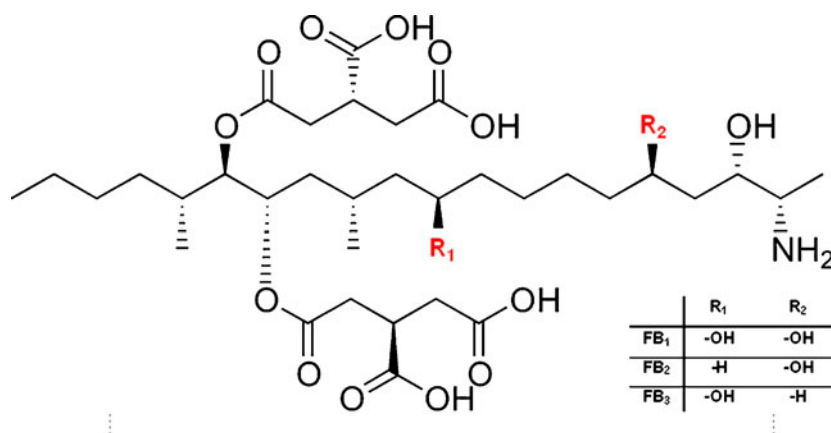


Fig. 11 Chemical structure of ochratoxin A

Fig. 12 The structures of FB₁ and two related metabolites



Other recent analytical procedures are lateral flow tests—membrane-based colloidal gold immunoassays for rapid detection of fumonisins B₁, B₂, and B₃. Lateral flow tests are applicable to analysis of corn, rice, and other commodities [190, 191] and are available commercially. MIP solid-phase extraction has been applied to pepper, rice, and corn flakes [192] and LC–fluorescence using post-column derivatization with *o*-phthaldialdehyde (OPA) and *N,N*-dimethyl-2-mercaptoethylamine [193] has also been used.

“Hidden (masked) fumonisins” are of current interest because they are not detected by the usual analytical methods. The issue of terminology has been discussed by Dall’Asta et al. [194]. One type is non-covalently bound and is an associative interaction of fumonisins with matrix constituents. These hidden fumonisins can affect determination of free fumonisins when immunoaffinity columns are used for cleanup [195]. The term “bound fumonisins” applies to fumonisins covalently bound to proteins (PBFB), starch, and sugars after heat processing. Total bound fumonisin FB₁ (TBFB₁) is determined indirectly either by subtracting free FB₁ and HFB₁ found before hydrolysis from HFB₁ found after hydrolysis or by washing away free fumonisins with extraction solvent before the hydrolysis. There can be more bound fumonisins than free FB₁ in heat processed naturally contaminated foods [196, 197]. In vitro digestion has been used to estimate the bioaccessibility of TBFB₁ from naturally contaminated solvent-washed corn flakes as 37–64% [198]. So bound fumonisins should be regarded when evaluating exposure to fumonisins in a risk assessment.

Phycotoxins: paralytic shellfish poisoning toxins (PSTs), cylindrospermopsin (CYN) and β-methylamino-L-alanine (BMAA)

Updates on progress in the validation of analytical methods for marine and freshwater algal toxins are provided annually by James Hungerford [199]. Three of the many known phycotoxins are briefly discussed below.

Paralytic shellfish poisoning toxins (PSTs), for example saxitoxins, gonyautoxins, and C-toxins (Fig. 13), are produced by marine dinoflagellates, e.g. *Alexandrium*, *Gymnodinium*, and *Pyrodinium*. They have now been found in freshwater cyanobacteria, e.g. *Cylindrospermopsis raciborski*, *Anabaena* spp., and *Aphanizomenon* spp. Official control of paralytic shellfish toxins (PSTs) remains a subject of major concern to regulators [200]; there is debate over whether the mouse bioassay, LC with pre-column oxidation (Fig. 14), or LC with post-column oxidation should be used for shellfish analysis. LC–MS based methods can also be used and may eventually supersede the oxidation and/or fluorescence methods [201]. Other approaches are being studied; two of these are based on the sodium channel, which is the target of the toxins, and another is use of antibodies [28]. Sensitive methods incorporating solid phase microextraction (SPME) have been used for analysis of drinking water [202].

Cylindrospermopsin (CYN) (Fig. 15) is one of three classes of cyanotoxins on the US Environmental Protection Agency Contaminant Candidate List III and, together with microcystins and anatoxin-a, may require regulation in drinking water [203]. A “guideline value” of 1 µg L⁻¹ has been proposed [204]. CYN has been found to be genotoxic

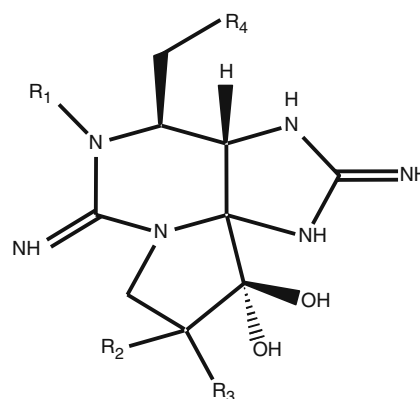
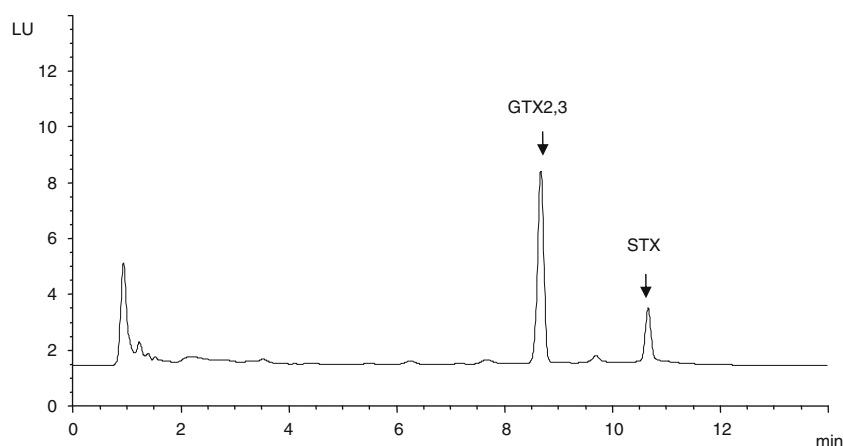


Fig. 13 Basic structure of PSP toxins

Fig. 14 Chromatogram obtained from naturally contaminated lobster tomalley sample containing GTX 2,3 (224 µg/100g) and STX (40 µg/100g). Determination by LC–FL after pre-column peroxide oxidation



in in-vitro systems and carcinogenic in mice [204]. Human poisoning (gastroenteritis) has occurred [204]. CYN is a metabolite of several other freshwater cyanobacteria belonging to the genera *Anabaena*, *Umezakia*, *Raphidiopsis*, and *Aphanizomenon* [204] and, more recently, *Lyngbya* [205]. CYN, originally regarded as a subtropical problem, is now occurring more frequently in European countries. Recent applications of LC–MS and LC–MS–MS for determination of CYN in lake or river water from, for example, the USA, Mexico, the Czech Republic, Italy, Germany, and Poland have been published [37, 203, 206–210]. Oehrle et al. [203] noted that single-ion monitoring would have resulted in a false positive for an Ohio River sample compared with tandem MS. LC–MS–MS was also recently used to find CYN in freshwater snails consumed by humans in Mexico [207]. An enzyme-linked immunosorbent assay (ELISA) is now commercially available [206]; however, overestimation compared with LC–MS was observed.

β-Methylamino-L-alanine (BMAA) (Fig. 16) is produced by some marine cyanobacteria (e.g. *Trichodesmium*) and by many other cyanobacteria, including *Nostoc* root symbionts [211, 212]. BMAA was detected in a seawater sample from a *Trichodesmium* bloom near Hawaii and was recently found in shrimps and other aquatic animals in Florida waters [213]. It has also been found (mainly

protein-bound) in cycad seeds, flying foxes, brain tissue of Alzheimer patients, and Chamorros in Guam who died of amyotrophic lateral sclerosis (ALS)/parkinsonism–dementia complex (PDC) [212]. BMAA is neurotoxic—it injures neurons in mice and induces motor system disease in monkeys. BMAA can be determined by LC of the fluorescent derivative formed with 9-fluorenylmethyl chloroformate (FMOC) or 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) or by LC–MS–MS [214]. Analysis of cyanobacteria and other biological samples for BMAA is complicated because it often occurs partly as a protein-bound form, so the sample must be hydrolysed to give additional free BMAA [212]. Recently, an LC–FLD method has been developed which is applicable to the analysis of blue green algae (BGA) food supplements, freshwater fish, and bottled water [215]. BMAA has been found in a Peruvian food, *Nostoc commune* [216], and recently in *Nostoc flagelliforme* used as the food item *fa cai* [217]; BMAA was not detected in a sample of genuine *fa cai* sold in Canada but the isomeric 2,4-diaminobutyric acid (DAB) was found; this is also a neurotoxin known to occur

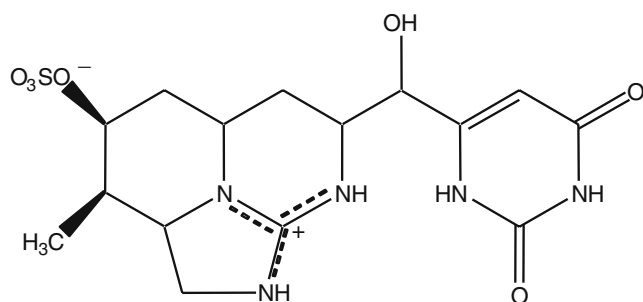
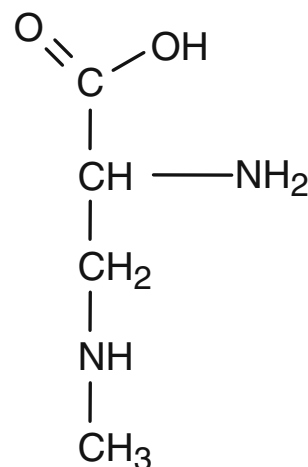


Fig. 15 Chemical structure of cylindrospermopsin

Fig. 16 Chemical structure of β-methylamino-L-alanine (BMAA)



in numerous cyanobacterial samples, as determined by LC–MS–MS [218, 219].

Allergens

For specific individuals with food allergies or sensitivity to gluten, consuming foods containing an allergen may be of concern. Recent estimates of numbers suffering from food allergies are approximately 4% for adults and 6% for children and have been increasing [220]. Some of these allergies will be outgrown, but the most severe typically remain throughout adulthood. Individuals with celiac disease number around 1% of the population, but this is thought to be a conservative estimate as many individuals go undiagnosed [221]. Most allergenic reactions occur to one of the nine priority allergens in Canada—peanuts, tree nuts, milk, egg, fish and/or crustaceans, sesame, soy, wheat, and sulfites. In both types of food sensitivity the only solution is complete avoidance of the food to reduce the risk of a reaction. Unfortunately, cross-contamination of other foods with allergens during processing remains a possibility and thus ongoing surveillance of products to verify labelling accuracy is a necessity. Analytical methods are needed to determine the validity of allergen label statements and assess the risk to the food-sensitive consumer. Immunological and DNA-based assays are the most developed methods for detection and quantification of food allergens. However, the preferred method is to detect the presence of the protein as it the direct cause of the allergic reaction. As a result, DNA-based methods have limited use. Immunological methods, for example ELISA and lateral flow devices (dipsticks) have become important tools for detection and quantification of food allergens. Commercial assays are available from a variety of companies for most of the priority food allergens. These methods have improved since their introduction in the late 1990s; however, there are still some limitations in their application including the lack of reference materials, knowledge of processing and matrix effects, and a harmonized validation procedure specific to protein quantification in food matrices.

Processing effects and food matrices can have a significant affect on the ability of an ELISA to detect the proteins of interest. In a recent study it was shown that roasting peanuts resulted in a significant decrease in the detection of proteins and the ability to determine peanut content. In addition, a prolonged baking time (26 min compared with the standard 16 min) resulted in reduced detection by a factor of 5–10 [222]. This decrease in the ability of the ELISA to detect the proteins of interest could be because of changes in the solubility of the proteins, changes in protein structure, or changes in the epitopes. Interestingly, heat treatment by roasting of peanuts has the opposite effect and actually increases the allergenic poten-

tial of peanuts [223]. A recent study of egg proteins has shown there is a similar decrease in the ability of the ELISA to detect the proteins of interest in salad dressing [224]. This matrix is likely to have a high vinegar component and it has been shown that vinegar also reduces the allergenicity of eggs [225]. In the latter investigation it seemed that egg protein detection correlates with egg protein allergenicity when exposed to vinegar, in contrast with the results obtained after roasting peanuts. Unfortunately, the correlation between allergenicity and ELISA response is difficult to determine beforehand and information on food matrix and processing effects is needed for confidence in a result. The development of incurred materials that have a known amount of allergen that has undergone industrial processing steps would be invaluable in validation of a method.

The development of reference materials, for example incurred materials or any material used to evaluate the effect of processing and food matrix on the response from an ELISA, will depend on the availability of material that has been fully characterized. The continuing lack of these materials for the development and validation of ELISA methods is partly because of the difficulty in deciding what will make a good reference material for a given allergen or commodity. The lack of recognized reference materials means that most kit manufacturers supply a calibration standard developed for their own particular assay, which is likely to be different from the calibration standard from another ELISA manufacturer. These differences in protein content in the calibration standards sometimes result in different final results; this is very evident from interpretation of results from ELISA assays that determine gluten content. Most commercial ELISA methods for gluten rely on two different antibodies, the R5 antibody which reacts to a five-amino-acid sequence found in the gliadin fraction of wheat [226] and the omega-gliadin antibody which reacts to the omega-gliadin fraction of wheat [227]. The R5-ELISA is calibrated by use of a purified gliadin material based on multiple wheat cultivars while the omega-gliadin assay is calibrated by use of a wheat material from NIST [228]. The R5-ELISA also responds to gluten from barley and rye because these proteins also contain the five amino acid peptide sequence, but the omega-gliadin assay has low cross-reactivity (5%) towards barley hordeins and typically results in different estimates of gluten content if the source is barley. Interestingly, the R5-ELISA is likely to overestimate the gluten content of barley because it relies on a 1:1 prolamin-to-glutenin ratio to calculate total gluten, which is not valid for other grain [229].

The difficulties associated with the analysis of gluten and those associated with some processed proteins when using ELISA methods emphasize that immunological assays are not perfect. Both false-positive and false-

negative results can occur, which reveals the need for a conformational method of analysis. Mass spectrometric analysis of proteins does not have these specific drawbacks and if the proteins are soluble and can be digested, typically by trypsin, positive identification can be achieved, typically with only one peptide. Analysis of proteins in biological systems has developed rapidly since the discovery that these large molecules can be introduced into the gas phase and measured by mass spectrometry [230]. In study of food allergens a significant body of work has been conducted on peanut proteins. Early work using mass spectrometry was performed either to characterize all of the proteins present in a sample [231] or those determined, in other investigations, to be allergenic [232]. Initial work using mass spectrometry as a tool to investigate the presence of allergens in different food matrices was confirmatory in nature, with specific peptides being used to confirm the presence of allergenic proteins in the samples. One of these early studies relied on finding peptides specific to the allergenic peanut protein (Ara h1) and then using a combination of LC and tandem mass spectrometry to confirm the presence of peanut allergenic proteins [233]. In another study LC coupled to high-resolution time-of-flight tandem mass spectrometry was used to gather information on all of the peptides in the digested sample, and then determining if any food allergen proteins were present by use of a database search [234]. In the first example, knowledge of specific protein markers is needed to develop multiple reaction monitoring (MRM) methods. These methods have been used extensively for analysis of small molecules because they are very sensitive, and quantitative results can be obtained by use of isotope-dilution studies. These authors were able to confirm the presence of Ara h1 protein spiked at a level of 10 ppm into an ice cream matrix and in a subsequent study were able to confirm the level of the same protein at a level of 2 ppm in dark chocolate [235]. A difficulty in using one or two peptides for confirmatory or quantitative analysis is that if these peptides were altered in some way by processing they would become invisible to the method and a false negative result would be obtained. In the latter example, in which all the digested peptides are monitored, processing would not be such a problem because there would probably be additional peptides from the allergenic food that were not altered and could be used for identification. However, this method would be very dependant on the level of other proteins in solution and higher-protein-content samples would be likely to mask the peptide signals from the allergenic food, producing a false-negative result. In either type of analysis the experimental workflow requires enzymatic digestion of the proteins into smaller tryptic fragments before analysis. These digestions are performed using trypsin, which cleaves the protein at lysine and

arginine residues that are not adjacent to proline [236], an assumption that has been challenged [237]. This digestion must be efficient and reproducible for reliable qualitative and quantitative analysis of proteins. Like ELISA methods, mass spectrometry will benefit from the use of reference materials, incurred materials, and method-validation procedures. One difficulty in the validation of these methods is the choice of peptides and proteins to target. For a given allergenic food there are many targets that could be used for qualitative and quantitative analysis, but these targets can vary within cultivars and thus whole food reference materials will be vital [238, 239]. Mass spectrometric methods for detection, confirmation, and quantification of food allergens have not been developed to the extent of commercial ELISA methods, but these types of validation studies are starting to appear in the literature [240]. The promise of mass spectrometric methods of analysis, whether confirmatory or quantitative, could alleviate some of the problems associated with ELISA methods, including processing effects, but a procedure for harmonized multi-lab investigations will be essential to the production of results of which we can be confident, as they will be for ELISA methods [241].

Summary and outlook

The contaminants and allergens discussed above have a wide range of chemical and physical properties, ranging from lipophilic to hydrophilic, from volatile to non-volatile, and from small molecules, for example chemical contaminants, to large allergenic proteins. In addition, many of these analytes have unknown toxicological or allergenic effects in humans and, therefore, maximum allowable levels set by government regulatory agencies are also driven by the achievable limits of detection. Therefore, matrix-independent methods and low quantification limits are still required to gather surveillance data about the occurrence and background levels of both recognized and newly identified contaminants in foods in order to estimate human daily intake for risk assessment. Clearly, the choice of appropriate extraction and cleanup methods using modern SPE techniques in combination with chromatographic methods, usually with MS detection, are crucial for success. There is still a need for appropriate reference materials, particularly for allergens.

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