How Safe is Safe? Analytical Tools for Tracing Contaminants in Food

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A growing world population up to 9.7 billion by 2050 will increase the demand for food. This will require higher crop production globally, by enhancing productivity through optimised methods, fertilisers, agrochemicals and pesticides. In order to comply with regulations on food safety, manufacturers of food and beverages must carefully control contaminants such as pesticides, mycotoxins and heavy metals. Protection against fraudulent products and improper food ingredient labelling is also essential. Analytical instrumentation technologies and methods such as mass spectrometry coupled with liquid chromatography (LC), gas chromatography (GC) and inductively coupled plasma (ICP) provide the tools to support and guarantee food safety with consumer, animal and plant protection.

Introduction

Food safety is a major concern for the European population, with many food scandals being reported in the past few years, such as the horsemeat scandal in 2013 when traces of horsemeat were found in products sold in supermarkets. Deep frozen lasagne claiming to contain beef was found with no declaration of horsemeat or improperly declared horsemeat content – whereas the actual horsemeat content was up to 100%. In another case, mineral oil and plastic parts were detected in chocolate products. In spring 2016 an environmental institute in Munich found elevated concentrations of glyphosate in beer, Europe´s favourite alcoholic beverage.

To strengthen confidence in food products the European Commission aims to assure a high level of food safety and animal & plant health within the EU through the farm-to-fork principle. This strategy implements effective system controls and evaluates compliance with EU standards as well as evaluating EU imports from third party countries.

European citizens are entitled to know how their food is produced, processed, packaged, labelled and sold. The central goal of the European Commission's Food Safety policy is to ensure a high level of protection of human health regarding the food industry — Europe's largest manufacturing and employment sector.

Food Control is a Fundamental Issue

Over the last century, the global population has quadrupled, in 1915, there were 1.8 billion people, today there are 7.3 billion people and by 2050 it is estimated that the world population will reach 9.7 billion. This growth, along with rising incomes in developing countries (which causes dietary changes such as eating more protein and meat) increases the global demand for food with food demand expected to grow anywhere between 59% and 98% by 2050 [1]. This will require higher crop production on a global scale by enhancing productivity through optimised methods, fertilisers, agrochemicals and pesticides.

The use of herbicides, insecticides and fungicides reduces crop losses, both before and after harvest, however, residues resulting from the use of plant protection products may pose a risk to human health and require a legislative framework to monitor food contamination. National programs for pesticide monitoring in the US, Europe and Japan have set Maximum Residue Levels (MRL's) or tolerance information (EPA) for pesticides in food products. A default value of 0.01 mg/kg is applied for MRL enforcement, requiring highly sensitive and specific analytical

Table 1: Analytical conditions for LC and MS

Analytical system stream 1 (underivatized samples)	
LC system	Nexera MX (Shimadzu, Japan)
Analytical column	Hypercarb (100 mm x 2.1 mm, 5 µm)
Mobile phase A	1 % acetic acid
Mobile phase B	Methanol + 1 % acetic acid
Injection volume	5 µL
Column temperature	35 °C
Analytical system stream 2 (FMOC-derivatized samples)	
LC system	Nexera MX (Shimadzu, Japan)
Analytical column	Raptor C18 (100 mm x 2.1 mm, 2.7 µm)
Mobile phase A	5 mM ammonium acetate
Mobile phase B	Acetonitrile
Injection volume	10 µL
Column temperature	35 °C
MS	
MS system	LCMS-8060 (Shimadzu, Japan)
Ionisation	HESI (positive/negative)
Nebulizer gas flow	3.00 L/min (N_2)
Drying gas flow	5.00 L/min (N_2)
Heating gas flow	15.0 L/min (Air)
DL temperature	150 °C
Block temperature	400 °C
Interface temperature	325 °C

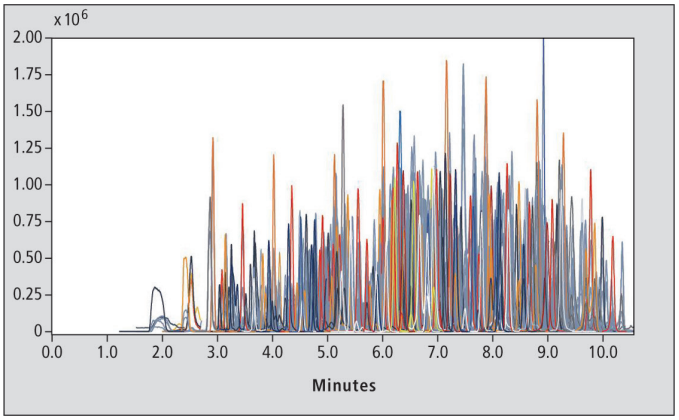

Figure 1: MRM chromatograms of 646 pesticides spiked into a mint extract at 0.01 mg/kg (Up to 3 MRMs per compound and 5 msec polarity switching time).

Table 2: Quantification ranges of pesticide compounds with derivatisation and without derivatisation

Compound	Quantification Level [ng/mL]	
	Without derivatization	With derivatization
Glyphosate	5 - 100	5 - 100
Glufosinate	5 - 100	10 - 100
Aminomethylphosphonic acid (AMPA)	5 - 100	Not quantified
3-Methylphosphinico propionic acid (MPPA)	10 - 100	Not quantified

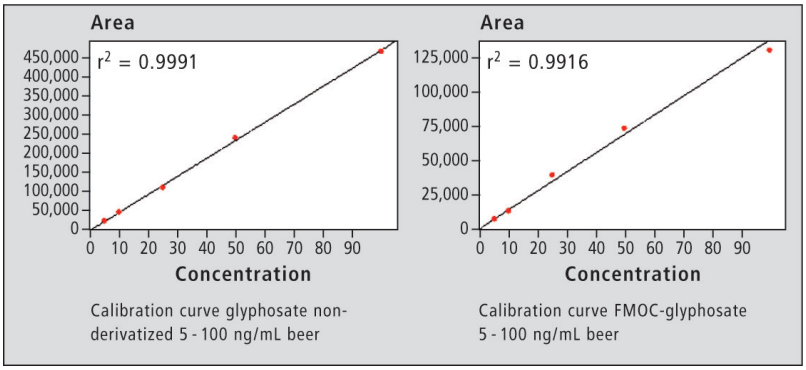

Figure 2: Calibration curves of glyphosate, glufosinate, AMPA and MPPA determined in duplicate obtained from beer after sample pre-treatment. R^2 was better than 0.99 for all calibration curves.

technologies for monitoring the steadily increasing number of pesticides [2,3,4].

Pesticide Analysis

The Shimadzu LCMS-8060 triple quadrupole mass spectrometer features high sensitivity and speed for accelerated method development workflows and increased pesticide monitoring programs. Using the Shimadzu Pesticide MRM Library (including information on 766 certified reference materials) a single multi-residue LC-MS/MS method has been developed for the detection of 646 pesticides (3 MRM transitions for over 99% targeted pesticides resulting in 1,919 transitions in total, with a polarity switching time of 5 msec).

Glyphosate is currently one of the most common pesticides used worldwide. Despite its approval by regulatory bodies all over the world, concerns persist regarding its potential harm to humans and the environment. In June 2016, the European Commission extended the license for glyphosate use by 18 months, after member states failed to achieve a qualified majority in favour of or against the executive's proposal, despite being identified as a possible carcinogen by the International Agency for Research on Cancer (IARC) in March 2015.

Based on new research results, the European Chemical Agency (ECHA) came to a different conclusion on 15th March 2017. The ECHA's Committee for Risk Assessment (RAC) confirms the current harmonised classification of glyphosate as a substance causing serious eye damage and being toxic to aquatic life with long-lasting effects. But the RAC concluded that the scientific evidence available did not meet the required criteria to classify glyphosate as a carcinogen, mutagen or as being toxic for reproduction. Glyphosate will therefore still be found in fruit and vegetables as well as in drinking water and beer soon [5].

Determination of Glyphosate and Related Substances using LC-MS/MS

Glyphosate is challenging to analyse chromatographically due to its high polarity, however a well-established method [6] can be employed which includes a derivatisation step with 9-fluorenylmethyl chloroformate (FMOC) followed by LC-MS analysis. Unfortunately, this is time-consuming and expensive and is also susceptible to errors. A further drawback is that not all highly polar and structurally similar pesticides, for example glufosinate's metabolite 3-methylphosphinico propionic acid (MPPA), can be derivatised with FMOC. On the other hand, a sample pretreatment without derivatisation as described in EURL-SRM QuPPe-Method (5.6.4 Method 1.3) [7] which is not limited to glyphosate detection is therefore desirable since it will be quicker and more cost-efficient and will enable the simultaneous detection of other highly polar pesticides and their metabolites. This method also includes some disadvantages as chromatograms obtained using a new column show poor chromatographic behaviour due to strong interactions of analytes with active sites. The column needs conditioning with QuPPe extracts of e.g. spinach. This masking of the active sites is temporary and the activity of the column gradually increases with the injection of solvent or diluted extracts. Following a sequence of injections with low or no matrix load will typically raise the need for intermediate conditioning with extracts to restore the column [7].

To develop an optimised routine method for the quantification of glyphosate, its metabolite aminomethylphosphonic acid (AMPA), glufosinate and its respective metabolite MPPA in beer samples, the derivatisation method was compared to a non-derivatisation method. A total of 24 different kinds of beer were analysed. The use of a high sensitivity mass spectrometer for both methods (LCMS-8060 coupled to a Nexera UHPLC, both from Shimadzu) avoids the need for an additional sample concentration step such as solid phase extraction (SPE), thereby reducing analysis time and cost.

Sample preparation for LC-MS/MS method and analytical conditions

1 mL methanol (MeOH) was added to 1 mL beer, vortexed and centrifuged for 15 min at 12,000 rpm. 500 µL of the supernatant was used as an underivatised sample. For derivatisation, 25 µL EDTA-borate buffer and 75 µL FMOC were added to 500 µL of the supernatant. After incubating at 50°C for 60 minutes the reaction was stopped by adding 30 µL of 0.2% phosphoric acid. Finally, 125 µL of water was added.

The derivatised and underivatised samples required different chromatographic conditions which are listed in Table 1.

Results of LC-MS/MS Measurements

Two methods (with and without derivatisation) have been evaluated for the quantification of glyphosate and glufosinate in beer, without the use of internal standards AMPA could be quantified using the FMOC derivatisation whereas MPPA could only be quantified with the underivatised method.

Therefore, neither of these methods is suitable for the detection of both metabolites at the same time with adequate sensitivity.

Calibration curves obtained for the compounds are shown in Figure 2, and the corresponding chromatograms in Figure 3. Analytical results for glyphosate in 24 different beer samples are listed in Table 2. Both methods show comparable results. None of the other compounds could be detected in the samples.

Both methods permitted the quantification of all target compounds at or below 0.01 mg/kg ($\triangleq$ 10 ng/mL), below the European Union maximum residue levels (MRL) for all compounds. The non-derivatisation method shows comparable sensitivity to the FMOC derivatisation method, but sample preparation is much easier and quicker. Glyphosate was detected at a concentration above LOQ in more than 30% of the commercially available beers tested,

Table 3: Analysis results for glyphosate in beer

	Glyphosate	FMOC-Glyphosate		Glyphosate	FMOC-Glyphosate		Glyphosate	FMOC-Glyphosate
Sample	Conc. (ng/mL)	Conc. (ng/mL)	Sample	Conc. (ng/mL)	Conc. (ng/mL)	Sample	Conc. (ng/mL)	Conc. (ng/mL)
Beer 1	< LOQ	< LOQ	Beer 9	11.93	12.13	Beer 17	< LOQ	< LOQ
Beer 2	< LOQ	< LOQ	Beer 10	< LOQ	< LOQ	Beer 18	35.17	27.25
Beer 3	< LOQ	< LOQ	Beer 11	< LOQ	5.34	Beer 19	< LOQ	< LOQ
Beer 4	17.33	23.11	Beer 12	6.23	5.63	Beer 20	< LOQ	< LOQ
Beer 5	9.85	13.46	Beer 13	14.25	14.69	Beer 21	< LOQ	< LOQ
Beer 6	< LOQ	< LOQ	Beer 14	20.6	23.61	Beer 22	< LOQ	< LOQ
Beer 7	< LOQ	< LOQ	Beer 15	< LOQ	8.46	Beer 23	9.97	9.67
Beer 8	< LOQ	< LOQ	Beer 16	< LOQ	7.24	Beer 24	12.78	16.14

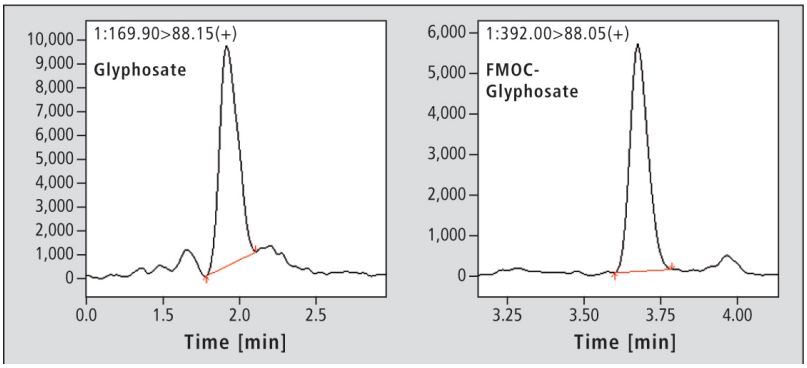

Figure 3: Chromatograms of a 15 ng/mL beer sample – 15 ng/ml glyphosate/ FMOC- glyphosate

Table 4: EU concentration limits for Mycotoxins

Mycotoxin	EU limit in µg/kg
Aflatoxins (B1, B2, G1 and G2)	4 - 15
Aflatoxin B1	2 - 12
Aflatoxin M1	0.05
Ochratoxin A	2 - 10
Patulin	25 - 50
Deoxynivalenol	500 - 1,750
Nivalenol	Not specified
Zearalenone	20 - 400

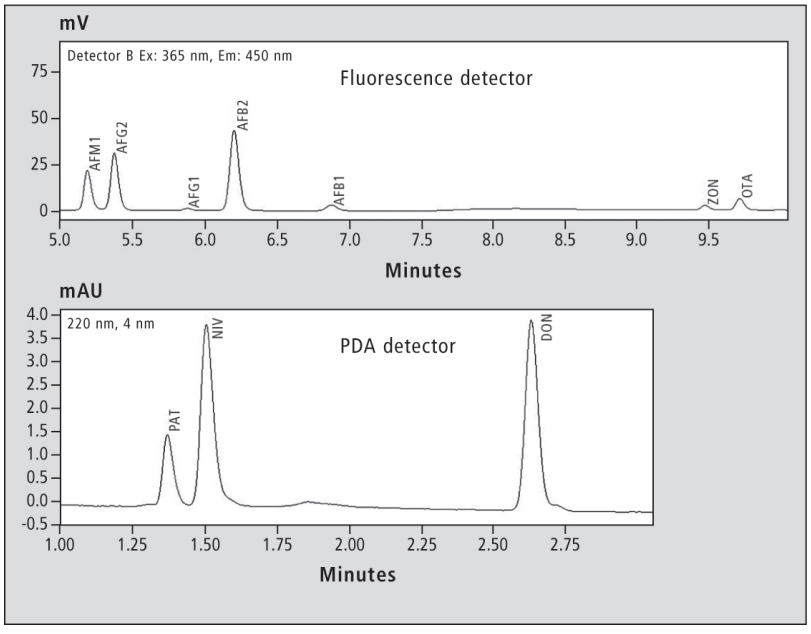

Figure 4: Mycotoxins detected by fluorescence and PDA detection

however the positive samples contained less glyphosate than the MRL of the commodities (e.g. barley, hops) [8].

Mycotoxin Analysis

The European legislation for mycotoxins in food, which are hazardous substances for humans when ingested, consists of two main regulations: The Commission Regulation 1881/2006 setting maximum levels for certain contaminants in foodstuffs, and the most recent mycotoxin related update by Commission Regulation 1058/2012 setting maximum residue limits for aflatoxins, ochratoxin A, patulin, deoxynivalenol, zearelenone, fumonisins, T2 and HT2 toxin in a variety of different products. Mycotoxins are not destroyed by temperature treatment, and are barely influenced by cooking, freezing or digestion. These properties make the investigation and quantitation of mycotoxins in food very important.

Aflatoxins are toxic and carcinogenic. Aflatoxin B1 the most common type of aflatoxin present in food products is one of the most genotoxic and carcinogenic species [2]. Ochratoxin is produced by penicillium and aspergillus species and is a contaminant in alcoholic beverages such as beer and wine. Patulin, produced by p. expansum, aspergillus, penicillium, and paecilomyces fungal species, is often found in mouldy fruits and vegetables. Zearalenone is produced by fusarium species and may be present in crops; AFM1 is often found in milk products.

In order to comply with the European regulation on food safety, manufacturers of food and beverages must control the quantities of these contaminants, so sensitive methods for detection of mycotoxins in complex matrices are essential.

Mycotoxin Screening System

AFB1, AFB2, AFG1, AFG2, AFM1, OTA, ZON, DON, NIV, and PAT were investigated using a mycotoxin screening system employing the Shimadzu i-Series integrated UHPLC system. The five commonly tested analytes (Aflatoxins, ZON, OTA, NIV and DON) in malt products were extracted and analysed in spiked and non-spiked beer samples from different batches.

PAT in apple juice and grape juice was also tested separately.

EU limits for Mycotoxins are shown in Table 4. Mycotoxins detection after UHPLC was performed using a combination of fluorescence and photodiode array (PDA) detection. The chromatograms of the standard mixture with PDA and fluorescence detection containing all 10 mycotoxins is shown in Figure 4.

The EU MRL for mycotoxins as specified by EU standards are the strictest in the world [9]. Mycotoxin standards were prepared using the concentration specified in the EU control criteria to determine the mycotoxin levels in the food samples under investigation contain mycotoxins. A simple, fast one point calibration was sufficient to assess compliance with the EU control criteria. Samples were created using the calibration standard and the food samples, and the subsequent results show the mycotoxins concentrations in the food samples and whether they complied to the EU control criteria.

The samples tested (apple juice, grape juice and two batches of beer) either contained no mycotoxins or were well below the EU criteria. The spiked samples showed mycotoxin concentrations above the EU criteria.

For all mycotoxin standards, the limit of detection (LODs) and limit of quantitation (LOQs) were determined as shown in Table 5. The LODs and LOQs were approximately 50% of the EU MRL.

Determination of Heavy Metals Using ICP-MS

Heavy metals such as cadmium, chromium and lead are natural components of the earth's crust and are typically present in our environment at various concentration levels. They enter the human body via food, drink and air. Some of these heavy metals, the so-called trace elements such as

Table 5: Limits of detection (LODs) and limits of quantitation (LOQs) for the mycotoxin analyser method.

	AFM1	AFG1	OTA	AFB1	AFG2	AFB2	ZON	PAT	DON	NIV
LOD [ppb]	0.25	4	0.1	0.0025	0.025	0.00015	25	13	10	80
LOQ [ppb]	0.75	12	0.3	0.0075	0.075	0.00045	75	50	20	200

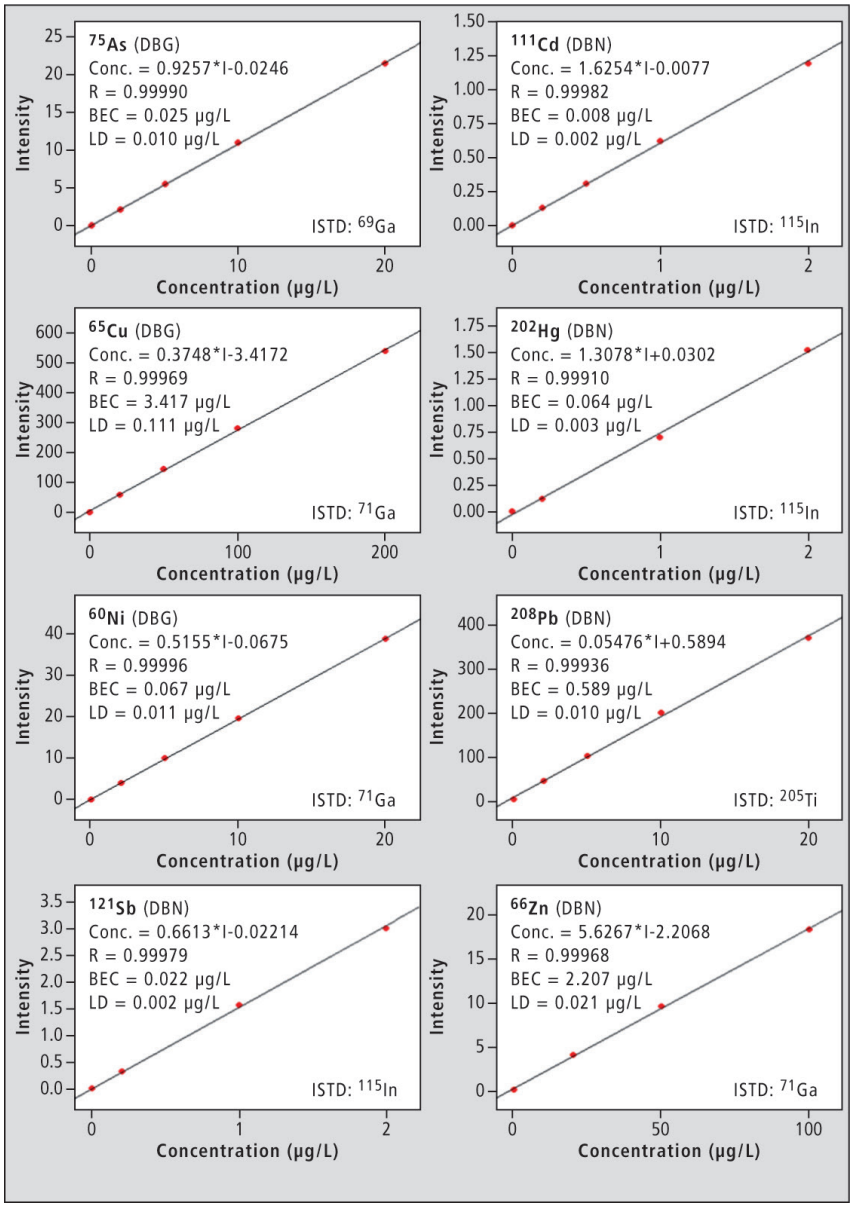

Figure 5: Calibration curves of 65Cu, 202Hg, 60Ni, 63Sb, 66Zn, 75As, 111Cd, 208Pb

chromium, iron, cobalt, copper, manganese, zinc and tin are in low concentrations essential to the human body, as they are important for the metabolism. At higher concentrations however, they are toxic and harmful to humans. Heavy metal poisoning may occur from contamination of drinking water from lead transfer pipes, air contamination from industrial emissions or ingestion via the food chain in the form of contaminated vegetables, meat and fish. Drinking water and wastewater are monitored continuously according to the European drinking water regulation and the law for indirect introduction of wastewaters.

Determination of heavy metals is done using Atomic Absorption-(AAS), Inductively Coupled Plasma Optical Emission-(ICP-OES) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS). ISO 17294-2:2016 specifies a method for the determination of 62 elements in drinking water, surface water, ground water, wastewater and eluates using an ICP-MS such as the Shimadzu ICPMS-2030.

Metal Elements in Beer

Next to drinking water, the most consumed and best controlled food on a global scale, beer is one of the world's favourite bottled beverages. The annual per capita consumption is approx. 70 L in European countries. The quality standards for the analysis of beer are defined by the Central European Commission for Brewing Analysis (MEBAK) and the European Brewery Convention (EBC) which represents the technical and scientific interests of the brewing industries in European countries.

These regulations include the principles and methods for the analysis of raw materials, semi-finished goods, by-products and finished products, additives and technical supplies, containers and packaging materials with the aim of standardisation, primarily in the brewing and malting industries. The analysis methods of MEBAK are available in several application books and include the determination of elements like copper, zinc, sodium, potassium, calcium and more, anions such as nitrate and sulphite as well as organic components such as ethanol, glycerin and others [10]. The determination of these trace metals in beer is required since they may be toxic in the human body in higher concentrations and have an influence on the brewing process. The elemental distribution differs significantly depending on the soil, water, cereal, hops, yeast and anthropogenic sources such as environmental pollution and agricultural treatment by fertilisers, pesticides and fungicides.

Last but not least, the metal content in beer may be influenced during production, processing, bottling and storage. During the brewing process, raw materials and processed products are often in contact with various materials such as stainless steel, copper, glass and other equipment for extended periods of time.

Determination of copper is important since high concentrations are disadvantageous to the colloidal stability and taste of the beer. The same applies to zinc, which is an essential trace element that influences the yeast metabolic processes such as protein synthesis and nucleic acid metabolism. Typical concentration levels of copper and zinc in beer are 0.2 mg/L [11].

Furthermore, the determination of arsenic, antimony, cadmium and lead is important, as these elements are toxic when present in beer or the brewing water. The source of these elements in beer and other alcoholic beverages can be attributed to the contamination of raw material and/or technological processes.

Antimony and Arsenic in Water, Beer and Soft Drinks

Arsenic is released into beer from a filtering material called Kieselguhr or diatomaceous earth, used to remove yeast, hops and other particles and give the beer a crystal clear appearance. Diatomaceous earth consists of fossilised remains of diatoms, a type of hard-shelled algae that lived millions of years ago. It is widely used in beer and wine filtering and is an ingredient in other products [12].

Another element under scrutiny is antimony (Sb). Annual consumption of antimony trioxide in the United States and Europe is approximately 10,000 tons and 25,000 tons respectively. The main uses are as a flame retardant synergist in combination with halogenated materials and as a catalyst in the production process for polyethylene terephthalate (PET) bottles. Elevated concentrations of Sb have been found in beverages such as cola drinks and orange

Table 6: ICPMS-2030 measurement parameters

Parameter	Setting
RF generater power	1.2 kW
Plasma gas	8 L/min
Auxilliary gas	1.1 L/min
Carrier gas	0.7 L/min
Nubulizer type	MicroMist
Sampling depth	5 mm
Spray chamber temperature	5 °C
Coll. cell gas flow (He), DBG mode only	6 mL/min
Quantified isotopes	^{75}As, ^{111}Cd, ^{65}Cu, ^{202}Hg, ^{60}Ni, ^{208}Pb, ^{121}Sb, ^{66}Zn
Internal standards (ISTD)	^{69}Ga, ^{71}Ga, ^{115}In, ^{205}Ti

Table 7: Distribution of 6 elements in beer

Element	Beer 1	Beer 2	Beer 3	Beer 4	Beer 5
^{75}As	2.05	3.49	2.39	1.46	0.39
^{111}Cd	0.07	0.05	0.07	0.08	0.15
^{65}Cu	29.10	28.70	40.50	38.20	22.40
^{60}Ni	2.25	5.62	1.53	2.58	4.47
^{208}Pb	< LQ	< LQ	< LQ	< LQ	< LQ
^{121}Sb	0.39	0.19	2.15	0.45	0.55
^{66}Zn	5.28	23.90	5.04	2.83	29.20

juices which are stored in PET bottles, as the Sb migrates from the plastic to the liquid and accumulates in the drinks. The migration process is accelerated in alcoholic beverages. Vodka samples from glass and PET bottles have been compared according to their Sb-levels where it was found that the Sb concentration in vodka from a PET bottle can be up to 20 µg/L compared to less than 1 µg/L in a glass bottle [13].

The maximum allowable concentration of Sb in drinking water is 5 µg/L. Since the latest development in the beer industry is the introduction of 0.33 L, 0.5L and 1 L PET bottles on supermarket shelves with a variety of beers from European countries, analytical investigations are in process of evaluating antimony concentrations in beer also.

High Sensitivity ICP-MS

For the simultaneous quantitative determination of inorganic elements in beer, ICP-MS is the preferred quality control tool. ICP-MS offers high sensitivity (trace detection), a wide dynamic range and high sample throughput. Even though beer is regarded as a difficult matrix due to the high number of constituents, the Shimadzu ICPMS-2030 octopole collision cell assures high accuracy for all element measurements. Using helium as a collision gas and running in Kinetic Energy Discrimination (KED) mode, this cell suppresses most of the spectroscopic interferences (polyatomic interferences). Efficiency of interference suppression and sensitivity are improved using a cooled cyclonic chamber and well-controlled torch positioning.

Preparation of Beer Samples and ICP-MS Setup

For this study, samples of two commercially available beers were evaluated. The two beer samples analysed were undiluted and aspirated after degassing using the measurement parameters listed in Table 6. An internal standard solution containing ^{71}Ga, ^{115}In and ^{205}Tl was added using the automatic internal standard addition kit.

Concentrations of Ni, Cd, Sb and Pb in the undiluted beer were determined using a calibration curve method. For each element studied, calibration curves were generated using 4 standards in the concentration range from 2 to 10 µg/L. The standards were prepared using a matrix-matched solution containing 5% ethanol.

The calibration curves in Figure 5 show that all correlation coefficients were better than 0.999, and low levels of detection limits (LD) were achieved. They were calculated automatically by LabSolution-ICPMS software with 3σmethod. The data listed in Table 7 demonstrates that ICPMS-2030 is an ideal tool for trace contaminant analysis in beer.

Conclusion

Contaminants like pesticide residues, mycotoxins and heavy metals may occur in our food from a variety of different sources. These are in the focus of European food and safety authorities, and are controlled by national and international regulations. Analysis of relevant chemical contaminants is therefore an essential part of the food safety policy of the European Commission to ensure the highest level of protection of human health.

Modern hyphenated analytical techniques such as chromatography (LC-MS), spectroscopy and mass spectrometry can determine these contaminants in complex food matrices with high sensitivity at ultra-low concentration levels in order to keep the food and beverage chain safe.

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