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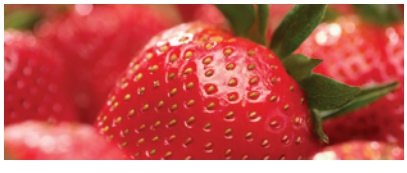

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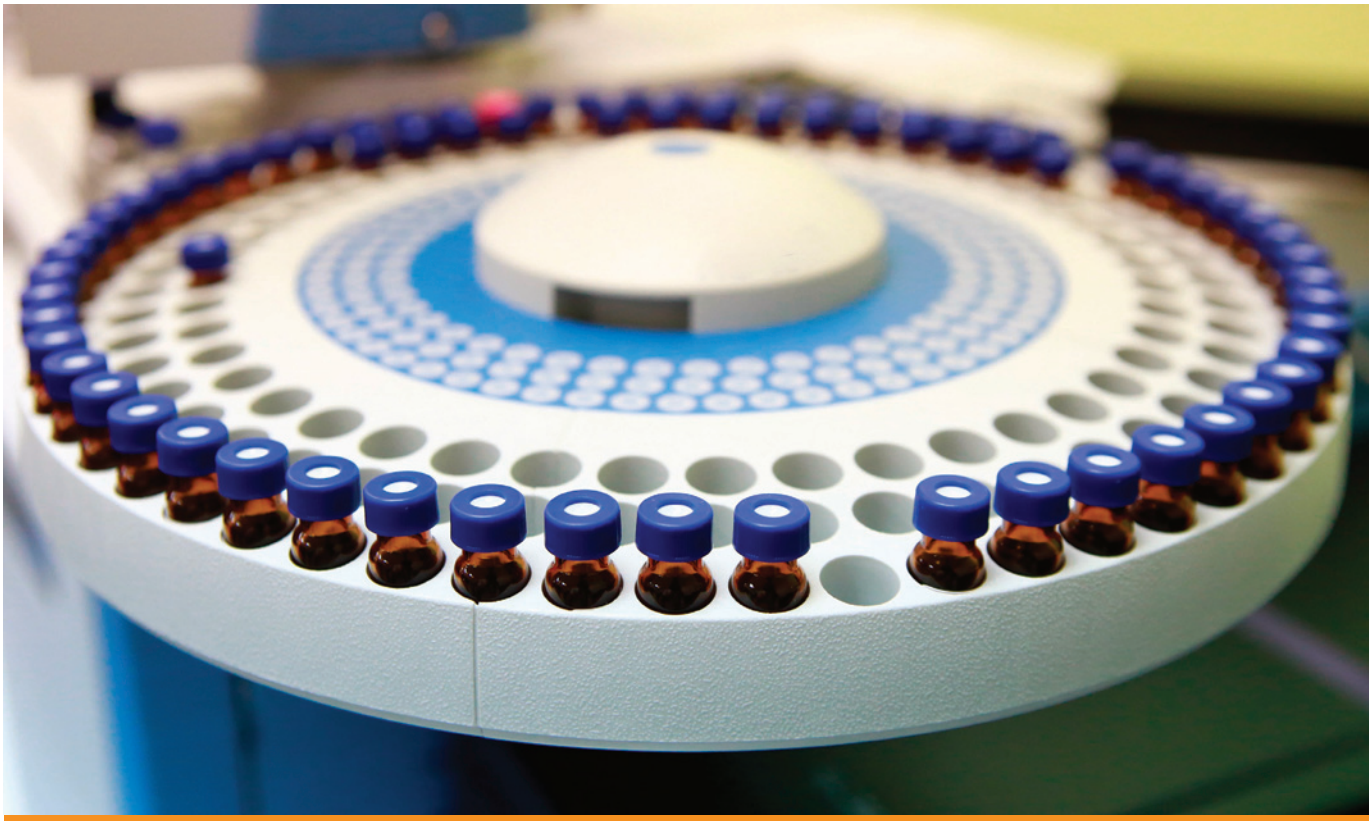

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Rapid Classification of Beef Aroma Quality Using SIFT-MS

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Aroma is an important characteristic in the acceptance of beef by consumers, and preference is often culturally dependent. Traditional grading of aroma quality has been carried out using sensory analysis of very limited numbers of samples, due to the inherent costs and the lack of appropriate technologies to replace the human olfactory system. Since meat aroma is derived from various volatile organic compounds (VOCs) that impart favorable or unfavorable qualities, there is potential to apply selected ion flow tube mass spectrometry (SIFT–MS) to facilitate wider scale, economic grading of carcasses. SIFT–MS is a direct analysis technique that can provide both high sample throughput and selective analysis of the chemically diverse volatiles that contribute to the aroma. This paper describes an investigation of the applicability of SIFT–MS to beef grading. Classifications of beef aroma were provided by a trained sensory panel. SIFT–MS was used to analyze the same samples for aroma volatiles. Application of multivariate statistical analysis to the combined data set demonstrated that SIFT–MS discriminates premium quality beef from eight sensory defects, and, therefore, could be applied as an instrumental grading tool obviating sensory panel grading.

Vaughan S. Langford, Murray J. McEwan, Tracey Cummings, Nicola Simmons, and Clyde Daly

There are several factors that contribute to the acceptability of a beef cut to the consumer, but most important is its flavor. Certain volatile organic compounds (VOCs) impart favorable or unfavorable characteristics to the flavor, and these can be detected using a variety of analytical techniques. Beef flavor is made up from a significant number of volatile compounds, largely arising from the cooking process. These flavor compounds are generated in the Maillard reaction, lipid oxidation, and interactions between them during the cooking process (1–7), and from vitamin degradation (1,8). A wide range of volatile flavor compounds are produced during the cooking of beef, and, if an understanding of the flavor compounds is to be found, many different volatile compounds should be monitored. Further, the aroma of cooked beef can be complex, and many hundreds of volatile flavor compounds have been identified (2,3,6,9-13). For example, a list of 90 VOCs have been monitored as flavor compounds in cooked beef, using the technique of gas chromatography–olfactometry (GC/O) (2,14). These compounds include aldehydes, ketones, alcohols, hydrocarbons, and pyrazines. Many of the traditional techniques for monitoring beef flavors are based on gas chromatography-mass spectrometry (GC–MS), often in conjunction with olfactometry. However, analysis using gas chromatographic techniques are slow and require expert operation, so they are impractical for process applications.

Recently, direct mass spectrometric methods have been developed that eliminate time-consuming chromatographic analysis. In this paper, we apply one of these methods: Selected Ion Flow Tube Mass Spectrometry

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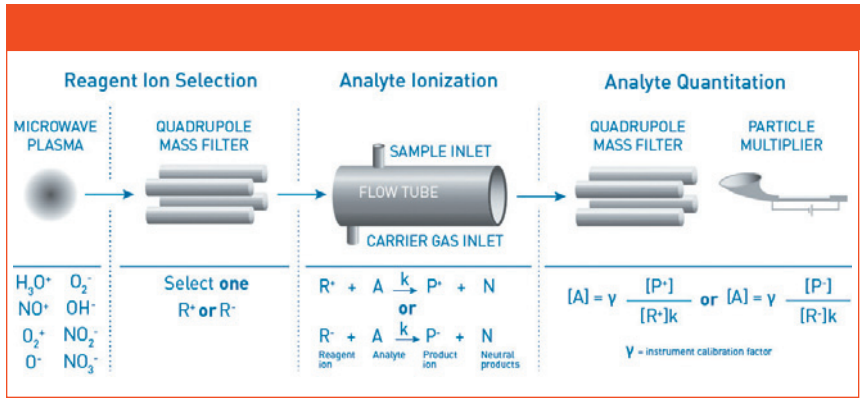

Figure 1: Schematic diagram of SIFT-MS – a direct chemical-ionization analytical technique.

Table I: Reagent ions and product ions mass-to-charge ratios (in Daltons) used to quantify target compounds (Table III). To avoid unnecessary complications, not all reagent ion products of each volatile are shown. Secondary product ions shown in parentheses.

Compound	Reagent and product ions used			Reference for reaction ion
	H_3O^+	NO^+	O_2^+	Chemistry (see end of paper)
Acetaldehyde	45 (63)			23
Acetone	59 (77)	88		23
Ammonia	18		17	24
Butanone (methyl ethyl ketone)		102		23
Dimethyl sulfide		62	62	25
Ethanol	47 (65, 93)			26
Ethyl acetate	89 (107)	118		27
Ethyl butyrate	117 (135)	146	71	28
Hydrogen sulfide	35			29
Indole		117	117	30
Methanol	33 (51)			26
Methyl mercaptan	49			29
n-Propyl acetate	103	101		28
Pentanal	87 (105)	85		23
Skatole		131	131	30
Trimethylamine	60			24

(SIFT–MS), a rapid, highly sensitive analyzer of whole air, to the detection of VOCs from various New Zealand beef samples. The rapid analysis provided by SIFT-MS has the potential to quickly identify premium quality carcasses early in the production process.

Methods

SIFT–MS

SIFT–MS (15,16) is a real-time analytical technique for direct, comprehensive gas analysis to ultra-trace levels (17). Data obtained by SIFT–MS instruments compare well with the leading chromatographic method for volatile organic compound (VOC) analysis, GC–MS (18).

SIFT–MS uses soft, precisely controlled chemical ionization, coupled with mass spectrometric detection (Figure 1), to rapidly quantify VOCs to low part-per-trillion concentrations by volume (pptv). Eight chemical ionization agents (reagent ions) are now available in commercial SIFT–MS instruments: H_3O^+, NO^+, O_2^+, O^-, O_2^-, OH^-, NO_2^-, and NO_3^- (19). These reagent ions react with VOCs and inorganic gases in well controlled ion-molecule reactions, but they do not react with the major components of air (N_2, O_2, and Ar). This enables SIFT–MS to analyze air at trace and ultra-trace levels without pre-concentration.

Rapid switching between the eight reagent ions provides very high selectivity. The key benefit of the additional ions is not primarily in the number of reagents ions, but in the multiple reaction mechanisms that provide additional independent measurements of each compound, delivering unparalleled selectivity and detection of an extremely broad range of compounds in real time.

In this paper, a Voice200 SIFT–MS instrument (Syft Technologies, Christchurch, New Zealand; www.syft.com) was utilized in selected ion mode (SIM). The compounds targeted together with the masses used for quantitation are summarized in Table 1. The three standard positively charged reagent ions were utilized to achieve selective analysis.

Samples

Sensory profiling

To create a range of flavor profiles, samples were sourced either from different types of cattle, or by modifying processing and storage conditions for vacuum packet primal cuts:

- normal production of prime grass-fed cattle
- high pH beef from both prime and bull carcasses
- manipulation of product by additional aging/chilling at higher temperatures to develop undesirable storage-related flavors
- sourcing product with abnormal flavors from both bulls and cows.

Previous studies have shown that beef from older bull and cow animals can be associated with "barnyard" and "milk" notes, respectively. Beef with an elevated final pH (high pH) relates to carcasses that have insufficient glycogen at the point of slaughter to enable the normal post mortem glycolytic cycle to progress. This condition has been associated with "sour" or metallic-like notes. Therefore, samples with these attributes were included in the sample selection provided by Carne Technologies (Table 2).

Samples were collected and stored frozen prior to sensory analysis, then thawed and minced prior to cooking and serving. Sensory panelists trained by Carne Technologies to recognize and assess

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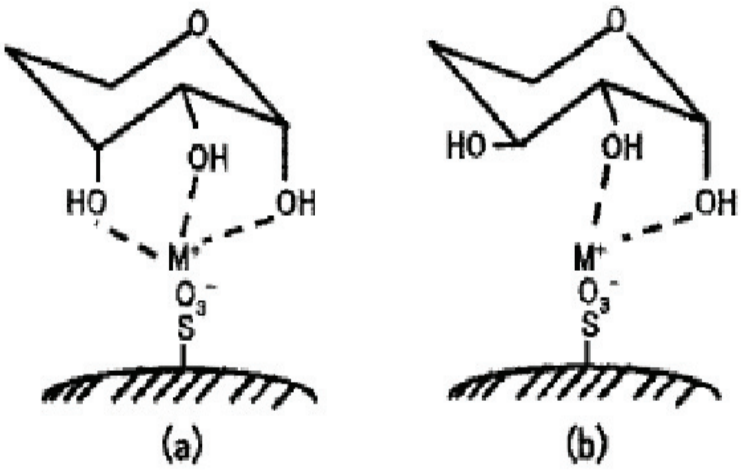

Sample : 5µL, 0.1% (w/v) each

1. Maltotriose
2. Maltose
3. Glucose
4. Lactic Acid
5. Glycerol
6. Acetic Acid
7. Ethanol

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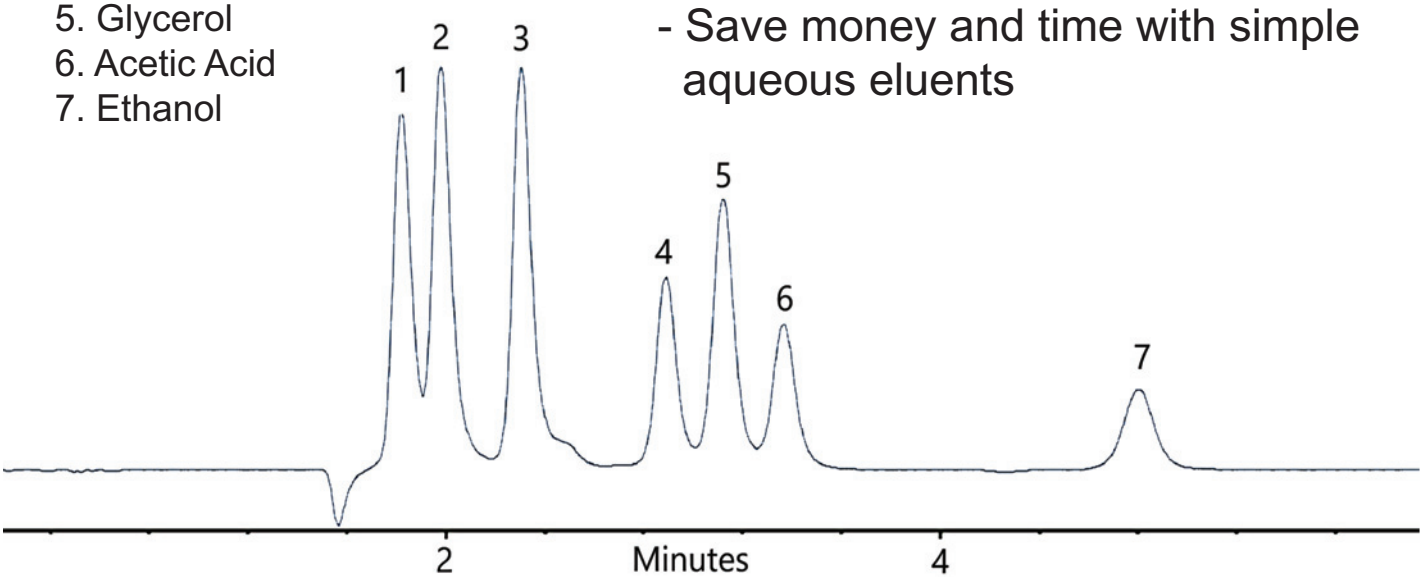

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Table II: The New Zealand beef samples analyzed in this study together with a description of their origin, and – where appropriate – the flavor defect attributed by the sensory panel.

Sample name (as supplied)	No. of samples supplied	Replicate samples analyzed	Description
Prime beef	40 in 8 packs of 5 samples each	8 (one from each pack, with portions taken from each sample)	40 prime beef from grass-fed cattle. Samples aged for 21 days
Bull 1	1	5	Defective "bull" flavor, type A
Bull 2	1	5	Defective "bull" flavor, type B
Cow 1	1	5	Defective "cow" flavor, type A
Cow 2	1	5	Defective "cow" flavor, type B
High pH 1	1	5	Defective flavor: "Heifer High pH"
High pH 2	1	5	Defective flavor: "R High pH"
Norm pH	1	5	Defective flavor: "R Norm pH"
Excessively aged (Over-aged)	1	5	Defective flavor: "Over-aged"

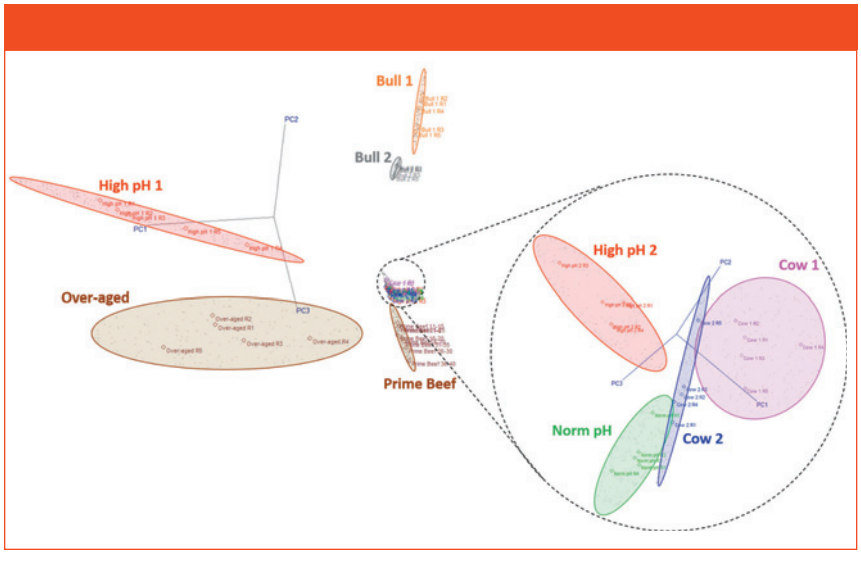

Figure 2: Class projections obtained from SIMCA multivariate analysis of the headspace data that are presented in averaged form in Table III. Each colored point in the class projections graph represents a replicate measurement. For clarity, the more congested region is expanded and rotated at right.

aromas and flavors were used to evaluate each sample. The attributes used by the sensory panelists consisted of meaty, beefy, barnyard, sour/acidic, milky, grassy/pasture, sweetness, offal, spoilage, rancid, storage, other/foreign (such as garlic or pepper), and aftertaste. Each attribute was scored on a 9-point scale where 0 is absent and 9 is intense/extreme. All samples, except "prime beef," exhibited different sensory defects.

Preparation for instrumental analysis

For SIFT–MS analysis, frozen samples were thawed at room temperature and finely diced. Samples of uncooked beef (20 grams) were placed in one-liter Schott bottles and capped with pierceable septa. Samples were incubated at 60 °C for one hour prior to analysis, using a SIFT-MS instrument equipped with a high-performance inlet (HPI) that is both passivated and heated (120 °C). The HPI provides a direct path from the sample headspace to the flow tube, minimizing loss of flavor volatiles.

Multivariate Statistical Analysis

The Selected Ion Mode (SIM) data from SIFT–MS were treated using multivariate statistical analysis to determine the ability of SIFT–MS to discriminate between the premium samples labelled "prime beef" and samples deemed defective by the sensory panel.

The multivariate statistical methodology applied in this work was Soft Independent Modeling by Class Analogy (SIMCA), which was developed by Wold in the 1970s (20). SIMCA applies principal component analysis (PCA) to the whole dataset and to each of the classes with the goal of creating a model that discriminates each class from the others. The Infometrix Inc. (Bothell, WA) implementation of the SIMCA algorithm in the Pirouette software package was employed here.

Three types of output from the SIMCA analysis are presented in this report:

- **Class projections:** These three-dimensional plots show how each sample falls with respect to the three most important principal components derived from PCA on the entire data set. Each user-defined class shows the sample with the same color and a "cloud" representing the calculated space in which all samples of the class are expected to lie. Better class separations lead to more confident assignment of unknown samples to a predefined class, if a suitable one exists.
- **Interclass distances:** These are a measure of the separation between classes, a value of three usually being considered acceptable for class separation (21). Sometimes, the class separability indicated by these distances is not apparent in the three-dimensional class projection plot.
- **Discriminating power:** This parameter helps identify variables that provide the most discrimination between the classes. A variable with larger discriminating power has greater influence on separating the classes than one with a small discriminating power. There does not appear to be a set threshold value above which this variable is considered good, because these values vary strongly with interclass distance.

Results and Discussion

The data obtained from SIFT–MS analysis of the headspace of New Zealand beef samples are summarized in Table 3. For each class of

sample, the replicate measurements have been averaged and the extent of variation indicated using two standard deviations of the mean. For most compounds, measurements show satisfactory repeatability given that analysis was carried out manually (since undertaking this study, application of automation to SIFT–MS has demonstrated significant repeatability improvements compared to manual analysis [22]). Limits of quantitation are typically below one part-per-billion by volume (ppbv) for headspace analysis using SIFT–MS. Direct analysis using soft chemical ionization means that all compounds were analyzed in a single, two-minute run for each sample. SIFT–MS requires no preconcentration or sample dilution, due to its wide dynamic range and its high robustness to moisture.

The top-rated prime beef samples generally lie in the mid-range of concentration values for the compounds targeted here, except for ethyl acetate, indole, and methanol, which are at the upper end. The repeatability observed for these premium steaks is particularly pleasing, because it illustrates that there is great consistency across the 40 animals that they were obtained from.

Defective beef samples tend to deviate significantly for several target compounds compared to the premium cuts. For example, the samples labeled "over-aged" and two of the "high pH" samples have elevated ammonia concentrations. The over-aged sample has elevated hydrogen sulfide and methyl mercaptan, both of which are extremely pungent; the latter is also detected in the "High pH 1" sample. This means that there is some potential for detecting particular defects by applying thresholds for a handful of markers.

However, certain defects (such as those found in the "Cow 1" and "Cow 2" samples) are not so readily distinguished from premium steaks based on thresholds of several marker compounds. Application of multivariate statistical analysis to the concentration data can be applied to achieve this. Figure 2 shows the class projections obtained when the full data set is processed using the SIMCA algorithm. Each colored point in this plot represents a replicate measurement. For clarity, the more congested region is expanded on the right-hand side of Figure 2. Table 4 summarizes the interclass distances obtained from the SIMCA analysis: all beef classes in this study are separable since the distances are all greater than three (21), including those that look marginal in the class projections plot ("Norm pH", "Cow 1", "Cow 2").

Table 5 summarizes the relative significance of various target compounds in discrimination of the beef samples: the larger the number, the greater the contribution. Ethanol dominates discrimination. Given that the flavor impact of ethanol is lower than the other target compounds, and that ethanol was the key discriminating volatile between grass versus pellet fed cattle (from a parallel, unpublished study), the data was reprocessed using the SIMCA algorithm with both ethanol and methanol removed (methanol was the second most significant discriminator in the feed study). The right-hand column of Table 5 lists the discriminating powers with this revised compound list, while Table 6 gives the interclass distances. The prime beef samples remain well separated from the defective samples, as do most of the defects from each other. However, the two "cow" samples ("Cow 1" and "Cow 2") are no longer completely separable with an interclass distance of 2.3, and "Cow 1" is not entirely distinguishable from the sample labelled "Norm pH".

Conclusion

The results presented here demonstrate that the SIFT-MS technique can effectively discriminate between prime and defective beef flavors when coupled with multivariate statistical analysis. The laboratory-based approach used here enables throughputs of about 30 samples/hour to be achieved. Even in its present form, SIFT–MS shows promise for wider scale flavor quality testing off-line compared to the traditional sensory approach.

Table III: SIFT-MS headspace concentrations (in parts-per-billion by volume, ppbv) of volatile compounds detected in the headspace of prime and defective beef samples averaged over the replicate measurements. Uncertainty is shown as two standard deviations of the mean (2SD) and "n.d." indicates not detected.

Compound	Prime Beef		High pH 2		Norm pH		Cow 1		Cow 2		Bull 1		Bull 2		Over-aged		High pH 1	
	Mean	2SD	Mean	2SD	Mean	2SD	Mean	2SD	Mean	2SD	Mean	2SD	Mean	2SD	Mean	2SD	Mean	2SD
methanol	1110	190	607	93	813	110	476	147	672	117	523	68	269	44	644	218	357	48
ethanol	145	159	65.1	45.2	85.5	41.9	141	24	223	64	57.7	22.2	15.8	48.6	16300	9200	18400	9600
acetone	937	263	635	141	932	153	1020	410	814	266	9470	1540	5000	633	2750	280	450	49
butanone	39.3	17.5	4.5	1.8	8.0	2.0	12.0	6.4	9.5	2.4	464	74	887	149	20.7	5.2	3.9	0.4
acetaldehyde	219	136	35.7	10.8	92.5	18.1	239	170	145	27	192	59	309	63	1760	608	276	116
pentanal	24.5	4.5	4.5	2.1	15.1	2.4	11.1	5.0	6.3	2.6	8.6	1.3	3.4	1.2	57.7	27.2	6.3	4.2
ethyl acetate	921	365	330	103	386	35	393	171	406	151	531	49	115	39	1080	227	295	53
propyl acetate	9.7	3.1	4.3	3.3	3.2	4.2	6.2	1.8	4.5	1.8	19.5	2.2	11.1	2.7	39.8	12.3	8.9	2.7
ethyl butanoate	18.9	16.4	0.7	7.9	4.1	15.1	17.6	15.0	19.0	20.8	41.2	12.2	24.6	7.0	73.4	27.8	12.4	5.1
hydrogen sulfide	1.2	0.5	0.8	1.1	0.5	0.3	0.5	0.7	0.7	0.9	1.0	1.0	1.5	0.9	5.8	3.0	1.3	1.5
methyl mercaptan	n.d.	---	n.d.	---	n.d.	---	n.d.	---	n.d.	---	n.d.	---	n.d.	---	21.4	11.5	23.4	16.2
dimethyl sulfide	66.2	16.9	26.4	4.9	40.3	6.0	37.2	12.2	64.3	10.6	57.9	13.3	329	86	34.4	6.4	26.0	4.9
ammonia	175	37	579	354	159	41.7	138	88	178	36	179	59	109	97	786	442	405	145
trimethylamine	54.4	12.3	41.1	8.4	54.7	8.4	63.3	24.8	48.6	14.1	552	98	302	47	169	16	30.0	2.3
indole	21.7	21.4	12.0	12.0	9.4	10.2	9.8	14.8	15.0	5.3	9.6	2.3	9.8	5.7	20.9	5.9	3.5	1.9
skatole	4.8	2.8	2.9	2.3	2.5	2.9	3.3	1.8	3.1	1.6	4.1	0.9	4.2	1.5	15.6	7.4	2.5	1.4

The goal, however, is to take this demonstrated flavor-screening potential to an in-line or near-line application. To achieve this, sample throughput needs to be enhanced twentyfold, to meet the needs of modern beef processing lines. This requires both optimization of the SIFT-MS analytical method and rapid heating of the beef sample to facilitate faster release of flavor compounds. A possible rapid sampling approach could utilize the i-knife, which was invented for surgical applications, but has recently been evaluated for meat (31).

Author Contributions

Nicola Simmons, Clyde Daly, and Vaughan Langford designed the experiments. The late Tracey Cummings coordinated sensory analysis and selected samples for instrumental analysis. Langford conducted the instrumental analysis and processed the data. Langford and Murray McEwan wrote this article.

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Table IV: Interclass distances obtained from SIMCA multivariate analysis of the beef headspace data in Table III using the full compound list. The orange shading highlights the separation between premium and defective samples, while the gray shading indicates the region with smaller separations.

Sensory descriptor	Prime Beef	High pH 2	Norm pH	Cow 1	Cow 2	Bull 1	Bull 2	Over-aged
High pH 2	15							
Norm pH	8.2	14						
Cow 1	8.5	5.6	4.1					
Cow 2	7.0	7.3	4.3	3.3				
Bull 1	51	57	30	45	64			
Bull 2	35	41	32	26	43	26		
Over-aged	56	60	57	56	55	61	58	
High pH 1	174	226	226	166	191	216	210	13

Table V: Discriminating powers (DPs) from SIMCA multivariate analysis of the replicate SIFT-MS headspace data (Table III). Calculations were made for the full compound list and for the list without ethanol and methanol.

Compound	Discriminating power	
	All targets	No ethanol or methanol
Ethanol	185000	—
Butanone	6360	12300
Acetone	2340	8140
Trimethylamine	1600	3300
Methanol	1550	—
n-Propyl acetate	1100	1200
Ethyl butanoate	836	1120
Skatole	642	1580
Dimethyl sulfide	547	715
Indole	479	583
Ethyl acetate	370	1050
Acetaldehyde	331	1580
Pentanal	109	792
Hydrogen sulfide	103	374
Methyl mercaptan	74	66
Ammonia	47	456

Table VI: Interclass distances obtained from SIMCA multivariate analysis of the beef headspace data in Table III using the compound list with ethanol and methanol removed. The orange shading highlights the separation between premium and defective samples, while the gray shading indicates the region with smaller separations.

Sensory descriptor	Prime Beef	High pH 2	Norm pH	Cow 1	Cow 2	Bull 1	Bull 2	Over-aged
High pH 2	29							
Norm pH	18	17						
Cow 1	8.9	8.9	2.9					
Cow 2	6.6	21	3.3	2.3				
Bull 1	15	64	36	60	28			
Bull 2	56	102	59	42	57	32		
Over-aged	20	30	28	15	28	76	44	
High pH 1	16	5.8	12	10	10	137	93	37

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A Simple and Effective ICP-MS Method Capable of Detecting Trace and Major Elements in Milk Samples

Photo credit: Joe Zugic Photography

To address the challenges associated with a broad, complicated food and beverage supply chain, innovative solutions for contaminant detection are continually being sought. Easy-to-operate instrumentation and methodology can be adopted over the entire food supply chain, allowing contaminated food samples to be identified more readily. Here, we describe a simple method capable of identifying and quantifying a diverse range of elements, including trace metals that are toxic in low concentrations, and also the major elements that are essential to healthy sustainable life. Inductively coupled plasma mass spectrometry (ICP-MS) is the definitive technique used to identify and quantify potential metal contaminants; here it has been applied to the metals present in samples of milk powder and evaporated or condensed milk. The ICP-MS method used enabled the detection of metals with quantitation limits to the order of parts per billion. It was found that all milk samples contained elemental concentrations below those levels directed by regulation, with milk powders containing the highest concentrations of major metal elements.

Mass spectrometry has increasingly become associated with the detection of contaminants and nutrients in food, overtaking other less common methods of detection. Under the umbrella of mass spectrometry are inductively coupled plasma mass spectrometry (ICP-MS) and liquid chromatography and tandem mass spectrometry (LC–MS/MS), which have both been adopted increasingly by researchers throughout the world to identify and quantify contaminants and nutrients in food. As demonstrated by the 2017 fipronil contamination scare (1), there is an industrial necessity to provide reliable analysis methods that can be applied by any food scientist within any processing facility. In tandem with the detection of contaminants, food must also be nutritious, supplying consumers with the essential components of life, such as the metals that are involved in important biological processes.

Elements are the building blocks of life, and we need to ensure that we receive the right mixture of elements at the appropriate dosage for our continued sustainable development and healthy growth. Of the 118 elements on the periodic table, the majority are metals. For humans, receiving the appropriate levels of certain metals is vital to our existence. Iron, for instance, is an essential component of hemoglobin, and assists in transporting oxygen throughout the body. Other major elements include sodium, potassium, magnesium, copper, and zinc, all of which facilitate a range of biological functions and roles within the human body, making them nutritionally essential. Therefore, in order to function appropriately, we need to be sure we are accumulating the right mixture of elements at the appropriate levels. Dosage of any element is important; an element must be present at a safe enough level to provide functionality, but not so high that it becomes toxic. This means reliable quantitation is essential.

However, there are also those elements that are toxic even in low concentrations.

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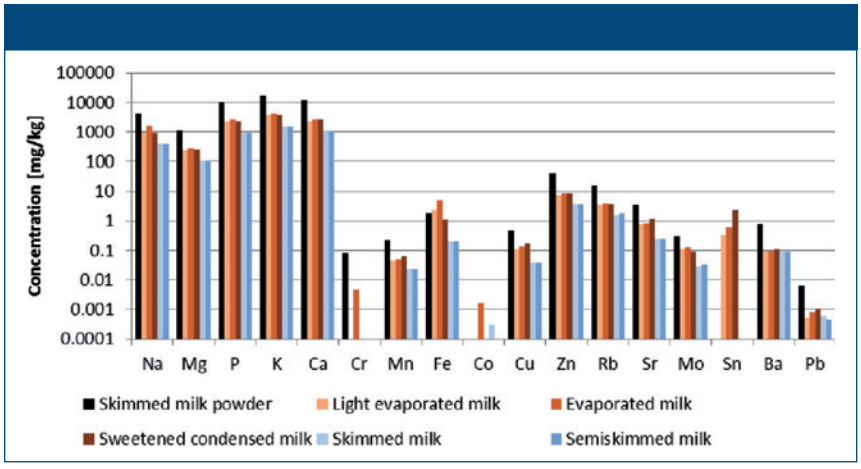

Figure 1: Results of analysis for store-bought samples (milk powder in black; evaporated milks in shades of orange; UHT milks in shades of blue-grey). Values below the LOQ have not been plotted.

Table I: ICP-MS setup parameters

Component Parameter	Type or Value
Nebulizer	PFA-ST
Spray chamber	Glass cyclonic at 2 °C
Injector	2.0 mm id quartz
Sample uptake rate	260 µL/min
Mixing tee	On-line addition of internal standards
RF power	1600 W
Collision flow 1	3.8 mL/min (for As, Se, Ge)
Collision flow 2	4.7 mL/min (for all remaining elements)
AMS dilution	10 x

For instance, the metalloid arsenic (2), and heavy metals such as mercury (3), lead (4), and cadmium (5) have been associated with a variety of health issues. While heavy metals occur naturally, their release into the environment is often a result of anthropogenic activities that are commonly industrially related (6), which can facilitate their dispersion into the food chain. To address the variety of health issues associated with heavy metal contamination, government or independent health experts have imposed maximum levels of metals in foodstuffs to further protect the public.

ICP-MS itself is becoming renowned as the method of choice for metal analysis across a variety of applications from pharmaceutical development and cancer research (7) to environmental and food analysis (8,9). Specifically considering its use in the food industry, ICP-MS has been used across a variety of applications such as arsenic detection in rice (10). One of the specialties of ICP-MS is its intrinsic use of plasma that completely ionizes the entire sample, ensuring the detection of all metals or analytes. Methods such as atomic absorption spectroscopy and ICP-OES have been successfully applied to the analysis of metal elements within samples of food (11). However, both these methods lack either the multi-element detection capabilities or the sensitivity that ICP-MS offers.

For food samples, the ability to identify and quantify the various metal elements within a given sample is essential both for contaminant detection and accurate nutrient reporting. Here, we describe a fast and simple method capable of detecting a dynamic range of elements to a high level of reliability and accuracy within various samples of milk with variable fat levels. Milk is well known as a source of nutrition in the early development of children. During these early stages, confidently knowing the quantities of nutritional elements being ingested is critical to healthy growth. This underpins the need for a useful methodology that can provide simple solutions to food scientists across the globe (12).

Experimental

Six samples of milk were purchased from local stores for analysis. Samples were chosen to provide a concise coverage of various types of milk with a varied concentration of fat. The forms of milk used were: skimmed milk powder (non-fat), light evaporated milk (4% fat), evaporated milk (9% fat), sweetened condensed milk, skimmed milk (ultra-high temperature (UHT) milk, <0.5% fat), and semi-skimmed milk (UHT, < 2% fat). For experimental controls, three certified reference materials were also obtained. Two European Reference Materials (ERMs) were obtained from the Joint Research Centre of the European Commission, denoted as ERM-BD 150 and ERM-BD 151. The National Measurement Institute of Japan (NMIJ) sample, NMIJ 7512-a, was obtained from GL Sciences B.V.

Milk samples of varying weights (5 g of UHT milk, 2 g evaporated milk, 1 g condensed milk, and 0.5 g milk powder) were digested with a combination of concentrated 2.5 mL nitric acid (Fluka, TraceSelect Ultra) and 2.5 mL 30% hydrogen peroxide (Sigma-Aldrich, H_2O_2 ≥30%, for Ultratrace analysis). Samples were made up to equivalent volume by the addition of water, which was altered to account for the varying concentrations in the different types of milk (0 mL for UHT milk, 3 mL for evaporated milk, 4 mL for condensed milk, and 5 mL for milk powder). Samples were then heated using a Titan MPS Microwave Sample Preparation System using standard 75 mL vessels. Digests were transferred to 50 mL autosample tubes and spiked with 10 µL of 1000 mg/L gold solution before being made up to 50 mL with deionized water.

Analyses were performed using the NexION 2000 P ICP-MS instrument (Perkin Elmer) with sample detection achieved using a collision mode to reduce elemental interference. The technical parameters for the instrument are reported in Table I. No modifications were made to the instrument and the default sample introduction system was used.

Results and Discussion

Detection of Major and Trace Elements in Milk Samples

Before we discuss the analysis of store-bought milk samples in detail, the methodology of sample preparation and

Table II: Analysis of NMIJ 7512a milk powder

Element	NMIJ 7512a		
	Measured (mg/kg)	Certified (mg/kg)	Recovery
Na	1847	1870	99%
Mg	804	819	98%
P	5499	5620	98%
K	8231	8410	98%
Ca	8204	8650	95%
Mn	0.879	0.931	94%
Cu	4.59	4.66	99%
Zn	40.5	41.3	98%
Rb	8.67	8.93	97%
Sr	5.68	5.88	97%
Mo	0.213	0.223	95%
Ba	0.436	0.449	97%

comparisons between the control and current methods should be explained in further detail. The first important step in preparing samples for analysis is the removal of the fat content. Milk samples vary in the quantity of fats. For instance, skimmed milk powder is considered to contain no fat, whereas evaporated milk typically contains 9% fat. Excess fat in the samples will negatively affect the performance of the ICP-MS. Therefore, excess fat must be broken down prior to sample analyses to ensure quality and consistency between samples and the uninhibited detection of the elements.

To break down fats, the sample is digested. While there are several conceivable methods of achieving this, the method employed here involves a mixture of nitric acid and hydrogen peroxide. This mixture breaks down the proteins and makes the sample more viscous. It is then necessary to heat samples, which facilitates digestion. The method used here involves the use of a microwave to ensure uniform heating across the entire sample. An alternative could involve using heating blocks, however, this might result in uneven heating.

Following digestion, the certified reference materials are analyzed to demonstrate the accuracy of the methodology being used. The results of NMIJ 7512-a milk powder sample and ERM-BD 150 and ERM-BD 151 skimmed milk powders are reported in Tables II and III, respectively. Recovery rates (the difference between the measured and certified samples) are in the range of 89–107%, 93–105%, and 94–99% for the three CRM samples, respectively. These excellent recovery rates demonstrate an overall agreement between the control experimental levels and the presently employed methodology, meaning we can be confident in the elemental breakdown of the milk samples.

Next, the limits of quantitation (LOQ) must be discussed further. These limits are essential in order to identify the limitations of the instrumentation and determine whether results obtained are trustworthy. The limits of UHT milk samples were calculated following recommendations of the Commission Regulation (EC) No 333/2007 (13) as 10 times the standard deviation of 10 consecutive blank measurements. Milk powder LOQs were taken as 100 times the limit of the UHT samples.

One of the challenges of using ICP-MS is in the potential for the interference of elements that can lead to false positive element detection. This is a common issue, and is the result of cross-reactivity between various other species. For instance, $^{40}Ar^{16}O$ is a common interfering species of ^{56}Fe (14). In this example, to prevent false detection of iron, the argon oxide analyte needs to be removed. This is where collision cells become useful. Collision cells work on the principle that the interfering ion, in this case ArO^+, is much larger than the analyte, in this case Fe^+. Upon ionization, both species travel through a stream of inert gas. The larger analytes are more likely to collide with gas particles that remove them from the mass spectrometer instrument. The intensity of all analytes is reduced, including the desired analyte as a result of a low proportion of collisions with the inert gas stream. However, any analyte intensity reduction is offset by the reduction in the intensity of the interference.

Analytes of any form are then compared against regulation approved levels to ensure that the food is safe. Levels of specific metals must be within defined limits, typically sub parts per million. As defined by the Commission Regulation (EC) 1881/2006, the regulated levels for lead and tin in milk are 0.02 and 50 mg/kg, respectively (15). In this instance, it was possible to detect metals to the level of singular parts per billion, well below the relevant limits. The measured LOQs for lead and tin in milk powders are 0.0017 and 0.012 mg/kg, respectively, several orders of magnitude below the limits. In addition, the LOQ for mercury was found to be 0.0083 mg/kg, indicating the potential to detect ultralow levels of mercury in milk. LOQs to this order of magnitude below the regulatory limits add credence and confidence to the results of the experiment.

Using the methodology outlined in the experimental section, the store-bought samples were analyzed to identify and quantity a range of elements. This included the so-called essential major elements such as sodium, magnesium and calcium, amongst others, and also the toxic trace elements such as mercury, cadmium and lead. Measured concentrations for the various samples of milk are reported in Figure 1. Any sample with a reported measurement below the LOQ has not been plotted.

From Figure 1, it is clear that the concentrations of major elements are highest in milk powder samples, followed by condensed and evaporated milk samples and finally are lowest in the UHT samples. Certain spikes in samples can potentially be explained as a result of the storage method of the milk. For instance, the elevated levels of tin in evaporated and condensed milk have been hypothesized to be a result of their storage in tin-steel cans. This phenomenon is not unique to milk, and in fact has previously been detected in other examples of canned foods (16).

Fundamentally, this method offers an advantageous analytical technique for the analysis of metal elements over previous instrumentation and methodology due to its

Table III: Analysis of ERM-BD 150 and 151 skimmed milk powders

	ERM-BD 150				ERM-BD 151		
Element	Measured * (mg/kg)	%RSD*	Certified (mg/kg)	Recovery	Measured (mg/kg)	Certified (mg/kg)	Recovery
Na	4074	1.5	4180	97%	4127	4190	98%
Mg	1225	1.9	1260	97%	1242	1260	99%
P	10368	2.4	11000	94%	10829	11000	98%
K	16343	1.6	17000	96%	16766	17000	99%
Ca	12499	1.4	13900	90%	12927	13900	93%
Mn	0.274	3.9	0.289	95%	0.286	0.29	99%
Fe	4.72	4.3	4.6	103%	49.7	53	94%
Cu	1.04	1.2	1.08	96%	5.05	5.00	101%
Zn	45.3	1.7	44.8	101%	45.5	44.9	101%
Se	<LOQ	…	0.188	…	<LOQ	0.19	n/a
Cd	<LOQ	…	0.0114	…	0.100	0.106	94%
Hg	0.0640	8.5	0.060	107%	0.545	0.52	105%
Pb	0.0170	4.3	0.019	89%	0.200	0.207	97%

*Result of 7 individual measurements

twofold advantages. First, it presents a simple method to reliably quantitate a dynamic range of elements that include both trace toxic metals and those essential for health and well-being within milk samples. Secondly, it demonstrates the ability to implement a relatively simple methodology for the employment of metals detection.

Summary

This method provides an analytical technique for the analysis of metal elements in milk demonstrating a twofold advantage. First, it provides a simple method for quantitative analysis over a wide dynamic range of elements, including both toxic metals and those metals essential for health and well-being. Secondly, it demonstrates a simple methodology for a wide spectrum detection of metals.

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Comprehensive Identification of Migrating Compounds from Plastic Food Packaging Materials Using High-Resolution Mass Spectrometry

Photo credit: Syda Productions / stock.adobe.com

In order to ensure the continued use of plastic packaging in food, the packaging used must be safe for contact with food products, and not cause contact contamination. Of rising concern is the role of non-intentionally added substances (NIAS). Therefore, this study assesses whether two commonly used plastic packaging materials, polyethylene and low-density polyethylene plus nylon, are safe for use. Their potential migrants are analyzed using data-independent acquisition and liquid chromatography coupled with high-resolution accurate mass spectrometry (HRAMS). Using automatic detection capabilities and filtering procedures, MS and tandem mass spectrometry (MS/MS) data were processed to find chemical formulae, and structures and toxicity information for the most abundant analytes in a given sample. Through this method, 26 migrating compounds, mainly cyclic oligomers, were identified. Using the toxicity rules set out by Cramer, 19 of these 26 compounds were identified as having moderate or high toxicity.

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Food packaging is a major market segment in the global food industry, often compared in size to that of the pharmaceutical industry (1). Over one third of all food packaging comprises materials made from plastic (1,2), which over the past several decades has extended to different plastic materials, copolymers, and additional ingredients.

Packaging itself is essential to our modern society. As our food supply chain has grown to become more global, plastic packaging has been instrumental in ensuring that the food reaching consumers is safe to eat. Packaging helps to preserve food by protecting it from light, humidity, oxygen, foreign compounds, mechanical influences, and microbial contamination, all of which help to improve shelf life (1). Two polymers used frequently in food packaging include polypropylene and polyethylene, with the latter being used in various forms including low-density polyethylene (LDPE), linear low-density polyethylene (LLDPE), and high-density polyethylene (HDPE) (3).

However, while food packaging protects from external contaminant introduction, there is increasing concern about the potential of chemical contamination from the plastic packaging itself. In fact, food contact migration is reported as one of the largest sources of food contamination, and is approximately 100–1000 times higher than that caused by pesticide residues (3,4). This migration can occur from multiple sources, including from contact with the internal face of the packaging, from diffusion and partition processes within multi-layered plastics, or even from adhesives.

The potential health implications that plastic contamination poses to the consumer have resulted in the control and supervision of plastic materials in multiple countries. For instance, the European Union has imposed Regulation EU 10/2011, which has established specific rules for what plastic materials can be applied safely to food packaging, and lists specific compounds that are authorized for use in plastic formulations and manufacturing (5). But, this regulation does not take into account unintentional sub-

Table I: Source parameters in positive polarity

Ion source gas 1	40 psi
Ion source gas 2	50 psi
Curtain gas	25 psi
CAD gas	7 psi
Temperature	450 °C
Spray voltage	5500 V
Resolution power of the TOF system	32,000 FWHM (for *m/z* 200)

Table II: Data-independent acquisition parameters used in the full scan mode

Accumulation time	0.2 s
Declustering potential	80 V
TOF start mass	100
TOF stop mass	950

Table III: MS/MS mode parameters

Accumulation time	0.2 s
TOF start mass	50
TOF stop mass	950
Generic collision energy	35 ±15 V
Isolation windows in Q1	10, divided as follows: 100–185, 184–270, 269–355, 354–440, 439–525, 524–610, 609–695, 694–780, 779–865, and 864-950
Total cycle time	0.78 s

stances, often referred to as *non-intentionally added substances* (NIAS), meaning they frequently do not appear in lists of permitted ingredients. NIAS may be introduced through a variety of processes: a result of interaction between ingredients, degradation of material components, or from impurities within the raw material (6). As a result, the migration of non-authorized substances must not exceed a level of 0.01 mg/kg of food or simulant.

To meet these strict criteria, highly sensitive and advanced analytical instruments and techniques are needed to test samples for the presence of NIAS. For nonvolatile chemicals in particular, liquid chromatography coupled to high-resolution accurate mass spectrometry (LC–HRAMS) has a proven history identifying nontarget compounds (7). In addition, nontargeted screening approaches assist in identifying potentially unknown contaminants compared to other acquisition methods. This technology has already been successfully applied to proteomics and metabolomics (8), as well as clinical and forensic toxicology (9).

The present study investigates the migration of compounds from two different multilayer plastic packaging materials commonly used in fruit puree and juices. Using an LC–QTOF instrument, coupled with data-independent acquisition, non-targeted peaks were identified and concentrations were compared against those dictated by EU regulation. Following the successful identification of molecular formulae and structures, the toxicity of these structures was assessed

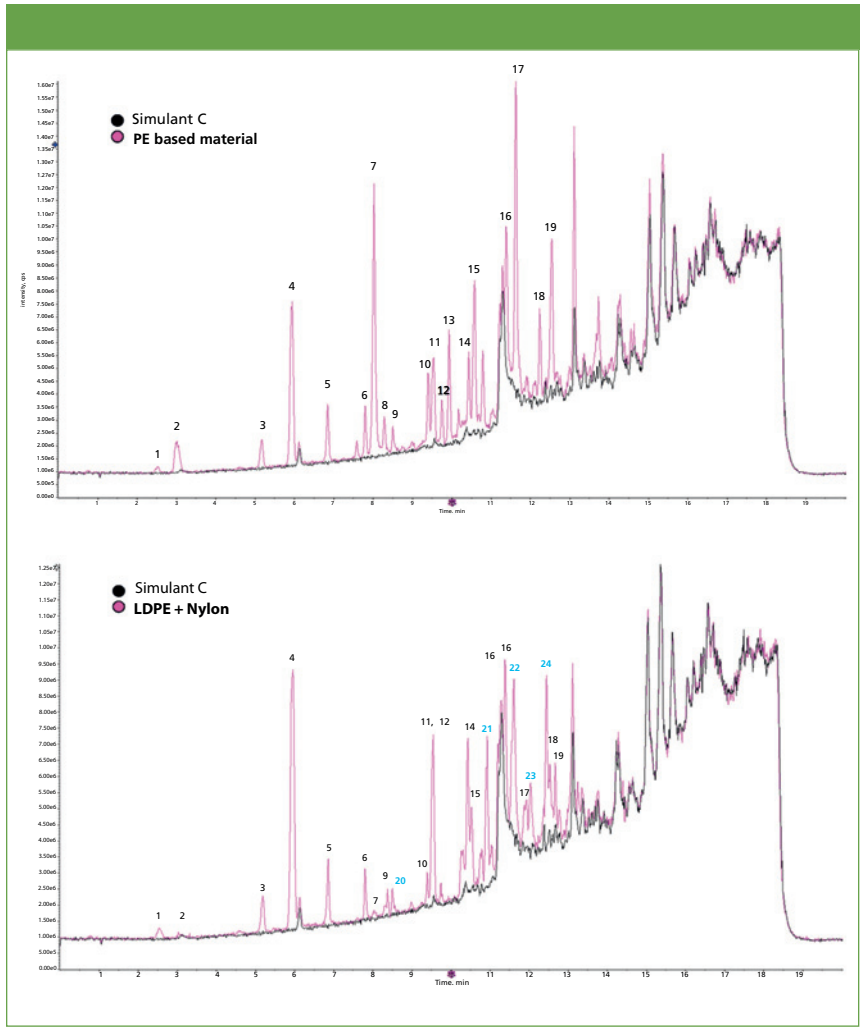

Figure 1: Spectra of the two materials obtained using simulant C. The most abundant peaks are identified using compound ID numbers, the identities of which are reported in Table IV.

to determine the potential harm such NIAS pose to consumers.

Experimental

Method and Apparatus

Two commercially available multilayer plastic materials, used for the packaging of purees and juices from fruits and vegetables, were purchased over the internet for testing. The two multilayered materials used were: a polyethylene based material, and an LDPE plus nylon material.

The migration test was performed according to the procedures established by EU Regulation 10/2011. The standardized test conditions, that simulate the long-term storage of food at or below room temperature, involve subjecting the material to 10 days of contact time with a contact temperature of 40 °C. Migration tests were performed using 1 dm^3 surface area. This surface area was chosen as the regulation has previously established that materials can now be manufactured in such a way that the material is not releasing more than 10 mg per 1 dm^3 of the plastic material.

Two simulants were used to measure the impact of material contact: simulant B, which consists of a solution of 3% acetic acid in water (w/v), and simulant C, which is a solution of 20% ethanol in water (v/v). For each material, 10 x 10 cm of plastic material was added to a 50 mL PTFE tube with 35 mL of the relevant simulant, in which carbendazim-d3 and malathion-d10 were added to the simulants as internal standards for quality control purposes. Three replicates and one control were performed. Each tube was placed in an incubator for 10 d at 40 °C. Afterwards, one aliquot of each sample was directly injected and analyzed by LC-HRAMS along with a calibration curve of ε-caprolactam.

The liquid chromatography separation was carried out using an Exion LC (Sciex). Mobile phase A was made up from 98% water and 2% methanol, and mobile phase B was made up from 98% methanol and 2% water. Both phases contained 5 mM of ammonium formate and 0.1% formic acid. Separation was carried out using a Zorbax Eclipse Plus C8 column (Agilent) with a length, diameter and particle size of 100 mm, 2.1 mm and 1.8 µm, respectively. The column was thermostatted at 35 °C. The mobile phase gradient started from 80% of mobile phase A, which was maintained for 2 min. From 2 to 15 min, the amount of mobile phase B increased linearly to 100%, which was then maintained for 2 min. The mobile phase was then changed to 80% A, maintained for 3 min for re-equilibration, leading to a total running time of 20 min. An X500R (SCIEX) mass spectrometer equipped with Turbo V Source with a Twin Sprayer probe was used for acquisition. The data-independent acquisition tool SWATH Acquisition was used to analyze the data. A mixture containing 10 compounds with masses in the range of 132.9049–2034.6255 g/mol was used for calibration. SCIEX OS 1.3 was used for qualitative and quantitative analysis. The source parameters for the mass spectrometer are reported in Tables I–III.

Control samples were run in similar conditions, which are left to stand for 10 days at 40 °C in the PTFE tube. Control samples were then compared with the three samples and used to filter out chemical peaks where the difference in intensity of the peaks was deemed to be negligible.

Results and Discussion

Identifying Migrant Compounds

A data-independent non-targeted acquisition mode was used to analyze the analytes. This approach segments the full scanned mass range into smaller segments for simpler MS/MS analysis, reducing the complexity of the fragmentation spectra. This allows for a retrospective analysis of the data, while ensuring that overlapping analyte peaks are not missed. This analysis proved vital in this study, owing to the complex overlap of spectra.

Using the strategy outlined in the experimental section, a total of 3149 spectral features were identified in the polyethylene-based material. Using the filtering strategy, the number of elucidating ions decreased

from 3149 to 57 (which is characteristic of polyethylene packaging material), which then further decreased to only 21 ions after focusing on the most abundant compounds (those with a signal intensity above 1 x 10^6). However, to date, there is no library of NIAS substances to screen against the identified compounds, and none of the relevant ions were present in the commercial MS/MS spectral library used (SCIEX). MS/MS libraries are constantly evolving but still only cover a limited number of analytes. For those compounds whose MS and MS/MS had the lowest associated error, probable structures were constructed using ChemSpider and then modelled in silico to compare fragmentation patterns against those observed. Owing to the numerous possible structures, chemical intuition and prior knowledge of the substance was essential to rule out many database structures.

From the two packaging materials, a total of 26 migrating compounds were identified from the screening method. Of these, 21 were assigned a plausible structure, which was later confirmed by fragmentation and bibliography agreement. A further 3 migrants were assigned a plausible structure based on fragmentation data, but these could not be confirmed. The final two structures could only be assigned molecular formulae, with not enough information collected to accurately assign structures. Spectra for the two materials using simulant C are reported in Figure 1. The summarized identity of those compounds identified is summarized in Table IV.

Most migrants were identified in both materials. The only compound listed in Regulation EU 10/2011 was caprolactam, a monomer of nylon (compound 2). It was found that caprolactam was present at 42 mg/kg in the polyethylene material and 9.33 mg/kg in the LDPE + nylon material. As European legislation permits concentrations of only 15 mg/kg, the migrant compound caprolactam is present at more than two-fold higher concentrations in the polyethylene-based material (5). In addition, four cyclic oligomers of caprolactam were detected in both materials corresponding to the dimer, trimer, tetramer, and pentamer (compounds 4, 3, 5, and 6 respectively). This phenomenon is not surprising as oligomers up to the octamer have previously been identified in other migration studies (3, 9, 10).

Two other substances were identified: the plasticizer bis (2-methoxyethyl) adipate (compound 9) and diethyl 5-({[(2,4,5-trimethoxybenzoyl)oxy]acetyl}amino) isophthalate (compound 15). These were the results of the best matches based on identified chemical structures and fragmentation patterns.

All other compounds identified were classified as NIAS, with the majority being oligomers. The main migrants were oligomers from polyurethane adhesives. Cyclic ester oligomers were found to be made up of the monomers adipic acid (AA), phthalic acid (PA), diethylene glycol (DEG), monoethylene glycol (MEG) and neopentilglycol (NPG) in the combination 1:1 (AA-DEG, PA-DEG), 1:2 (PA-DEG-DEG), 2:2 (AA-MEG-AA-MEG, AA-DEG-AA-DEG, PA-DEG-PA-DEG), 1:1:2 (AA-MEG-AA-DEG, AA-DEG-PA-DEG) or 1:1:1:1 (PA-MEG-AA-DEG, PA-DEG-AA-NPG). However, once again most of the oligomers were not present in any library database

Table IV: Compounds identified in the polyethylene and LDPE materials using simulants B and C

ID	Elemental Composition	Mass / g mol-1	Compound	TC
1	$C_{12}H_{22}N_2O_2$	227.1754	1,8-diazacyclotetradecane-2,9-dione	I
2	$C_6H_{11}O$	114.0913	Caprolactam Total Specific Migration Limit: 15 mg/kg (RD 10/2011)	III
3	$C_{18}H_{33}N_3O_3$	340.25947	Caprolactam trimer 1,8,15-Triazacyclohenicosane-2,9,16-trione	III
4	$C_{10}H_{16}O_5$	217.10705	Caprolactam dimer 1,4,7-Trioxacyclotridecane-8,13-dione	III
5	$C_{24}H_{44}N_4O_4$	453.34353	Caprolactam tetramer 1,8,15,22-Tetraazacyclo-octacosane-2,9,16,23-tetrone	III
6	$C_{30}H_{55}N_5O_5$	566.4276	Caprolactam pentamer 1,8,15,22,29-Pentaazacyclopentatriacontane-2,9,16,23,30-pentone	III
7	$C_{12}H_{12}O_5$	237.07575	3,4,6,7-Tetrahydro-2,5,8-benzotrioxacycloundecin-1,9-dione	I
8	$C_{16}H_{20}O_7$	325.12818	3,6,9,12,15-Pentaoxabicyclo(15.3.1)henicosa-1(21),17,19-triene-2,16-dione	III
9	$C_{12}H_{22}O_6$	263.14891	Bis(2-methoxyethyl) adipate	I
10	$C_{16}H_{24}O_8$	345.15439	1,6,11,16-tetraoxacycloicosane-2,5,12,15-tetrone	I
11	$C_{18}H_{28}O_9$	389.18061	AA-MEG-AA-DEG	III
12	$C_{20}H_{32}O_{10}$	433.20682	AA-DEG-AA-DEG	III
13	$C_{22}H_{28}O_{10}$	453.17552	AA-DEG-PA-DEG	III
14	$C_{20}H_{24}O_9$	409.14931	PA-MEG-AA-DEG	III
15	$C_{20}H_{27}NO_{10}$	490.17077	Diethyl 5-({[(2,4,5-trimethoxybenzoyl)oxy] acetyl}amino) isophthalate	III
16	$C_{24}H_{24}O_{10}$	473.14422	PA-DEG-PA-DEG	III
17	$C_{19}H_{33}O_8$	404.22789	22 database possibilities	N/A
18	$C_{23}H_{30}O_9$	451.19626	PA-DEG-AA-NPG	III
19	$C_{21}H_{29}NO_8$	424.19659	83 database possibilities	N/A
20	$C_{10}H_{16}O_4$	201.11214	1,6-dioxacyclodecane-7,12-dione	I
21	$C_{20}H_{32}O_9$	417.21191	AA-DEG-AA-DEG -O	III
22	$C_{22}H_{28}O_9$	437.18061	PA-DEG-AA-DEG - O	III
23	$C_{20}H_{32}O_8$	401.21699	1,6,13,18-Tetraoxacyclotetracosane-2,5,14,17-tetrone	I
24	$C_{24}H_{24}O_9$	457.14931	PA-DEG-PA-DEG - O	III
25	$C_{10}H_{18}O_6$	235.11761	AA-DEG + H_2O	I
26	$C_{22}H_{30}O_{11}$	471.18609	PA-DEG-AA-DEG + H_2O	III

consulted, and therefore identification was made based on a bibliographic search of their molecular masses. All oligomers were identified in the literature except for 3,6,9,12,15-Pentaoxabicyclo(15.3.1)henicosa-1(21),17,19-triene-2,16-dione, an ester whose appearance was attributed to the conceivable combination of monomers present in the materials.

Only four substances were present only in the LDPE and nylon material. These are labelled as peaks 20–24 in Figure 1. Compounds 21, 22 and 24 had not been fully described in the literature, and there is currently no thorough hypothesis or explanation for the formation of these compounds in the present study. However, the peaks displayed similar characteristics to other oligomers already identified in the simulants. Peak 20 was identified as 1/6-dioxacyclodecane-7,12-dione and is attributed in the literature to the presence of polyurethane adhesives used to laminate packaging multilayer materials (11). Compound 23 was identified as 1,6,13,18-tetraoxacyclotetracosane-2,5,14,17-tetrone, also found in compostable adhesives (12).

The two peaks that were not identified are those labelled as 17 and 19 in Figure 1. It was only possible to assign the most probable chemical formula, identified as $C_{19}H_{33}NO_8$ and $C_{21}H_{29}NO_8$. Due to the number of potential structures in the databases (22 and 83 possibilities respectively), it was not possible to identify a specific structure.

The chromatographs for the migration of compounds for simulants B and C are overall very similar. The overlapping chromatographs are shown in Figure 2. Only two peaks were identified as exclusively present in simulant B, labelled as compounds 25 and 26. Based on individual masses, these molecules were identified as the cyclic oligomers AA-DEG and AA-DEG-PA-DEG with a molecule of water.

Packaging Material Toxicity

The toxicity of individual NIAS was then assessed to determine whether those compounds identified in the analysis are hazardous to public health. A bibliographic search for their toxicity found that the majority have not been registered in EU regulation. Thus, a theoretical assessment was performed using the Threshold of Toxicological Concern (TTC) approach (13–15). TTC is based on Cramer rules, which assigns the toxicity of compounds based on the molecular structure into the following categories: low (class I), moderate (class II), and high (class III). Cramer has also devised a maximum recommended intake value for each compound class of 1.8, 0.54, and 0.09 mg/person/day, respectively. Using these rules, only 7 of the identified compounds were identified as low toxicity, with the results for all compounds summarized in Table IV.

Due to the potential toxicity these compounds pose, their presence in plastic materials intended for food could generate a risk for consumers. Given the peak

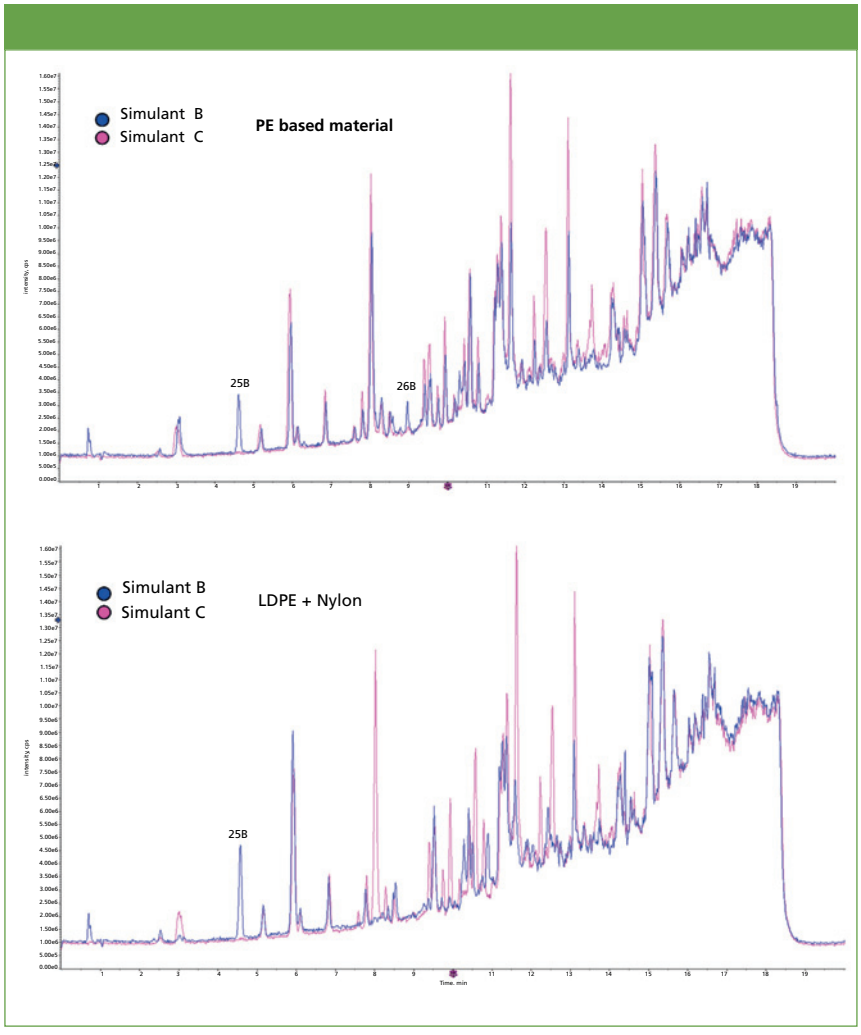

Figure 2: Overlapping spectra of the two materials using simulants B and C. Compounds 20–24 are only observed in simulant B and are identified in Table IV.

intensity, and the high presence of caprolactam, the polyethylene-based material could pose a higher risk to consumers compared to the LDPE material.

Conclusions

This study sought to identify the potential migrating substances from food packaging contact. Two packaging materials were tested, polyethylene and low-density polyethylene plus nylon. A total of 26 substances were identified in this study, combining those identified in the LDPE and polyethylene materials: 21 have been assigned a confirmed structure, three have a tentative assigned structure and the final two only have assigned molecular formulae. The majority of the compounds are considered by Cramer's rules to be of moderate or high toxicity meaning their presence should be restricted to low concentrations. Only one compound identified is listed in the Regulation (EU) 10/2011: caprolactam, which was found to be above suggested concentrations in the sample of polyethylene tested. This work has shown the relevance and importance of evaluating NIAS in food contact materials to ensure consumer and food safety.

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Immunoaffinity Solid-Phase Extraction with HPLC-FLD Detection for the Determination of Aflatoxins B2, B1, G2, and G1 in Ground Hazelnut

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Aflatoxins, a group of mycotoxins, are highly toxic substances and very harmful to human health. Consequently, the European Commission has set very low maximum levels for aflatoxins in various foodstuffs. Aflatoxin B1 is considered to be extremely toxic to human health, but also the aflatoxins B2, G2, G1, and the milk-derived derivatives M1 and M2 are in focus and demand rigorous analytical control. Trace-level quantification of these toxins requires very sensitive and reliable analytical methods. This work describes the determination of four aflatoxins in ground hazelnut by immunoaffinity solid-phase extraction (SPE)–based sample preparation and subsequent HPLC measurements with fluorescence detection without pre- or post-column derivatization. The use of a selective immunoaffinity-SPE and fluorescence detection enables very low detection limits to fully meet the requirements. The separation was achieved on a C18 column within 4 min under isocratic condition. Good linearity and recovery rates qualify the method as a fast, reliable and selective solution for the quantitative determination of aflatoxins in ground hazelnut.

Sylvia Grosse, Mauro De Pra, and Frank Steiner

Mycotoxins are naturally occurring fungal toxins that were first found in the fungus *aspergillus flavus.* The aflatoxins (AFs) are one prominent subgroup, of which 20 naturally occurring forms are known. AF–B2, AF–B1, AF–G2, and AF–B1, as well as the milk-derived variants AF–M1 and AF–M2, are the major compounds out of this group. Nuts, grains, herbs, and spices are common sources of aflatoxin contamination, while the M derivatives are found in dairy products. In particular, AF–B1 is considered to be a very potent carcinogen, which mainly affects the liver, and may therefore cause liver cancer, as well as chronic hepatitis, jaundice, cirrhosis, and impaired nutrient metabolism (1). Due to their high thermal stability, they are not destroyed during the production process or cooking procedures (2).

The European Commission has set various maximum levels for aflatoxins in several foods under consideration of their consumption and use. The maximum level for aflatoxin B1 ranges from 2 to 12 µg/kg for foods used for direct consumption or as ingredients, with the exception of baby food products with a maximum level of 0.10 µg/kg. Secondly, the sum of AF–B2, AF–B1, AF–G2, and AF–G1 must not exceed 4 to 15 µg/kg (3). As a result, a sensitive and accurate analytical method is required to control the levels of these toxins in various foodstuffs.

Whereas thin-layer-chromatography (TLC) was previously used for the determination of B and G aflatoxins, nowadays high performance liquid chromatography (HPLC) coupled to fluorescence detection (FLD) with and without derivatization, as well as mass spectrometry (MS), is commonly applied (4). While MS provides additional peak identification potential, it is a relatively costly technique that requires a certain level of qualification of the analyst, and is therefore not available in all food analysis laboratories in emerging countries. On the other hand, derivatization techniques require additional instrumental

effort as well, and may also negatively affect method robustness and transferability.

A powerful alternative to increase the method specificity and its limit of quantitation (LOQ) is the use of highly specific immunoaffinity solid-phase extraction (SPE) to selectively extract and enrich the aflatoxins prior to the HPLC–FLD analysis (5). With this sample preparation method, low LOQ values with direct fluorescence detection can be achieved, even in highly complex and fatty matrices.

This article describes the determination and quantification of AF–B2, AF–B1, AF–G2, and AF–G1 in ground hazelnut with immunoaffinity SPE purification followed by HPLC–FLD analysis without derivatization. The applied technique allows sufficient trace level detection for this matrix far below the European Commission regulations.

Experimental

Chemicals and equipment

Methanol (LC–MS grade), acetonitrile (LC–MS grade), hexane (HPLC grade), phosphate buffered saline (PBS) buffer pH 7.2, acetic acid (LC–MS grade), and sodium chloride were obtained from Fisher Scientific. The aflatoxin mixture consisting of B2, B1, G2, and G1 was purchased from Sigma Aldrich. AflaClean Select Immunoaffinity SPE cartridges were purchased from LCTech GmbH.

Preparation of Standards

Quantification was performed by standard addition calibration to correct matrix influences, due to the absence of a hazelnut sample that was free of any aflatoxin content. The aflatoxin stock solution (stock solution 1), containing AF–B2 and AF–G2 with a concentration of 857 µg/kg, and AF–B1 and AF–G1 with a concentration of 2856 µg/kg, was used to prepare the calibration standards by diluting stock solution 1 with 1% acetic acid in the ratio 1:100 (stock solution 2).

Stock solution 2 was added to a purified sample extract after immunoaffinity SPE (see "Preparation of Samples," below) to obtain different calibration levels, as listed in Table I. The recovery experiment was carried out by adding 113 µL of the aflatoxin stock solution 1 to 20 g of the ground hazelnut sample prior to sample preparation. The resulting concentration of the recovery sample is 1.7 µg/kg for G2 and B2, and 5.65 µg/kg for G1 and B1, when 100 % recovery is assumed.

Preparation of Samples

Spiked and nonspiked samples were prepared in triplicates. Then, 20 g of ground hazelnut sample and 2 g sodium chloride were weighed into a 200 mL bottle and 100 mL 80% methanol and 50 mL hexane were added. The solution was stirred for 15 min, and then filtered through a folded filter. Thereafter, 14 mL of the lower liquid phase was mixed with 86 mL PBS buffer pH 7.2 and 50 mL was loaded onto the immunoaffinity SPE cartridge. Afterwards, the cartridge was washed with 10 mL of water. Elution was carried out with 2 mL methanol, with the first 1 mL applied to the cartridge and allowed to react for 5 min. The extract was diluted 1:2 with 1% acetic acid before it was filtered through a 0.2 µm regenerated cellulose membrane in the HPLC vial for injection.

Instrumentation

The Thermo Scientific Vanquish Flex UHPLC system consisted of a quaternary pump, split sampler, column compartment and fluorescence detector, equipped with a standard bio flow cell with 8 µL volume. Chromatographic separation was performed on a 100 x 3 mm, 3 µm Thermo Scientific Acclaim C18 column, using a 4 min isocratic method of 50% water, 30% methanol and 20% acetonitrile with a flow rate of 0.5 mL/min. The injection volume was set to 20 µL. The active preheater and the column temperature were held at 30 °C constantly. The FLD excitation and emission wavelength were set to 365 nm and 450 nm, respectively, while the lamp was operated in high power mode with a sensitivity of 8. The data acquisition and processing was performed with Thermo Scientific Chromeleon 7.2.8 chromatography data system (CDS) software.

Results and Discussion

During the method development process, various aqueous and organic mobile-phase combinations were tested in order to achieve sufficient baseline separation of the four toxins AF–B2, AF–B1, AF–G2 and AF–G1 on the C18 column. The best result could be obtained with 50/30/20 water/methanol/acetonitrile (v/v/v) mixture with a flow rate set to 0.5 mL/min. At this point, no immunoaffinity SPE clean-up prior to HPLC-FLD analysis was performed. Figure 1 shows the separation of the aflatoxin mixture with excitation at 365 nm and emission at 450 nm. Some peaks were eluted before the first target aflatoxin G2. These unknown peaks are impurities in the standard solution that have not been

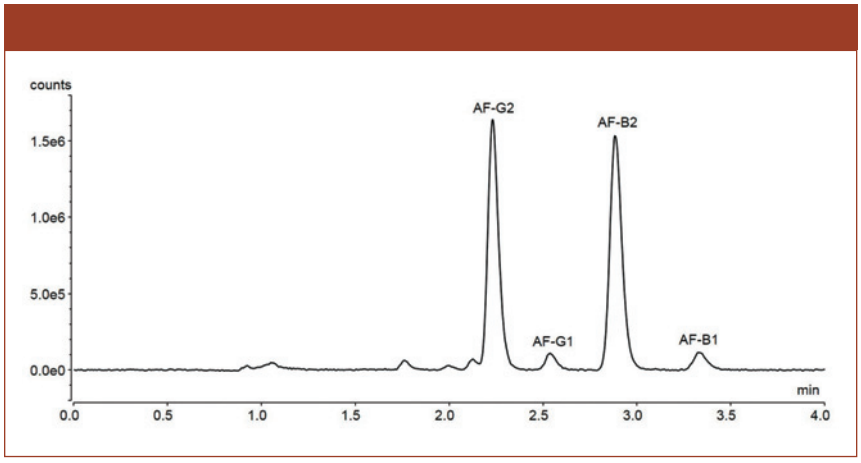

Figure 1: FLD chromatogram of AF–G2, AF–G1, AF–B2, and AF-B1 at concentrations of 0.9 µg/kg for AF-G2 and AF-B2, and 2.9 µg/kg for AF-G1 and AF–B1.

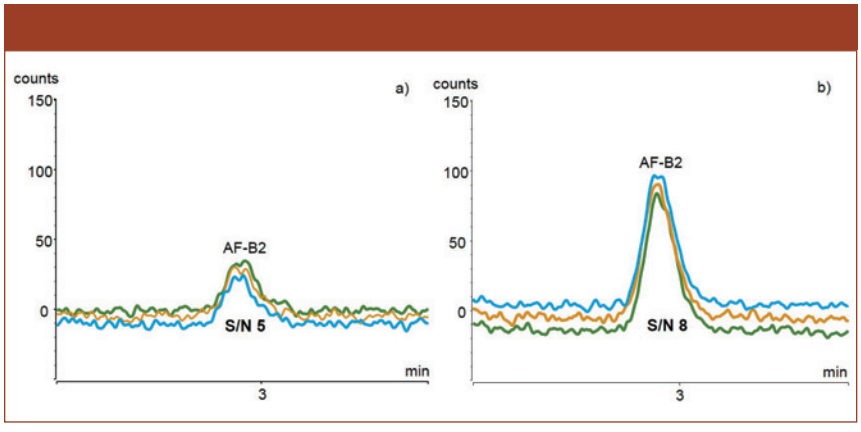

Figure 2: Exemplary chromatograms of aflatoxin B2 (triplicate injection) for the determination of a) LOD and b) LOQ.

Table I: Calibration levels and added concentration in µg/kg to a purified sample extract

Calibration Level	Toxins G2, B2 Concentration [µg/kg]	Toxins G1, B1 Concentration [µg/kg]
1	0.2	0.7
2	0.4	1.4
3	0.9	2.9
4	1.3	4.3
5	1.7	5.7
6	2.1	7.1

further identified. All aflatoxin analytes are baseline separated within 4 min.

Calibration was performed with the standard addition method. The original sample, which was found to already contain all analytes, was set to zero amount, which results in a negative x-axis intercept. In this way, the calculated amount of the analytes corresponds to the absolute amount of the negative x-intercept. Linearity (R^2) was found to be in the range of 0.9920 to 0.9974, and the percentage of relative standard deviation of the retention times (%RSD t_R) were all below 0.2% (Table II).

Immunoaffinity SPE purification has proven to be a very specific sample preparation method for ground hazelnut to achieve low LOD and LOQ levels. For the determination of LOD and LOQ values, one aliquot from each of the three sample extracts was pooled, diluted to a S/N ratio of 3 for LOD and S/N ratio of 10 for LOQ and injected three times. Figure 2 shows the resulting chromatogram of AF–B2 as one example. A full overview of the calibration results is given in Table II. Reliable quantification can be achieved with the applied method to safely determine the maximum levels of 5 µg/kg for AF–B1 and 10 µg/kg for the sum of all four aflatoxins, as regulated by the European Commission.

After immunoaffinity SPE very pure extracts are obtained, as the chromatogram in Figure 3 demonstrates. The highly specific purification allows a separation of interfering matrix components, while enriching the target analytes. Some matrix peaks can be observed in the first 2 min, but no interferences in the target analyte region from 2 to 4 min are present. Neither the nonspiked sample extract nor the spiked (recovery) extract, where the standard solution was added before the sample preparation, show a peak in front of the toxin G2. In contrast, an impurity can be detected in the calibration standard, with the standard solution being added to the sample extract after the SPE cleanup (Figure 4). This clearly highlights that immunoaffinity SPE provides a distinct advantage in eliminating impurities present in the standard solution and in improving LOD and LOQ levels.

Furthermore, the overlaid chromatograms in Figure 4 show that no carryover in the blank injection was observed, even after injection the highest calibration point at a concentration of 2.1 µg/kg for AF–G2 and AF–B2 and 7.1 µg/kg for AF–G1 and AF–B1.

Table III summarizes the quantitative results with recovery rates of each compound and calculated sample amounts (corrected by recovery rate).

The applied method provided excellent recovery rates for the toxins G2, B2, and B1 (95–100%) but somewhat inferior for AF–G1 with 72%. The shelf life of the cartridges specified by the manufacturer should be several months, if properly stored. The cartridges used in this study were close to their expiration date, possibly causing the lower AF–G1 recovery.

The major analyte of interest is the highly toxic AF–B1 and found to be present in the sample at an averaged concentration of 3.4 µg/kg (±1.3 µg/kg). The European Commission has set a limit of 5 µg/kg for this compound, which is just above the

Table II: Percentage RSD of retention times (%RSD t_R) (n=13), calibration range, and linearity, LOD and LOQ with standard deviation (S.D.) (n=3)

Compound Name	%RSD t_R	Calibration Range [µg/kg]	R^2	LOD [µg/kg] ±S.D.	LOQ [µg/kg] ±S.D.
AF–G2	0.09	0.2–2.1	0.9970	0.075 ± 0.008	0.185 ± 0.017
AF–G1	0.17	0.7–7.1	0.9947	0.931 ± 0.076	1.329 ± 0.066
AF–B2	0.09	0.2–2.1	0.9974	0.104 ± 0.013	0.206 ± 0.017
AF–B1	0.15	0.7–7.1	0.9920	1.056 ± 0.154	1.122 ± 0.061

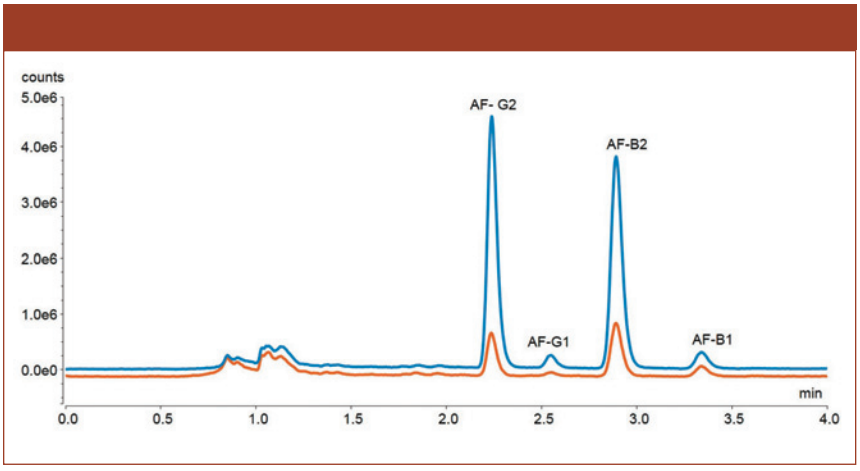

Figure 3: Overlay of spiked (recovery) sample (blue) and nonspiked (brown) hazelnut sample.

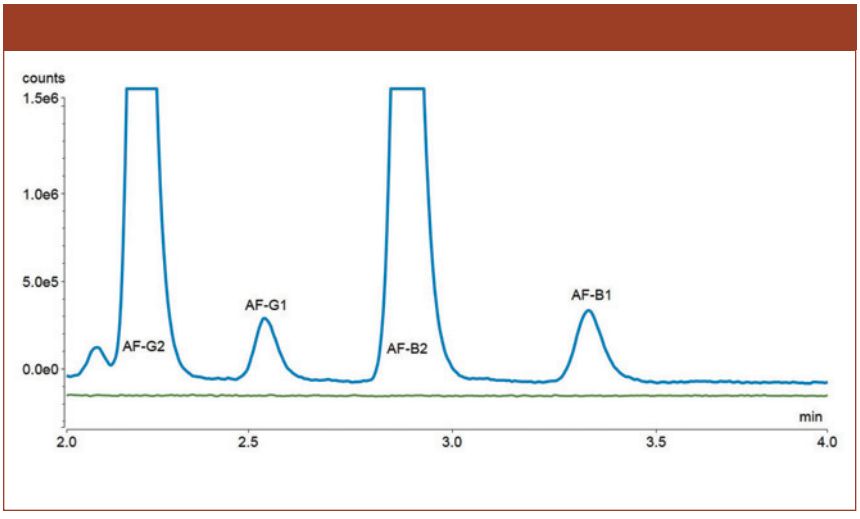

Figure 4: Zoomed overlaid chromatograms of processed sample spiked with the highest calibration concentration (blue) and consecutive blank injection (green).

Table III: Recovery and calculated sample amount results of ground hazelnut (averaged from three preparations) with standard deviation (+/- S.D.)

Compound Name	Recovery Rate [%]	Calculated Sample Amount (±S.D.) [µg/kg]
AF–G2	100	0.4 (±0.03)
AF–G1	72	2.2 (±0.1)
AF–B2	100	0.3 (±0.1)
AF–B1	95	3.4 (±1.3)

observed level. The sum of all four compounds should not exceed 10 µg/kg, and is calculated at an average of 6.3 µg/kg.

Conclusion

Aflatoxins are a group of mycotoxins, some of which are highly toxic to human health, and are known to cause various diseases. Therefore, the European Commission has set low µg/kg levels for aflatoxins in several foodstuffs used for direct consumption or as ingredients. Consequently, for their determination, a very sensitive and specific analytical method is required to enable trace-level detection of these analytes even in complex matrices. The combination of immunoaffinity SPE purification and enrichment with FLD detection without pre- or post-column derivatization offers a sensitive analytical method for the quantification of the aflatoxins G2, G1, B2, and B1 in ground hazelnuts. The applied method provides sufficient trace level detection performance down to 1 µg/kg for aflatoxins B1 and G1 and 0.1 µg/kg for B2 and G2, enabling aflatoxin analysis far below the tolerance levels defined by the European Commission. Good selectivity, linearity, and recovery for reliable quantitative results were observed, while the method run time of less than 4 min allows a high sample throughput.

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Detection and Quantitation of Pyrrolizidine Alkaloids in Diverse Food Matrices

Thousands of plants produce pyrrolizidine alkaloids as a natural defense mechanism against insects and small animals. The presence of these compounds in human food and animal feed, however, is a concern because of their potential threat to human health. Foods that are of particular concern include honey, certain herbs and teas, dietary supplements, and animal-derived products like milk and eggs. In an effort to better detect pyrrolizidine alkaloids in the food supply, Jean-François Picron, PhD, and his colleagues from Sciensano in Belgium, recently developed new ultrahigh-pressure liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) methods for the quantification of pyrrolizidine alkaloids in a range of food matrices. His group also completed dietary exposure assessments in support of Belgian and European regulatory guidelines. In this interview, Picron discusses this work.

Laura Bush

Q: Why is it important to study pyrrolizidine alkaloids in foods?

A: Pyrrolizidine alkaloids are emerging contaminants in food. These common secondary plant metabolites defend against herbivores and are produced by more than 6,000 plant species. They are thought to be one of the most widespread plant toxins in the world.

1,2-unsaturated pyrrolizidine alkaloids are carcinogenic and genotoxic, and they can affect wildlife, livestock, and humans through contaminated food consumption. Cases of human poisoning, mainly involving acute and chronic liver damage, have been documented.

The European Food Safety Authority (EFSA) has published different opinions about the risk of pyrrolizidine alkaloids to human health since 2011, but their efforts have been hampered by a lack of analytical methods. So, it was very important to develop analytical methods that could accurately quantify very low levels of pyrrolizidine alkaloids in a wide range of food items, in order to cover the diet as completely as possible.

Q: You developed a method to quantify pyrrolizidine alkaloids in food. Can you briefly describe the method and why you chose the technique that you did?

A: In fact, we developed nine different analytical methods for different kinds of food matrices. The food matrices were grouped into nine families of foods with similar composition, such as fat or protein content.

All the analytical methods follow the same workflow: first, liquid acidic extraction of the analytes, and then solid-phase extraction of the crude extract to remove interfering compounds that could lead to a major matrix effect.

We chose liquid chromatography for the analysis. Pyrrolizidine alkaloids are found in two different forms: the tertiary amine and the corresponding N-oxide. Gas chromatography would have degraded the thermally unstable N-oxides, which is not the case with liquid chromatography. Moreover, ultrahigh performance liquid chromatography (UHPLC) instruments have great advantages over HPLC instruments for the quantitation of pyrrolizidine alkaloids because of their higher resolving power and greater sensitivity. The coupling of UHPLC with tandem mass spectrometry (MS/MS) enables high reliability and sensitivity, making this method the perfect choice for pyrrolizidine alkaloid analysis.

Q: How did you address the challenge of dealing with the wide range of food matrices? What other challenges did you face in developing the methods?

A: We targeted a diverse range of food items, such as honey, honey-based products, fresh meat, processed meat, dairy products, plant products (dry and wet), and infusions (teas). We had to adjust the type and concentration of the acid used for the extraction step for each matrix, as well as the stationary phase used for the solid-phase extraction step.

Another major problem of pyrrolizidine alkaloid analysis was that we had to find matrix blanks to build a calibration curve, which was tricky for some matrices. For example, we had to build artificial matrices in the lab for some matrices, such as inverted sugar syrup for the analysis of honey.

Another big problem for pyrrolizidine alkaloid analysis is the limited availability of analytical standards. There are more than 300 known toxic pyrrolizidine alkaloids, but standards are only commercially available for about 30 compounds. All those compounds were included in our methods.

The most complicated matrices were those with the highest protein content, especially cheeses. It took a long time to find a good compromise between sensitivity and recoveries, and we found that adding a lot of salt helped with protein denaturation.

As the human health risk of pyrrolizidine alkaloids is currently uncertain, and their prevalence in foods is uncertain, we had to target limits of quantification that were as low as possible to

detect the most pyrrolizidine alkaloids possible. With our methods, the limit of quantification for all matrices was below 1 ppb, and even as low as ppt levels for dairy products.

Another bottleneck for the analysis of pyrrolizidine alkaloids is the coexistence of many isomers. So, you have to carefully optimize your LC gradient. We were able to achieve that, and separate most of the isomers.

Q: You used your methods to study the presence of these compounds in products on the Belgian market. How did you conduct that study, and what did you find?

A: First, we established a structured sampling plan to cover the widest range of food items that also covers a complete diet. We bought and analyzed more than 1,300 food items available on the Belgian market, and our research delivered many interesting results.

For example, we reported for the first time high levels of europine in Mediterranean honeys, and thanks to our very low limits of quantitation in dairy products and meats, we suggested for the first time that N-oxides are present in animal-based products. Previous researchers suggested that the N-oxides were totally converted to the amine during the animal's metabolism. But, in fact, the previous analytical methods had lower sensitivity, so it was not possible to detect N-oxides with those methods.

On the other side of the contamination scale, we showed that plant-based food supplements were contaminated with ppm levels of pyrrolizidine alkaloids, even if they are not labeled as containing a pyrrolizidine alkaloid-producing plant. So, we found a wide range of contamination between ppt and ppm levels.

Also, the transfer rate between the contamination of pyrrolizidine alkaloids in herbal teas, from the dry material to the infusion, is incomplete. In fact, only about 20% of the contamination, depending on the compounds' structures, are effectively transferred to the infusion. This result is quite interesting, because, so far, EFSA has only applied a dilution factor from the dried extract, assuming a total transfer of pyrrolizidine alkaloids. This is quite good news for the risk evaluation.

Q: Your initial study led to a follow-up study on aromatic herbs. Why were the aromatic herbs studied separately? And what did you find in that part of the study?

A: In the main project, the study of aromatic herbs was not planned, but we were interested in analyzing some samples with our methods. The results of a few categories of dry mixes were very surprising. We found high levels of contamination in Italian mixes for pizza and pastas. So, we decided to conduct a follow-up project with a pragmatic approach.

The first step was to determine if aromatic herbs as such could produce pyrrolizidine alkaloids, and the results showed that they do not. After that, we obtained a large range of individual herbs and mixes. Most of them were contaminated, sometimes at ppm levels. Oregano was by far the most problematic herb with a high degree of contamination, sometimes with levels above 10 ppm. In this case, the contamination of oregano is clearly due to co-harvesting, because oregano, as such, cannot produce the contaminants. Some other research groups are currently studying the question of whether economic adulteration of oregano is leading to its contamination.

The contamination pattern in aromatic herbs was quite remarkable, and it was largely dominated by heliotrine-type compounds. In fact, the EFSA recently proposed to reduce the number of PAs to be monitored in food excluding the majority of heliotrine-type compounds. Maybe with this result, EFSA will reconsider the question because the contamination levels are quite significant.

Q: Once you had data about the contamination levels of these compounds in foods, how did you assess exposure levels and public risk, and what conclusion did you come to?

A: The analytical concentrations were linked to consumption data collected in a 2014 survey in Belgium. We calculated the mean PAs concentration for each kind of food item for the sum of the 30 targeted compounds.

The result showed that from the 90th percentile of the population, there is a significant increase in the intake of pyrrolizidine alkaloids. And if we compare the data with the previous EFSA exposure assessment, the median and the 95th percentile are quite comparable at the higher limits.

A notable point is that dairy products were, by far, the biggest contributor to one's intake of pyrrolizidine alkaloids, even though these matrices exhibited the lowest concentration levels.

For a risk assessment, it's important to use a margin of exposure approach for carcinogens and genotoxic compounds. The results showed that, if we use this approach, there is a health concern for about 10% of the population that is highly exposed to pyrrolizidine alkaloids.

Q: Do consumers need to worry about how much they're consuming of these compounds?

A: Consumers should adopt a varied diet. Change the brand of your milk and do not always drink the same tea. That way, you will probably dilute your potential exposure to pyrrolizidine alkaloid contamination.

Dr. Jean-François Picron obtained his Master Degree in Chemistry in 2007 from the Free University of Brussels (ULB), where he continued with a PhD Degree in organic synthesis of supramolecular receptors called calixarenes, and the studies of their physicochemical properties using NMR spectroscopy. In 2015, he joined the Organic contaminants and Additives» Unit of Sciensano, where he works in the frame of natural toxins, particularly pyrrolizidine alkaloids, by developing new UPLC-MS/MS methods for their quantification in a wide range of food matrices, with an additional focus on dietary exposure assessments to support Belgian and European regulatory guidelines. In 2018, he also became involved in biomonitoring studies for pesticide residues.

Measuring Mycotoxins

LCGC spoke to Rudolf Krska from the University of Natural Resources and Life Sciences in Vienna, Austria about the latest analytical techniques, and challenges, facing analysts involved in the evolving field of mycotoxin analysis.

Alasdair Matheson

Q. Mycotoxin analysis is a major field in food analysis at the moment. Why has mycotoxin analysis become important?
A: The occurrence of mycotoxins (secondary fungal metabolites) in various crops is a global concern, because it has significant implications for food and feed safety, food security, and international trade. Despite huge research investments, prevention and control of these toxic secondary metabolites remains difficult, and the agriculture and food industries continue to be vulnerable to problems of contamination. In addition, extreme weather conditions because of climate change are increasingly affecting the mycotoxin map in Europe and worldwide. The EU's Rapid Alert System for Food and Feed (RASFF) (EC, 2016) showed that of the total border rejections in 2015, 18.3% were due to mycotoxin contamination exceeding the EU legislative limits, accounting for the most frequently reported chemical hazard (1).

In recent years, research on cumulative risks, exposure, and long-term effects has raised awareness for the control of these health risks. As a result of the potential danger of mycotoxins to humans and livestock, strict regulatory controls determine the sale and use of contaminated food and feeds. Thus, grain and other foodstuff buyers increasingly demand more rigorous and timely food safety testing. Failure to achieve a satisfactory performance may lead to unacceptable consignments being accepted or satisfactory batches being unnecessarily rejected. Recent reports have also demonstrated that the range of mycotoxins present in the food and feed chains goes beyond the list of regulated toxins. In fact, some 140 different fungal metabolites have been found in feed and feed ingredients by the use of a multi-toxin method based on liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). As a result of such findings, the demand for quantitative and rapid screening tools for the determination of (multiple) mycotoxins has increased tremendously.

Q. What are the biggest challenges in mycotoxin analysis?
A: The biggest challenge in mycotoxin analysis is still the sampling issue. Despite recent available guidance (2), it is still a difficult and tedious task to obtain a representative sample. Appropriate extraction solvents matching the range of multiple mycotoxins to be determined is another crucial step followed by proper cleanup. The latter is dependent on the final determination step. The use of LC–MS/MS instruments, combined with optimized chromatographic separation, reduces the need for sample cleanup. This is especially true for regulated toxins, for which fully ^{13}C labeled internal standards can be used to compensate for matrix effects. Ensuring comparability of measurement results is another challenge, particularly for mycotoxin-commodity combinations for which no certified reference materials exist.

Q. What novel approaches have your group contributed to the field of mycotoxin analysis, and what advantages do they offer?
A: Within the frame of international efforts to obtain new and comprehensive data on the occurrence of multiple mycotoxins in food and feed chains, we have developed a unique multi-analyte approach based on LC–MS/MS. The developed analytical method, which does not require any cleanup, has continuously been extended and fully validated for the quantification of more than 300 fungal and bacterial metabolites, including all regulated mycotoxins in various cereals, food, and feed matrices. This highly cited mass spectrometric method is crucial, both to elucidate the occurrence of various mycotoxins potentially being present in agricultural and food commodities and to study their metabolization by plants, animals, and humans. Our mass spectrometric work has also included the development of a fast, easy to handle, and highly accurate isotope dilution mass spectrometric assay.

The latter has become feasible through the production of fully isotopically labeled mycotoxins by means of a patented technology developed in our laboratory, in cooperation with Romer Labs. We have also provided the first scientific proof of the occurrence of glucose-bound (masked) deoxynivalenol in naturally contaminated wheat and maize, as part of the plant's defense strategy to detoxify this most prevalent Fusarium mycotoxin. The potential threat to consumer safety from masked mycotoxins has, as a result, been addressed by institutions such as ILSI Europe (Washington, D.C., USA) and the European Food Safety Authority (EFSA) (Parma, Italy). Utilizing the power of the advanced multi-biomarker LC–MS/MS method, we have also been able to provide new insights into the human metabolism of Fusarium mycotoxins by identifying and quantifying appropriate biomarkers in human urine.

Within the frame of the EU-funded project MYCOSPEC, we have developed a mid-infrared spectroscopic sensing method using tunable quantum cascade lasers and thin-film waveguides obtained from the University of Ulm (Ulm, Germany). This novel method showed its great potential for the rapid on-site classification of contaminated wheat, maize, and peanuts at the concentration levels established in the EU for deoxynivalenol and aflatoxin B1, respectively.

Q. You are coordinator of an EU project www.mytoolbox.eu dealing with integrated management strategies to tackle mycotoxins. Can you tell us more about your role in this project and what this project involves?

A: In fact, there is still a pressing need to mobilize the wealth of knowledge from the international mycotoxin research conducted over the past 25–30 years, and to perform cutting edge research where knowledge gaps still exist. We believe that this knowledge needs to be integrated into affordable and practical tools for farmers and food processors along the chain to reduce the risk of mycotoxin contamination of crops, feed, and food. This is the mission of MyToolBox, a four-year project that has received funding from the European Commission, and which I have the pleasure to coordinate. It mobilizes a multi-actor partnership of academia, farmers, technology SMEs, food industry, and policy stakeholders to develop novel interventions aimed at achieving a significant reduction in crop losses caused by mycotoxin contamination. Besides a "field to fork" approach, MyToolBox also considers safe use options of contaminated batches, such as the efficient production of biofuels. Within a range of novel preharvest interventions, we will investigate the genetic resistance to fungal infection, cultural control, the use of novel biopesticides suitable for organic farming, competitive biocontrol treatment, and the development of novel modeling approaches to predict mycotoxin contamination. Research into postharvest measures includes real-time monitoring during storage, innovative sorting of crops using hyperspectral vision technology, novel milling technology, and the study of the effects of baking on mycotoxins at an industrial scale. Again, our multi-toxin screening method will be crucial to verify the success of all these intervention strategies.

Q. What areas of mycotoxin analysis will your group focus on next?

A: In the past few years, our team has continuously moved from the target analysis of individual mycotoxins to untargeted metabolite profiling and metabolomics of, ideally, all secondary metabolites that are involved in plant-fungi interactions. This methodology is based on in vivo stable isotopic ^{13}C-labeling and subsequent measurement of biological samples by full scan high-resolution LC–MS.

We plan to continue and expand our interdisciplinary and comprehensive strategy to study plant-fungi interactions and the metabolism of mycotoxins by moving our research to the next level, a fully integrated "omics-based" approach. In this context, we intend to pursue our efforts to characterize the analytically ascertainable metabolome of wheat and maize genotypes differing in their Fusarium resistance level, and ideally to link their metabolite profile to resistance criteria or markers. A major goal within this endeavor is the development of a standardized metabolomics platform to study primary and secondary metabolites produced by microorganisms and plants, and to understand the interactions between plants, fungi, mycotoxins, and other secondary metabolites at a molecular level.

Q. Does your group focus on other areas of food analysis using chromatography?

A: In the area of food and feed safety, my colleague, Professor Rainer Schuhmacher, and I aim for the fingerprinting of food and feed samples. The development of standardized conditions is a prerequisite for the development and establishment of robust fingerprinting methods. For the description of defined conditions, again in vivo ^{13}C-labeling of the matrix–for example, grains–is a promising approach, which can be achieved through growing plants under $^{13}CO_2$ atmosphere. We also intend to combine this approach with the labeling of tracers; for example, different food contaminants. This would facilitate the recognition of changes of the labeled contaminant itself, but also of the tracers or contaminants on the matrix as a result of, for example, food or feed processing. Moreover, the in vivo ^{13}C-labeling of the food or feed matrix would enable endogenous and exogenous compounds to be differentiated between. Stable isotopic labeling can be used to detect deviations of secondary metabolites of fungi, plants, and bacteria from normal patterns, flagging suspicious samples for further analysis and confirmation, and for a more accurate quantification and identification of compounds.

Q. Do you think the time will come when there will be no need for the chromatography component in food analysis?

A: In view of the amazing sensitivity and high resolution achievable with novel mass spectrometry, this is certainly a valid question. Nonetheless, chromatography will probably stay forever until, or unless, someone develops a radically different approach to separate complex mixtures. With the advent of small particles and ultrahigh-pressure LC (UHPLC), we can now process smaller amounts of samples faster than ever. And with the wealth of potential compounds, which we aim to quantify in our food and feed chain in highly complex matrices, separation remains as important as ever.

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Rudolf Krska is full professor of bioanalytics and organic trace analysis at the University of Natural Resources and Life Sciences, Vienna (BOKU), Austria. He is head of the Centre for Analytical Chemistry at the Department of Agrobiotechnology (IFA-Tulln) at BOKU, with a staff of over 50. From 2010–2015, he also served as head of the department. As a member of JECFA (Joint Expert Committee for Food Additives) of the FAO/WHO, he has evaluated the impact of trichothecene mycotoxins on humans. Krska was also member of the Working Group Fusarium of the European Food Safety Authority. He has received 10 scientific awards and is co-author of more than 280 SCI publications (h-index: 49). Currently, Professor Krska is coordinator of the European Commission funded project MyToolBox (Safe Food and Feed through an Integrated ToolBox for Mycotoxin Management), with 23 partners and a funding volume of more than 5 million Euro. Direct correspondence to: rudolf.krska@boku.ac.at

Streamlined Method for Pesticide Residues in High-Lipid Food Samples Using QuEChERS Extraction, LipiFiltr® Cleanup, and UHPLC–MS/MS Analysis

Bikash Bhattarai and Brian Kinsella, UCT, LLC

The easy to use, new LipiFiltr® push-through purification cartridge was designed to remove lipids from acetonitrile extracts. This application will outline the performance benefits achieved using the new LipiFiltr cartridges in applications involving multiclass, multiresidue analysis for pesticides in complex, high fat samples. Samples are extracted using a standard QuEChERS procedure, and an aliquot of the supernatant is simply pushed through the LipiFiltr purification cartridge using a disposable syringe. The purified extract is collected in an autosampler vial, and analyzed by LC–MS/MS. The ability to obtain significantly cleaner extracts, the ease of use, and the time and cost savings make the new LipiFiltr push-through cartridges an attractive cleanup option for laboratories conducting pesticide residue analysis in complex fatty samples.

Table 1: Extraction and analytical materials

ECQUEU7-MP	Mylar pouch containing 4 g $MgSO_4$, 1 g NaCl, 1 g $Na_3Cit•2H_2O$, and 0.5 g $Na_2Cit•1.5H_2O$
LPFLTR01	LipiFiltr Push-Through Cartridge
SLAQ100ID21-18UM	100 × 2.1 mm, 1.8-µm Selectra® Aqueous C18 UHPLC column
SLAQGDC20-18UM	10 × 2.1 mm, 1.8-µm Selectra® Aqueous C18 guard column
SLGRDHLDR-HP	High-pressure guard cartridge holder

Procedure

Sample Extraction

(a) Weigh 5–10 g of homogenized sample into a 50-mL centrifuge tube. Add 5 mL reagent water if necessary.
(b) Add 10 mL acetonitrile and internal standards.
(c) Shake or vortex samples for 5 min at 1000 strokes/min.
(d) Add ECQUEU7-MP packet to each sample and shake for 1 additional min at 1000 strokes/min.
(e) Centrifuge at ≥3000 rcf for 15 min.
(f) Attach LipiFiltr push-through cartridge to disposable syringe.
(g) Take 1.5 mL of supernatant into syringe barrel. Attach the plunger and gently push the sample through the LipiFiltr cartridge into an autosampler vial.

Instrumental

LC–MS/MS: Shimadzu Nexera X2 coupled with Shimadzu LCMS-8050
UHPLC column: 100 × 2.1 mm, 1.8-µm Selectra®Aqueous C18
Guard column: 10 × 2.1 mm, 1.8-µm Selectra Aqueous C18
Injection volume: 2 µL
Mobile phase A: H_2O containing 0.1% formic acid + 5 mM ammonium formate
Mobile phase B: Methanol containing 0.1% formic acid + 5 mM ammonium formate
Column flow rate: 0.45 mL/min

Results

Table 2: Gravimetric analysis

Matrix	Matrix Removal (%)
Beef	79.1
Black Olives	84.3
Avocado	54.7
Salmon	80.9
Chicken fat	71.7
Olive oil	61.5
Nuts	84.3
Swordfish	80.9

The performance of the LipiFiltr push-thru cartridges to remove fatty matrix was evaluated gravimetrically. The gravimetric analysis was done by collecting 2 mL of sample before and after cleanup in pre-weighed test tubes and heating them to dryness at 110 °C.

Conclusion

This application note demonstrates the performance benefits achieved using the new LipiFiltr cartridge in applications involving multiclass, multiresidue analysis for a wide range of pesticides (n = 189) in complex, high fat samples. Individualized recoveries obtained for each pesticide in a variety of matrices, such as avocado, olives, beef, and swordfish, can be found at unitedchem.com

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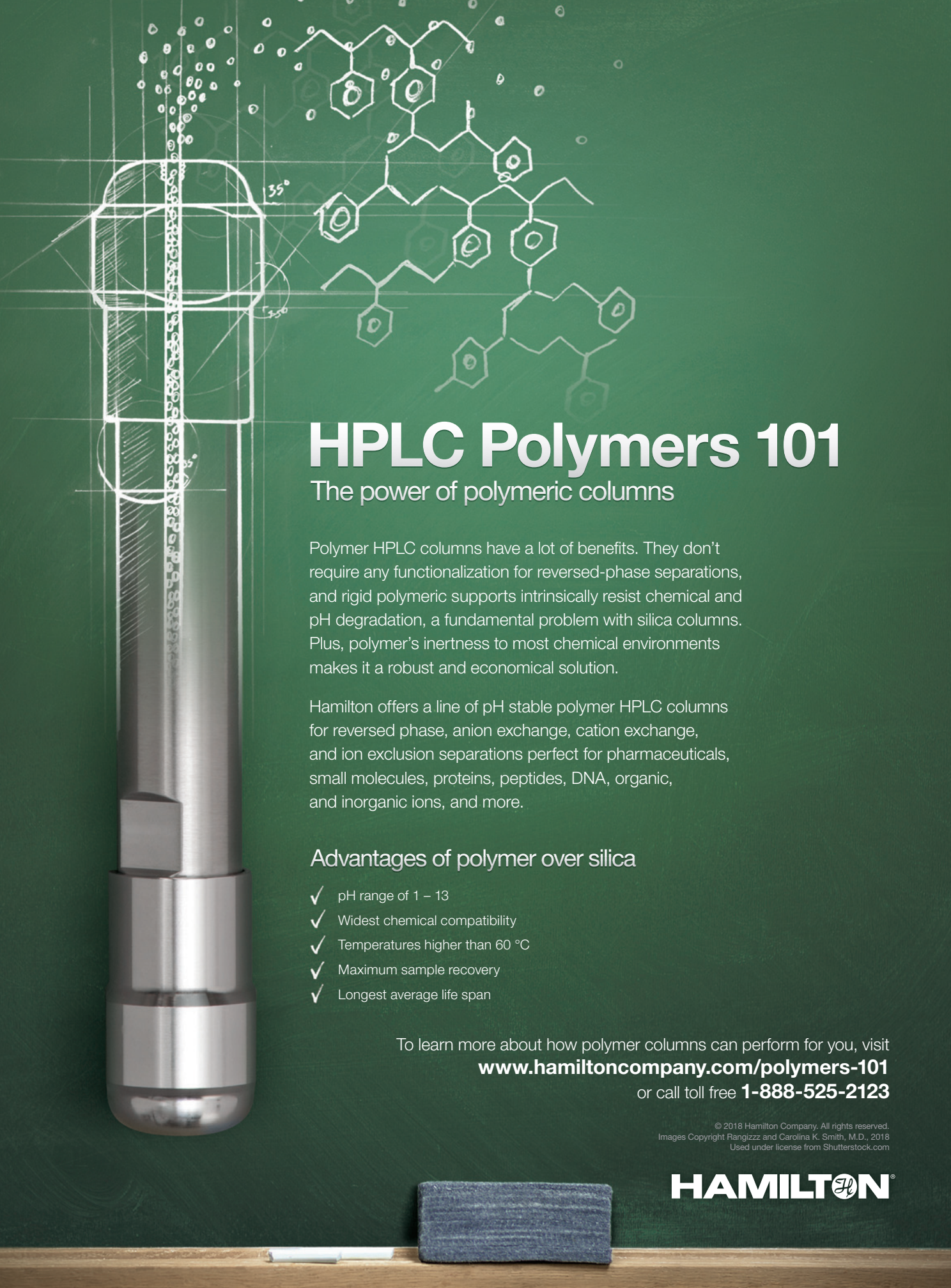

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