HUMAN HEALTH

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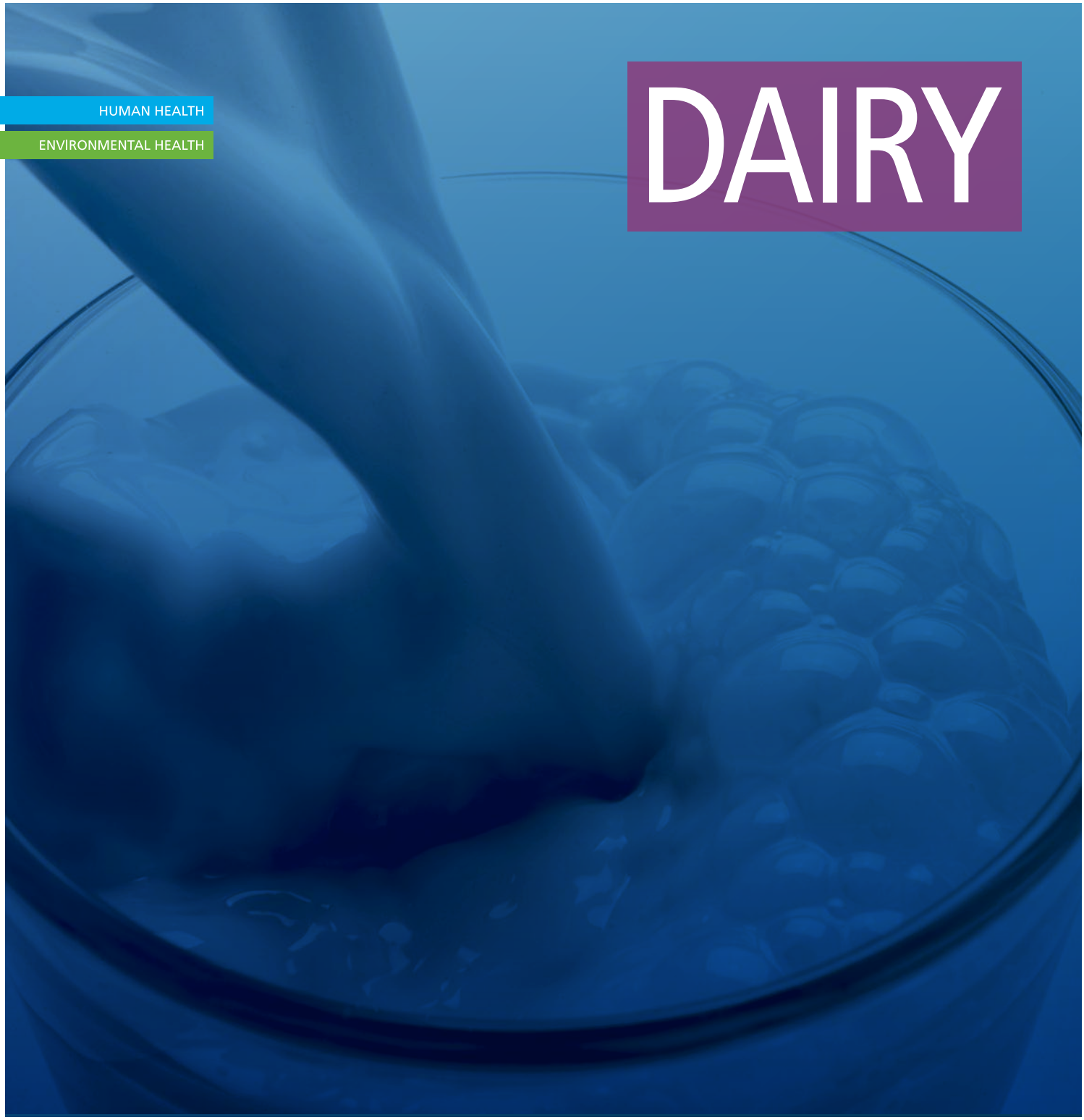
DAIRY



Dairy Compendium

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DAIRY

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APPLICATION NOTE

Gas Chromatography/
Mass Spectrometry

Author

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Solid Phase Extraction and GC/MS Analysis of Melamine Adulteration in Dairy Products

Introduction

In September 2008, melamine again made global headlines with contamination and adulteration of dairy products in China. This incident occurred about 18 months after melamine contamination of pet foods. During the initial melamine scare, gas chromatography/mass spectrometry (GC/MS) analysis was used successfully in testing finished food products as well as raw materials. This analysis is presented in the PerkinElmer application note "Screening for Melamine Adulteration in Protein-Based Foods by GC/MS"[1].

When the melamine in milk crisis began, similar test methods were used to test baby formula and other dairy products. The sample matrix of milk and dairy products is, however, much different than that of pet foods, with a much higher content of fat and sugar. This difference in matrix required that sample preparation methods be modified from those used in GC/MS analysis of pet foods. The major modification necessary is solid phase extraction (SPE) of the sample extract to remove the matrix of the milk. This paper will present the modifications necessary to successfully analyze dairy products for melamine with GC/MS. Additionally, GC/MS analysis of the data will support the method modifications.

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Experimental

The analysis of milk and dairy products requires a specific sample preparation. The techniques used in this application are a combination of an extraction procedure by Sigma-Aldrich®[2] and a modified derivatization reaction and analytical procedure presented in an FDA method[3].

Melamine samples were created in the lab by spiking full-fat milk with a melamine standard (50 µg/mL 50:50 acetonitrile:water) to a concentration of 1 µg/mL. The extraction procedure used follows:

1. Dilute 5 mL of spiked milk with 5 mL 100 mM phosphate buffer (pH 2.5) and 1 mL acetonitrile
2. Sonicate for 5 minutes in an ultrasonic water bath
3. Centrifuge at 3500 rpm for 10 minutes
4. Isolate the middle supernatant layer for SPE processing
5. Process 2.2 mL of the middle supernatant layer (equivalent to 1 mL milk sample) using SPE.

The SPE was carried out on a strong cation exchange cartridge, Discovery® DSC-SCX (500 mg/6 mL, Sigma-Aldrich). The cleanup procedure is as follows:

1. Condition and equilibrate SPE cartridge with 3 mL methanol followed by 3 mL 0.1% formic acid
2. Load sample (2.2 mL)
3. Wash SPE cartridge with 3 mL 0.1% formic acid followed by 3 mL methanol
4. Elute melamine from SPE cartridge with 4 mL 5% ammonia diluted in methanol
5. Evaporate 1 mL SPE eluent to dryness, in an autosampler vial, with nitrogen at 5 psi and 50 °C
6. Sample is ready for derivatization.

The dry sample is reconstituted in an autosampler vial with 200 µL of pyridine. Melamine is converted to trimethylsilyl (TMS) derivatives with the reagent Sylon-BFT (Supelco®) consisting of bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS); 300 µL of this solution is added and the sample is incubated at 70 °C for 45 minutes.

Following derivatization, the samples are ready for GC/MS analysis. The GC/MS system used in this paper was the PerkinElmer® EcoAnalytix™ Melamine Analyzer, based on the Clarus® 600 GC/MS, and the instrument parameters are summarized in Tables 1 and 2.

Table 1. Gas Chromatograph Conditions for Melamine-TMS Analysis.

Gas Chromatograph:	PerkinElmer Clarus 600		
Analytical Column:	Elite-5MS (30 m x 0.25 mm x 0.25 µm)		
Injection Port Type:	Programmable Split/Splitless		
Injector Temperature:	280 °C		
Injection Type:	Splitless		
Syringe Volume:	5 µL		
Injection Volume:	1 µL		
Injection Speed:	Fast		
Carrier Gas Type:	He		
Carrier Gas Program:	1 mL/min		
Oven Program:	**Temperature**	**Hold Time**	**Rate**
	75 °C	1 min	15 °C/min
	320 °C	2.67 min	End
Instrument Timed Events:	-0.5 min	Spl1 = 0 mL/min	
	1.0 min	Spl1 = 50 mL/min	

Table 2. Mass Spectrometer Conditions for Melamine-TMS Analysis.

Mass Spectrometer:	PerkinElmer Clarus 600 T
GC Inlet Line Temperature:	280 °C
Ion Source Temperature:	230 °C
Function Type:	Full scan
Full Scan Range:	*m/z* 50-450
Solvent Delay:	6 min
Full Scan Time:	0.2 sec
InterScan Delay:	0.05 sec

Results

Previously, it was demonstrated that the GC/MS method for melamine analysis can easily detect and quantify melamine below 0.1 µg/mL, 25 times less than the 2.5 ppm level established for melamine in food[1]. Earlier applications work confirmed the sensitivity of the method with the analysis of low-level standards between 1 and 10 ppb. Figure 1 demonstrates the analysis of a 5-ppb standard achieving a signal to noise (RMS) of greater than 25:1 (note: this analysis was run with a slightly different GC oven program and resulted in a later elution of the melamine peak).

The extraction procedure was carried out 8 times on a single batch of milk spiked with melamine at 1 ppm – Figure 2 demonstrates a chromatogram generated in this analysis. The samples were spiked at 1 ppm to test the precision and recovery of the SPE method at a level close to the regulatory level. The average RMS measured for the extracted melamine samples spiked at 1 ppm in milk was approximately 13,700:1 (n=8). This verifies that the method achieves sensitivity that will far surpass regulatory testing needs.

Additionally, the analysis of 8 different extractions of the same melamine sample yielded a precision of 3.45% RSD when comparing the measured peak area for the summed ions of *m/z* 327+342. This data establishes that both the extraction and analytical methods are very reproducible. The average peak area measured in the analysis of 1 ppm melamine extracts was 2.7 x 10^7 when compared to an average peak area of 2.3 x 10^7 for a 0.5 µg/mL standard (equivalent to 1 ppm in milk sample). The percent recovery of this extraction is approximately 120%. This recovery is on the high side of acceptable, but similar to the 112% recovery demonstrated in reference 2.

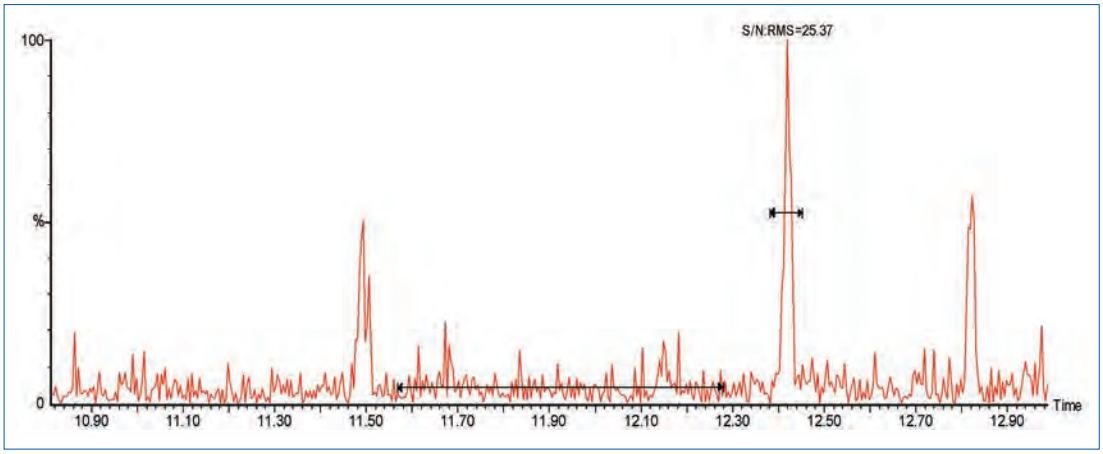

Figure 1. Chromatogram (extracted ion *m/z* 327+342) of the GC/MS analysis of a 5-ppb standard of melamine, demonstrating the sensitivity of the method.

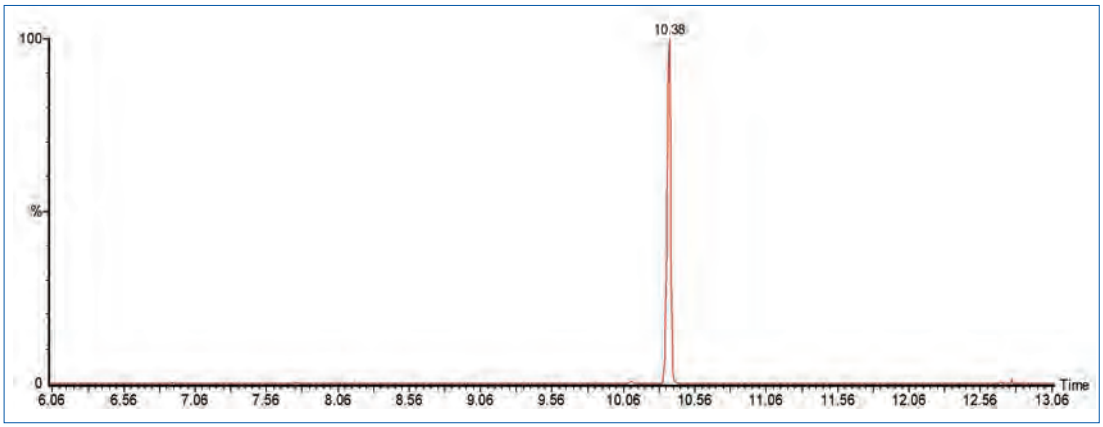

Figure 2. Chromatogram (extracted ion *m/z* 327+342) of the analysis of a milk sample spiked at 1 ppm with melamine.

Conclusion

The analysis of milk and dairy products for melamine requires the use of SPE to remove interferences caused by the high fat and sugar content of the matrix. A method including strong cation exchange, SPE, derivatization, and GC/MS analysis has demonstrated that melamine in dairy products can successfully be analyzed by GC/MS well below the limits required by regulation.

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007969_04

APPLICATION NOTE

Atomic Absorption

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Accurate Determination of Lead in Different Dairy Products by Graphite Furnace Atomic Absorption Spectrometry

Introduction

Milk is one of the basic food groups in the human diet, both in its original form and as various dairy products. The Chinese contaminated baby formula scandal in 2008 has increased public awareness of contamination possibilities, and has lead to tighter supervision of dairy products as China is faced with demands – both from home and abroad – to improve its food safety record. It is well-known that lead (Pb) is toxic and causes damage to the nervous system; it has a particularly detrimental effect on young children[1] and it has become a cause of major concern since the 1970s. As per World Health Organization (WHO) standards, the permissible limit of lead in drinking water is 10 µg/kg (parts per billion, ppb). Following an in-depth review of the toxicological literature, the Chinese guideline for maximum levels of lead content is set at 20 µg/kg (ppb wet weight) in infant formula (use of milk as a raw material measured by fluid milk diluted from powder, referring to the product ready-to-use) and at 50 µg/kg (ppb) in fresh milk, respectively.[2]

Lead analysis has traditionally been one of the major applications of graphite furnace atomic absorption spectrometry (GFAAS) worldwide. Currently, the Chinese regulatory framework approved standard methods for lead analysis has set GFAAS as the technique for the compulsory arbitration in food testing.[3] In order to ensure protection of consumers, analysis should be sensitive, efficient, and cost-effective so that more effective monitoring can be accomplished. Because GFAAS is a mature technique, it is well-understood and routinely used by technicians and suitable for this determination. Sample preparation is an important part of an analysis and yet can be time consuming.

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Generally, milk is an emulsion or colloid of butterfat globules within a water-based fluid. The exact components of raw milk vary by different animal species, but it contains significant amounts of lactose, fat, protein and minerals as well as vitamins. Due to the relative interference resulting from such a complex matrix, complete decomposition of milk samples prior to instrumental measurement by microwave or heating block acid digestion is generally recommended. This approach, however, is more time-consuming and poses a more rigorous requirement on quality assurance than simple dilution when concentrations of lead are to be determined at µg/kg level in the final solution which is extremely sensitive to reagent blank contribution and environmental contamination.

To overcome these issues, this work describes a simple and direct dilution method for sample preparation, followed by automated analysis using GFAAS. This method minimizes sample preparation, and also reduces potential contamination while still maintaining the speed of analysis.

Experimental Conditions

Instrumentation

A PerkinElmer® PinAAcle™ 900T flame and longitudinal Zeeman atomic absorption spectrometer (Figure 1) was used for the GFAAS measurements of lead (Pb) in different milk samples. The PinAAcle 900T spectrometer's transversely heated graphite atomizer (THGA) with Longitudinal AC Zeeman background correction provide a constant uniform temperature distribution across the entire length of the graphite tube. This allows a full implementation of the Stabilized Temperature Platform Furnace™ (STPF) technique in graphite furnace analysis where we can analyze complex sample matrices using aqueous standard solutions as calibration for suspended sample solutions to get accurate and precise results. Maximum atomic signals can be obtained with minimum memory effect and potential interference.

Figure 1. PinAAcle 900T atomic absorption spectrometer with AS 900 furnace autosampler.

The spectrometer was equipped with an AS 900 autosampler and a PerkinElmer Lumina™ single-element Pb hollow cathode lamp (Part No. N3050157) was used as the light source. A standard THGA tube (Part No. B0504033) and 1.2 mL polypropylene autosampler cups (Part No. B0510397) were used throughout for all measurement. The instrument was controlled by WinLab32™ for AA software running under Microsoft® Windows® 7 operating system. A summary of the PinAAcle 900T instrument settings is listed in Table 1.

Table 1. **Instrument settings for the PinAAcle 900T spectrometer.**

Parameter	Value
Wavelength:	283.3 nm
Slit Width:	0.7 nm
Lamp Current:	10 mA
Signal Measurement:	Peak Area
Measurement Type:	AA-BG
Integration Time:	5 s
Replicates:	3
Calibration Standard:	4, 10, 15, 20 µg/L
Sample Volume:	16 µL

Sampling

A total of 15 samples of six different dairy products were investigated in this study, representing all the main types of milk commercially available in China, including milk powder, skimmed milk powder, whole milk, low-fat milk, children's milk and yogurt. All the samples collected from the original packaging in a sealed clean polyethylene bag, were labeled and taken to the laboratory then kept refrigerated until analysis.

Sample Preparation

For the preparation of all solutions, ultrapure deionized (DI) water from a MiliQ-Element system (Millipore®, Milford, MA, USA) was used throughout. Concentrated nitric acid (69-70%), HNO_3, and hydrogen peroxide (30%), H_2O_2, were trace-metal grade or better (Jingrui Chemical Co., Ltd., Jiangsu, China). Metal-free polypropylene vials and pipette tips were pre-cleaned with diluted nitric acid (~5% HNO_3) and rinsed thoroughly with DI water before use.

For the subsequent GFAAS analysis, a solution containing 0.5% HNO_3 with 0.1% Triton X-100 (Part No. N9300260), a non-ionic detergent, was prepared daily both as a diluent and as a blank.

A 1-g sample of liquid milk or solid milk powder was accurately weighed and transferred into a 15-mL conical polypropylene tube (Part No. B0193233) which was subsequently diluted to make up the volume of 10 mL, and shaken vigorously for a few minutes to ensure homogeneity. The obtained suspension solution was immediately ready for GFAAS measurement using the autosampler. These suspensions were stable for more than 2 days. Even the more challenging total fat milk powder prepared by this rapid dilute-and-shoot procedure can be stable for this duration, which is sufficient for the inter-day variability check. The same procedure was used to prepare the blanks, and all the samples were prepared in duplicate on a routine analysis basis, unless stated otherwise.

For skimmed milk powders and fortified infant formulas whose protein content characteristics are modified by the manufacturing process, or for any milk powders with a higher protein content, any nitric acid addition will coagulate the dissolution resulting in a non-homogeneous suspension. In these cases, the milk-powder samples can be dispersed in 0.2 to 0.5% Triton X-100 solution, and a short 10-minute sonication will help disperse the milk powder into a more homogeneous solution that is stable for several hours, satisfactory for graphite furnace analysis.

For the validation by ICP-MS determination, a Multiwave™ 3000 high-pressure microwave digestion system (PerkinElmer, Inc., Shelton, CT) was employed to completely decompose the milk sample matrix using an acid mixture of HNO_3 and H_2O_2.

Calibration

As the concentration of Pb in milk samples is generally very low, all the reagents used must be of ultra-pure grade. Thus, Single-Element PerkinElmer Pure Plus Grade Standards (Part No. N9303748, lead in 2% HNO_3) and Matrix Modifiers (Part No. B0190635, 10% Pd as nitrate and Part No. B0190634, 1% Mg as nitrate) were recommended to be used. Calibration curves were constructed using online auto-dilution of a working stock lead standard solution of 20 µg/kg (ppb) by the AS 900 autosampler.

Method Validation

The performance of the procedure using GFAAS measurement was assessed by spike recovery and the evaluation of the Standard Reference Materials (SRMs) from National Institute of Standards and Technology (NIST®), NIST® 1549 Non-Fat Milk Powder, and China National Institute of Metrology (NIM), GBW08509a Skimmed Milk Powder. These two commercial lyophilized SRMs were treated as any dairy product sample.

In addition, these results were also compared to that obtained by the conventional mineralization-based procedures, followed by analysis using the NexION® 300X ICP-MS (PerkinElmer, Inc., Shelton, CT). The complete mineralization was carried out with the Multiwave 3000 microwave digestion system. Instrumental operating parameters for the ICP-MS measurements followed the routinely established protocols.

Results and Discussion

The temperature program for the analysis of lead is optimized to provide maximum matrix decomposition without loss of analyte. The furnace temperature program is given in Table 2.

Due to the challenging characteristics of the sample matrix, an additional drying step, using a special gas of dry compressed air, is recommended to eliminate the carbonaceous residues left after analyzing more than 50 samples in one single batch. The PinAAcle 900T spectrometer's TubeView™ color furnace camera is of great advantage in checking the position of the tip in the furnace, relative to the platform, which brings benefits in optimizing the drying and pyrolysis steps for the complex undigested milk matrix to ensure that no sample boiling or splattering occurred (Figure 2 – Page 4).

Table 2. **Furnace temperature program for the direct measurement of lead in milk samples using the PinAAcle 900T spectrometer with THGA tubes.**

Step		Temp. (°C)	Ramp Time (sec)	Hold Time (sec)	Internal Flow	Read Step	Gas Type
1	Drying	130	5	30	250		Normal
2	Drying	150	15	30	250		Normal
3	Drying	450	15	15	50		Dried Air
4	Pyrolysis	600	10	20	250		Normal
5	Atomization	1600	0	3	0	X	Normal
6	Clean-out	2500	1	5	250		Normal

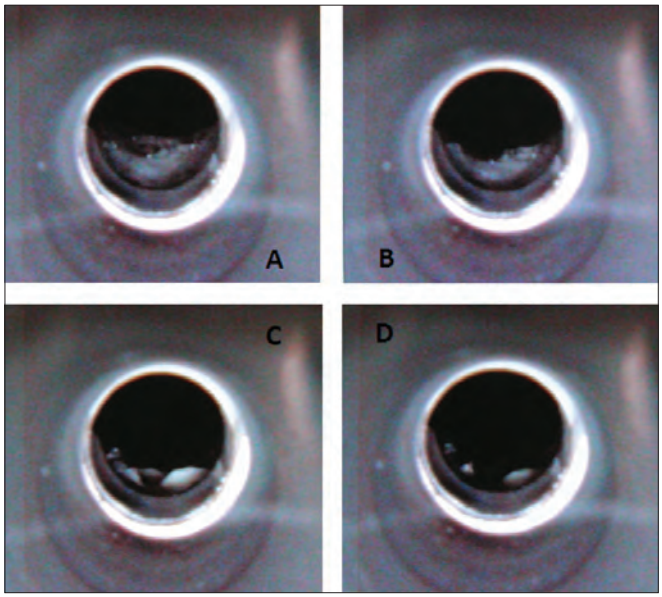

Figure 2. The drying steps of a complex undigested milk sample in the graphite tube, as seen using the TubeView color furnace camera.

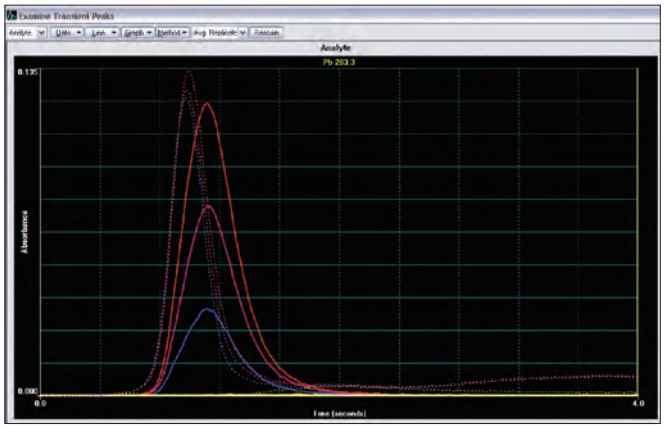

Figure 3. Overlay of typical lead atomic and background signal for the control material of skimmed milk powder. The solid blue line is from the control material of skimmed milk powder, the solid purple line is from the spiked control material, and the solid red line is from the standard at a concentration of 25 µg/kg, while the solid yellow line at the bottom is the reagent blank signal. Dashed lines represent the background absorption profiles.

Therefore, it helped in simpler and faster furnace (temperature) method development.

For Pb determination, complete mineralization of the milk components is not necessary when using the proven and established STPF technique with the patented THGA design which ensures uniform and consistent heating and high atomization efficiency, significantly reducing matrix interferences. All data were calculated from 3 replicate readings for each solution using peak-area (integrated absorbance) integration. Figure 3 depicts the overlay of typical peak profiles of the various solutions. One of the unique benefits of the STPF technique is clearly demonstrated here: even though the peaks may not appear at exactly the same time, the peak-area calculation still provides consistently accurate results.

To test the accuracy of the method, Pb was analyzed in the control material of non-fat milk powder from NIST® 1549 and skimmed milk powder from NIM GBW08509a. The high level of accuracy of the direct method is demonstrated by the good agreement of the results obtained in the analysis of the two SRMs with the certified values, as shown in Table 3. An estimation of analyte recovery was also obtained by spiking one of the SRM samples (GBW08509a) at the 50, 100, and 200% levels with the Pb single-element standard working stock solution, and the data, also collated in Table 3, demonstrates quantitative recovery.

***Table 3.* Results for the direct measurement of NIST® 1549 and GBW08509a by GFAAS (all in µg/kg).**

Sample	Certified Value	Spike Level	Expected Mean	Found Mean	Recovery (%)
NIST® 1549	19 ±3	0	19	19	101
GBW08509a	24 ±6	0	24	23	95
GBW08509a	24 ±6	12	36	35	96
GBW08509a	24 ±6	24	48	48	99
GBW08509a	24 ±6	48	72	71	98

Method detection limits (MDLs), defined as the analyte concentration in micrograms per kilogram (ppb) of dairy products which provides an absorbance reading statistically different from that of the blank, are calculated by dividing 3 times the standard deviation (SD) of the absorbance readings of the reagent blanks by the sensitivity. An impressive characteristic of this method, which uses a sample volume at 16 µL with 10-fold dilution factor, provides the MDL of 0.25 µg/kg (ppb). Thus, the MDL measured in the original dairy products is about two orders of magnitude below the expected level in the typical control materials (around 20 µg/kg). It indicates that this method could prove highly suitable for determining Pb in dairy products.

For additional independent comparative data against GFAAS analysis using this simple method, all collected dairy products were mineralized by conventional microwave total acid digestion, then analyzed for lead by ICP-MS. Table 4 (Page 5) shows the concentrations of Pb found in each dairy product sample.

Table 4. **Lead levels in commercially available dairy products determined by direct GFAAS analysis and conventional ICP-MS measurement (values are means ± SD, all in µg/kg).**

No.	SRMs/Samples	Certified Value	Measured Results GFAAS	ICP-MS
1	GBW08509a (Skimmed milk powder)	24 ±6	23.3 ±0.7	23.9 ±1.7
2	GBW10017 (Milk powder)	70 ±20	23.9 ±2.7	25.7 ±8.7
3	NIST® 1549 (Non-fat milk powder)	19 ±3	19.1 ±1.3	19.3 ±6.5
4	Milk powder	–	40.2 ±1.8	42.1 ±1.9
5	Skimmed milk powder	–	25.7 ±1.3	23.3 ±6.1
6	Whole milk (Brand 1)	–	4.46 ±0.32	4.57 ±0.60
7	Whole milk (Brand 2)	–	2.75 ±0.07	2.73 ±0.09
8	Whole milk (Brand 3)	–	6.13 ±0.07	6.78 ±0.49
9	Whole milk (Brand 4)	–	5.65 ±0.11	5.85 ±0.37
10	Low-fat milk (Brand 1)	–	2.34 ±0.09	2.39 ±0.38
11	Low-fat milk (Brand 2)	–	0.53 ±0.02	0.58 ±0.21
12	Drinkable children's milk (Brand 1)	–	1.70 ±0.09	1.73 ±0.22
13	Drinkable children's milk (Brand 2)	–	0.22 ±0.01	0.54 ±0.15
14	Drinkable yogurt (Brand 1)	–	1.89 ±0.16	2.02 ±0.18
15	Drinkable yogurt (Brand 2)	–	1.36 ±0.02	1.61 ±0.33

It is important to emphasize that there are no significant differences between the two independent testing methods, which further demonstrates the accuracy of the overall methods. However, the relative standard deviation (RSD) was generally higher for data obtained by ICP-MS analysis after conventional mineralization. This is most likely due to the dilution introduced during the digestion step used in the ICP-MS sample preparation. Even though the ICP-MS technique is more sensitive than GFAAS, the dilution of the extremely low levels of Pb present in the samples introduces additional uncertainty. Based on the results, it clearly appears that total digestion of matrix components is unnecessary with all these types of dairy-product samples, and it is more rapid and economical to run the samples with minimal preparation.

As is also shown in Table 4, the Pb concentration in one of the tested SRMs issued by State General Administration of the People's Republic of China for Quality Supervision and Inspection and Quarantine (AQSIQ), GBW10017 milk powder found in this study, is 23.9 ±2.7 µg/kg by direct GFAAS method and 25.7 ±8.7 µg/kg by total digested ICP-MS method, which are both significantly lower than the certified value (70 ±20 µg/kg). This difference has also been observed by other laboratories purchasing this reference material. Based on the higher value of standard deviation (20 µg/kg, 29% of error), the actual certified Pb result in this GBW10017 SRM issued by AQSIQ has yet to be ascertained and needs further investigation.

For an intuitive and illustrative comparison, the differences in Pb concentration and analytical precision are also presented in Figure 4 as a plot with error bar. Our results clearly affirm the great advantage of easy handling and precise analysis using direct determination of Pb concentration by GFAAS, since the need to measure Pb at such a low level (in µg/kg range) in the original dairy product samples requires extremely strict control of reagents, environment and process. This is very challenging, even for experienced professionals, due to the large dilution factor if undergoing the time-consuming and labor-burdened total digestion procedure, taking the poor match of experimental value with the certified value in the SRMs of GBW10017 as an additional proof.

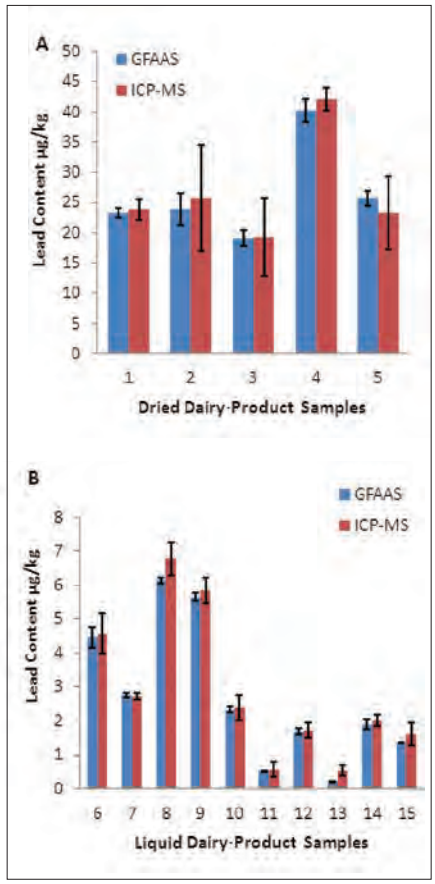

Figure 4. Comparison of lead levels in different dairy-product samples obtained by two independent test methods: A) dried milk powder samples; B) liquid milk samples.

Conclusions

In conclusion, a method involving simple sample dilution and automated PinAAcle 900T GFAAS detection can be successfully applied to the accurate measurement of Pb in different dairy products. Reduced sample handling minimizes the potential for losses or contamination. The advanced THGA technique keeps the risk of chemical interferences to a minimum, which provides a method detection limit well below the normal range of Pb that might be encountered. This method should also be applicable for analysis of samples with equivalent content of fat and complex matrices.

ICP-MS provides multi-element analysis and very high sensitivity. However, the high initial investment and more costly cost of ownership when compared with GFAAS may not offer the best choice for a simple single-element analysis. GFAAS offers not only high selectivity, sensitivity, and ease of operation, but also high tolerance to complex matrices. When coupled with simple sample preparation, it is consequently more appropriate for the trace level determination of a few toxic elements in dairy products as a routine monitoring technique in protecting human health.

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CASE STUDY

Food Fraud

Milk Authenticity – Organic vs Non-organic

With increasing concerns over contaminants in milk, both intentionally and unintentionally added, a growing number of people are switching to organic milk (sales of whole organic milk were up 17% between January and October of 2011 in the U.S. with reduced fat organic milk up 15%).[1] This surge in popularity, coupled with high food and fuel prices, has caused shortages in the supply of organic milk.[2] With demand therefore outstripping supply, and a gallon of organic milk costing anywhere from 25% to 100% more than conventional milk, the selling of conventional milk as organic is an attractive proposition to fraudsters. In the U.S. and E.U., the labelling of organic products has meant stricter policing of farming practices but this is not the case with all countries. Furthermore, with the growing export of organic milk powders, these fake organic milk powders can find their way into the West through distributors or through processed foods, such as chocolates, which will also command a higher price if claiming to be organic. While these substitutions invariably do not cause health problems it is still fraud, with consumers not getting what they paid for and hardworking organic farmers losing business and having profit margins eroded.

What is the Difference Between Organic and Non-organic Milk?

When producing organic milk, farmers must adhere to the following rules (These vary by country. Below are those for the U.S.):

- Grazing time – Farmers have to ensure that at least 30% of their cows' diet comes from pasture grass during a mandatory grazing season (no less than 120 days).[3]
- Antibiotic use – Organic dairy cows are not to be treated with antibiotics as a routine. If a cow should require an antibiotic, it's not allowed back in the milk production rotation until 12 months of antibiotic-free certification have passed.
- Bovine Growth Hormone (BGH) – Dairy cows from organic farms are not allowed shots of BGH.
- Pesticide use – The use of pesticides on an organic dairy farm is forbidden. The organic cow cannot consume pesticide-treated feed.[4]

How can we detect this?

Chromatography techniques, such as LC/MS and HPLC, can be used for detection if there are traces of pesticides, antibiotics or even growth hormones in the milk or animal feed. Measuring if the cows have been fed a predominately commercial feed diet, rather than fresh grass or silage, is more difficult. One of the techniques attempted to characterize organic vs. non organic milk is isotopic ratio mass spectrometry (IRMS). This technique has been shown to identify the type and even origin of feed used but requires large databases and has not been explored in enough depth to make definite conclusions. Recent work has been looking at levels of minor acids in the milk. One such study used GC/MS to measure levels of phytanic acid in organic and non-organic milk. As organic cows eat more fresh green matter, they consume more phytol (part of chlorophyll) which is broken down in ruminant's stomachs to phytanic acid. The study found that, on average, organic milk had double the phytanic acid levels than conventional (circa. 300 mg/100 g of milk as opposed to 150 mg/100 g of milk).[5] Another study proposed using hippuric acid which, again, was suggested to be found in higher levels if more grass and silage were consumed, though this study focused on goat's milk.[6]

Speeding up the Analysis

Invariably, most of these techniques involve some waiting time for separation to take place. A technique such as ambient ionization mass spectrometry provided by the AxION™ Direct Sample Analysis (DSA™) system integrated with the AxION 2 Time of Flight (TOF) mass spectrometer does not, as samples can be directly ionized and drawn straight into the MS. This means that some traditionally chromatography based applications, that did take up to an hour to analyze, can now take less than 30 seconds.

Experimental

Three organic and three conventional milk samples were purchased from a local supermarket. All milk samples were subjected to the same preparation in that 1 mL of milk was mixed with 2 mL of acetonitrile and 1 mL of methanol to carry out a protein precipitation. The samples were then centrifuged for 10 minutes at 7800 RPM. Finally, 1 mL of supernatant was diluted and then spiked with internal standard, d5-hippuric acid, to give final concentrations of 5 mg/L of internal standard in each sample. Ten µL of each protein precipitated sample were then pipetted directly onto the stainless steel mesh of the AxION DSA system ready for ionization and analysis. The DSA/TOF experimental parameters are shown in Table 1.

Table 1. **Experimental parameters for the analysis of hippuric acid in milk.**

DSA Parameters	Value
Heater Temperature	350 ˚C
Auxiliary Gas Pressure	80 psi
Drying Gas Flow Rate	3 L/min
Drying Gas Temperature	25 ˚C
Corona Current	-5 uA
TOF Parameters	**Value**
Mode	Pulse (Negative)
Mass Range	100-700 *m/z*
Capillary Exit Voltage	-100 V

Results

Figure 1 shows the mass spectra of an organic milk sample. It is clear that the dominate signals are from the hippuric acid and the deuterium substituted hippuric acid standard. If these peaks are examined in more detail and then overlaid, Figure 2, we can see that, from the area of the peaks, that the response from the hippuric acid is 1.692 times that of the deuterated standard. If this is repeated for conventional milk, Figure 3, we see that the ratio is closer to 1 at 0.932.

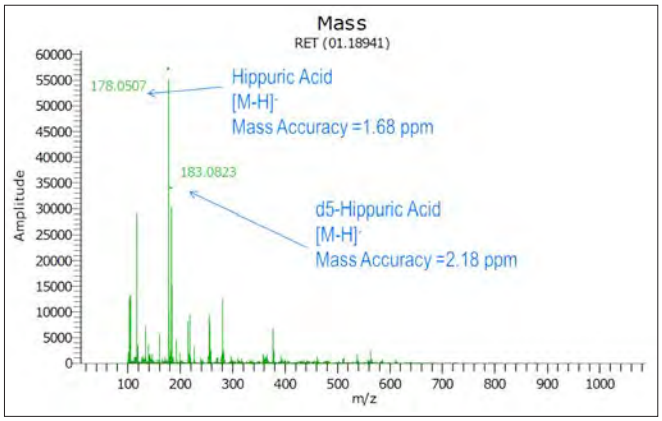

Figure 1. Representative mass spectra of the organic milk sample spiked with 5 ppm internal standard.

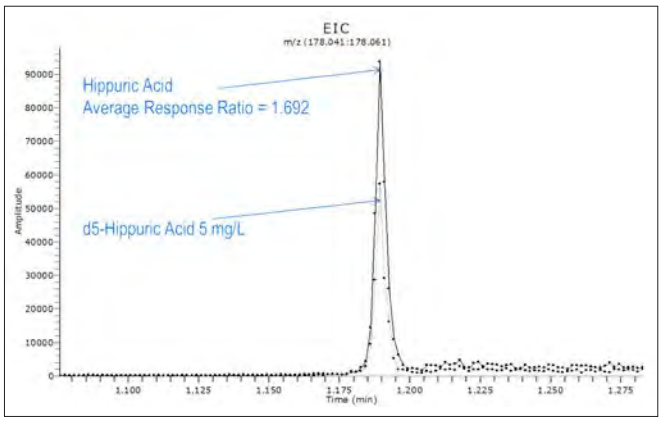

Figure 2. Representative Extracted Ion Chromatogram (EIC) of Hippuric Acid and d5-Hipopuric Acid (5 mg/L) for the organic milk sample.

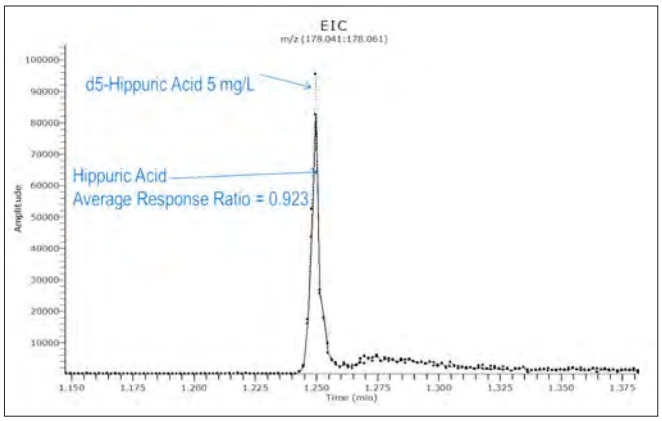

Figure 3. Representative EIC of Hippuric Acid and d5-Hipopuric Acid (5 mg/L) for the conventional milk sample.

Discussion

This was repeated for all 6 samples with the results shown in Table 2. It can be seen that for conventional milk, for all three samples, the hippuric acid concentration was, on average, a 1 to 1 ratio with the reference standard. Factoring in dilution, this means that the levels in the conventional milk were around 20 mg/L. For the organic milk samples 1 and 2, it was clear that the levels of hippuric acid were higher as hypothesized. These samples had around 35 mg/L which is approximately 1.75 times that of conventional milk. One organic milk sample had lower levels than even the conventional milk. An explanation for this could be that this particular sample was the supermarket 'home' or generic brand and was therefore 'not as organic as it suggests on the label'. To support this theory, a wider study would be needed, ideally splitting a herd of cows into two groups, feeding one an organic diet over a year and one a conventional feed based diet and measuring the hippuric acid levels in the resulting milk over the year similar to that studied for goats in a similar study.[6]

***Table 2.* Levels of hippuric acid in organic and conventional milk samples.**

Type of Milk	Response Ratio with respect to 5 mg/L d-5 Hippuric Acid	Hippuric Acid in Milk
Organic Milk Brand 1	1.692	33.48 mg/L
Organic Milk Brand 2	1.837	36.73 mg/L
Generic Organic Milk Brand 3	**0.864**	**17.28 mg/L**
Conventional Milk Brand 1	0.923	18.45 mg/L
Conventional Milk Brand 2	0.998	19.96 mg/L
Conventional Milk Brand 3	1.075	21.51 mg/L

Conclusion

This work has shown that it is possible to measure hippuric acid levels in milk by DSA/TOF MS using a reference standard to ascertain relative concentrations (rather than using a calibration curve). There is also evidence that the hippuric acid levels could be used to ascertain whether cows have been feed an organic diet however a much wider study would be needed to prove this conclusively. This opens up the possibly to study for pesticides, growth hormones, antibiotics and organic diet in one instrument and therefore have a definite check for organic compliance.

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APPLICATION NOTE

Near Infrared Spectroscopy

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DairyGuard: Augmenting Nutritional Testing of Milk Powder with Adulterant Screening

Introduction

Milk powder is one of the most widely traded food commodities, with over 2.5 million metric tons exported annually[1], and is used in a huge array of food products, from infant formula to baked goods and confectionary. Unfortunately, dairy products are also a frequent target of food fraud, with 137 cases of economically motivated adulteration worldwide recorded by the United States Pharmacopoeia in 2011-2012[2]. The value of milk powder is linked to its protein content, and standard methods for protein analysis rely on a simple nitrogen assay, with the protein concentration inferred from the nitrogen content. Consequently, the addition of chemicals rich in nitrogen can artificially increase the apparent protein and thus the price demanded.

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These known incidences of economically motivated adulteration have led to strict limits on melamine content. For example, the U.S. FDA states that melamine or cyanuric acid should not be present in foods at levels greater than 2.5 ppm, or 1 ppm for infant formula[3]. Establishing the absence of these materials above such levels requires highly sensitive techniques such as LC/MS/MS[4]. While it is important to have laboratory methods with the highest possible sensitivity, often such methods are comparatively expensive and time-consuming to run and this may limit their ability to obtain representative samples. Moreover, there are two additional concerns that are specific to economically motivated adulteration. The first is that ppm-level adulteration is not economically worthwhile, so genuinely adulterated samples are likely to have higher concentrations. For example, to increase the total nitrogen in skim milk powder by 0.16% (corresponding to an apparent protein increase of 1% total mass), it is necessary to add 2400 ppm of melamine. Second, and more troubling, is that while there are published cases of adulteration with melamine, "chemical space" is vast and there are many more high-nitrogen compounds that could potentially be used in the same way[5]. To stay ahead of the criminals, it's important to look beyond currently known adulterants and consider other possibilities.

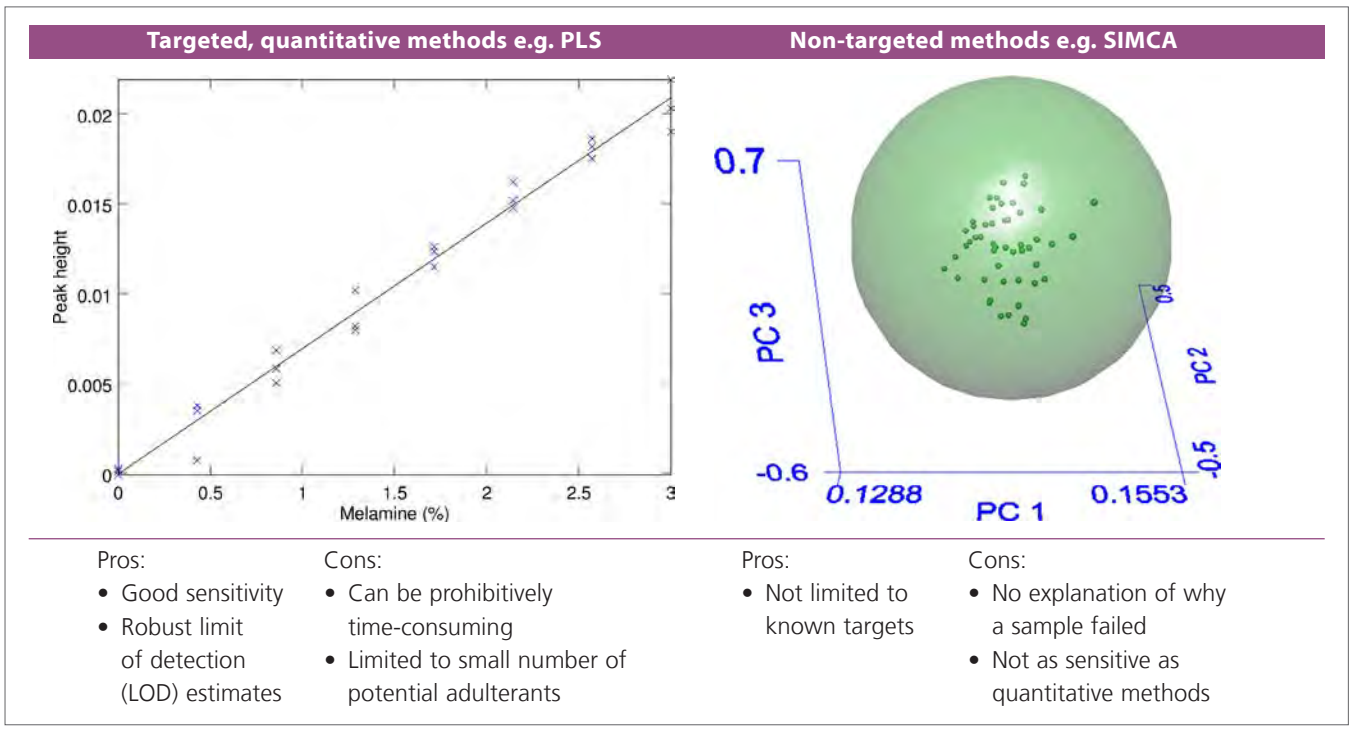
For these reasons, "fingerprinting" tools that measure the response of the entire sample without separation have a very important role to play in the fight against economically motivated adulteration. Near infrared (NIR) spectroscopy, in particular, is already very widely used in food, feed and agricultural industries for quantitative analysis of nutritional and quality parameters such as protein, moisture and fat. What is less well known is that it can be a superb tool for verifying the integrity of ingredient samples in the face of potential adulteration. NIR spectra can be measured in seconds, and contain information about the whole sample – including any adulterants present. There is no physical separation process at work, so the spectra must be processed with appropriate chemometric tools to differentiate the contributions of the milk powder matrix and any adulterants. In this note, we describe the use of the DairyGuard™ Milk Powder Analyzer and the novel Adulterant Screen™ algorithm to detect seven potential adulterants in milk powder at levels well below 1%, without any time-consuming PLS or other chemometric calibrations.

What is Adulterant Screen?

Previous applications of NIR to adulterant detection have utilized standard chemometrics tools (Figure 1). Quantitative methods using PLS regression have been developed for melamine and shown good performance. However, such targeted methods are only applicable to the adulterants they are calibrated for, and the calibration can be a very time-consuming process, involving the preparation of dozens to hundreds of samples with precisely known concentrations of the adulterant.

An alternative approach is to use a principal components analysis (PCA)-based method such as SIMCA, in which a model is built for the unadulterated material, and the quality of match of the sample spectrum to this model is used to determine whether the result is a pass or a fail. While this approach is truly non-targeted and potentially sensitive to any adulterant, there is no indication of why a failing sample has failed (no identification of the adulterant) and, because the method makes no use of the adulterant spectrum, the sensitivity cannot be expected to be as high as for a quantitative method.

Finally, methods that rely on conventional library searching – even with multivariate algorithms – suffer from an inability to model accurately the variation in the matrix, and are often used with commercial libraries that may be of limited applicability.



Pros:
- Good sensitivity
- Robust limit of detection (LOD) estimates

Cons:
- Can be prohibitively time-consuming
- Limited to small number of potential adulterants

Pros:
- Not limited to known targets

Cons:
- No explanation of why a sample failed
- Not as sensitive as quantitative methods

Figure 1. Schematic illustrations of existing chemometrics tools for adulterant analysis. Left: quantitative methods e.g. single peak height, or PLS regression. Right: non-targeted, factor-based methods e.g. SIMCA.

Adulterant Screen is a new algorithm designed specifically to address the problem of screening for potentially numerous adulterants in a complex matrix. It combines the generality and simplicity of non-targeted screening with some of the sensitivity benefits of a targeted approach.

How Does it Work?

The first step is to generate a library of spectra of samples of the unadulterated material, exactly as for SIMCA. This library should span as much as possible of the natural variation of the material – due to differences between batches, suppliers or processing parameters, for example. However, these are all "normal" samples: there is no need to obtain "high-leverage" samples (e.g. those with unusual nutritional parameters), as is often the case for quantitative methods for nutritional properties. The number of spectra required depends on the complexity of the matrix and the desired sensitivity: a typical library for milk powder might contain a few dozen samples.

The second step is to prepare a library of adulterant spectra. These are spectra of the pure adulterants: there is no need to create mixtures. With DairyGuard, this has already been done: a spectral library of 19 high-nitrogen agricultural and industrial chemicals is included with the system. Adding a new adulterant to the library is as simple as measuring the pure adulterant, and then copying the spectrum to the library folder.

These two sets of spectra are registered in the software, and the method is ready to use.

Performance Compared with SIMCA: physically Spiked Samples

Sixty-six samples of whole milk powder were prepared by spray-drying: forty-eight were used for the material library; twelve were used to prepare contaminated samples; and six used as blank controls. The following potential adulterants were used: melamine, urea, biuret, dicyandiamide, cyromazine and cyanuric acid. Each compound was ground finely then mixed thoroughly with the milk powder at concentrations of 0.2% and 2% mass. Samples prepared in a different manner (e.g."wet blending") may yield different results and require modified library spectra. Spectra were measured on a PerkinElmer DairyGuard Milk Powder Analyzer, which consists of a Frontier™ near-infrared (NIR) spectrometer using a NIRA II diffuse reflectance accessory. An accumulation time of 20s per sample at a resolution of 16 cm^{-1} was used.

A SIMCA method was built using PerkinElmer AssureID™ software at the default confidence level (99%). All 48 spectra from the material library were used.

Adulterant Screen was configured with 24 of the spectra for calibration and 24 for validation (threshold setting).

The results are summarized in Table 1 (Page 4), showing that Adulterant Screen provides significantly greater sensitivity than SIMCA.

The Adulterant Screen Algorithm

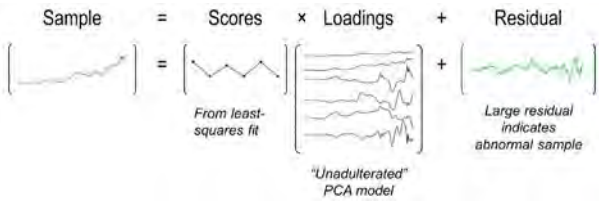
When a sample spectrum is scanned, the algorithm first compares it to a PCA model generated from the reference materials. This model is then augmented with each of the adulterant spectra in turn. If including a given adulterant in the model greatly increases the fit of the sample spectrum, it is likely that the adulterant is actually present in the sample. The algorithm also accounts for contamination with multiple adulterants, searching for every combination of up to three potential adulterants. The output of the algorithm is an estimated concentration, detection limit and confidence indicator for each adulterant in the library.

The concentration estimate is based on the relative intensity of the library spectrum of the adulterant and the amount found in the sample spectrum, without considering effective pathlength differences. As such, it is a semi-quantitative estimate. The detection limit estimate is expressed in the same terms. Finally, the reported confidence indicates the likelihood that the adulterant is actually present.

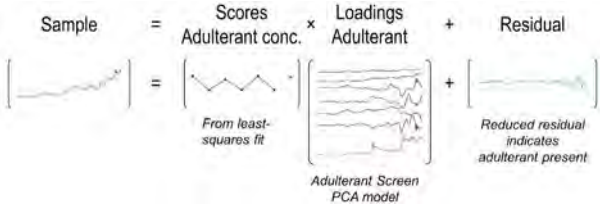
The confidence indicator and detection limit estimate are important because the sensitivity of the method is dependent on the similarity between the adulterant spectrum and the material spectrum. To validate detection limits, it is recommended to prepare a small number of samples with concentrations slightly above the estimated detection limits and verify that the adulterants are detected with high confidence.

If the sample is contaminated with some species that is not present in the library, or if the milk powder itself is a poor match to the original calibration data, the software will warn the user that unidentified components may be present.

Step 1. Residual calculated from standard PCA model.



Step 2. Residual calculated from augmented PCA model including adulterant spectrum.



Step 3. The residuals are compared against thresholds derived automatically from the validation samples. A detection limit is estimated and concentration and confidence scores assigned to the sample.

Table 1. Performance of SIMCA (with 99% confidence level) and Adulterant Screen for physically spiked whole milk powder samples. DCD = dicyandiamide; AS = Adulterant Screen. Incorrect results (false negatives) are highlighted. See the sidebar on prior page for an explanation of the detection limit and confidence estimates.

Sample	SIMCA pass?	AS pass?	AS level (%)	AS det. limit (%)	AS confidence
Blanks 1-6	Yes	Yes	Below LOD	-	-
Adulterants at 2%	No	No	All adulterants correctly identified		
Biuret 0.2%	Yes	No	0.19	0.15	Possible
Cyanuric acid 0.6%	Yes	No	0.37	0.25	Likely
Cyromazine 0.2%	Yes	Yes	0.017	0.11	Unlikely
DCD 0.2%	Yes	Yes	0.019	0.095	Very unlikely
Melamine 0.2%	No	No	0.21	0.12	Likely
Urea 0.2%	No	No	0.14	0.07	Likely

Both methods correctly recognized the blank samples as uncontaminated. SIMCA had no difficulty detecting adulteration at the percent level, and in some cases, 0.2% was sufficient to trigger a failure (melamine and urea). In terms of sensitivity, Adulterant Screen fared better, detecting cyanuric acid at 0.6% and biuret at 0.2% (but not cyromazine or DCD at 0.2%).

In addition to the improved sensitivity, Adulterant Screen also provided correct identifications for the adulterants: SIMCA merely indicated a pass or fail, without any indication as to the reason.

Enhanced Diagnostics

Spectrum™ software provides a suite of diagnostic tools for advanced users. An Adulterant Screen results screen is shown in Figure 2 below. Note that, while adulterants are listed for the blanks, the levels and confidence values are extremely low. The residual spectrum for one of the blank samples is shown: there is no evidence of any structure that may be associated with an unmodeled component.

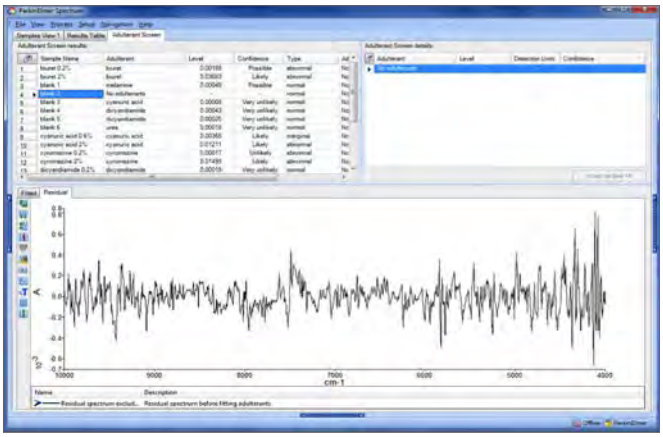


Figure 2. Typical results screen for Adulterant Screen in Spectrum Software version 10, showing the residual spectrum for an uncontaminated sample.

In comparison, the residuals from a contaminated sample (2% urea) show considerable structure (red trace in Figure 3). When the adulterant spectrum is included in the fit, most of this structure is eliminated (green trace).

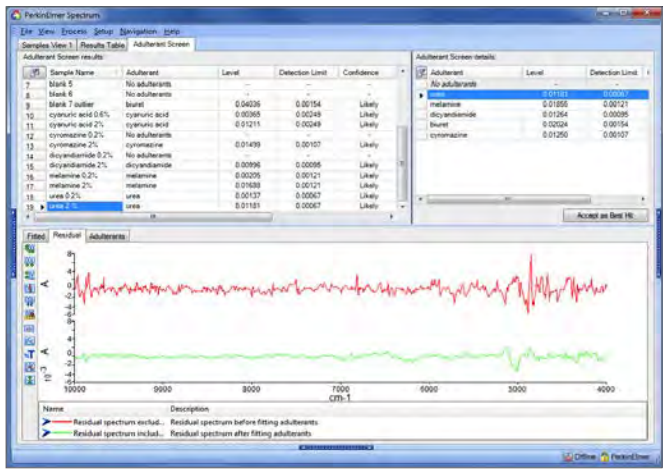


Figure 3. Residual spectra for a contaminated sample. Red trace: PCA residual, showing evidence of unmodeled components. Green trace: Adulterant Screen residual, showing a much improved fit.

Even if the structure in the residual is caused by the presence of an adulterant, it is not generally true that the structure will be recognizable as the spectrum of the adulterant. There will usually be both positive and negative spectral features in positions corresponding to absorption bands of both the uncontaminated material (milk powder) and the adulterant.

To provide a more interpretable spectrum, Adulterant Screen estimates the spectrum of the adulterant from the sample spectrum, using a least-squares fit to both the uncontaminated material and the adulterant reference spectrum. Particularly for chemical adulterants with distinctive spectra, good agreement between the extracted adulterant spectrum and the library spectrum is a strong indicator that the adulterant is really present. Conversely, if bands from the library spectrum are missing in the extracted spectrum, this may indicate that the sample is adulterated with something that is not present in the library. Figure 4 shows the extracted (black) and library (red) spectra for the 2% urea sample. Every peak in the library spectrum is matched by a peak in the estimated spectrum, so we can be confident that urea really is present in the sample.

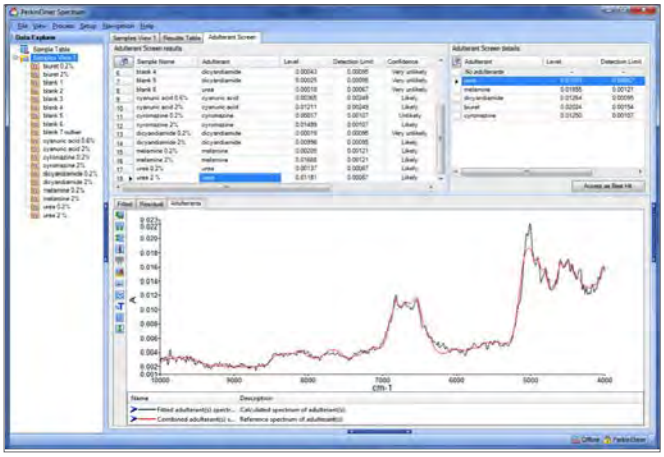


Figure 4. Estimated (black trace) and library (red trace) adulterant spectra for a sample contaminated with 2% urea.

This mode of results visualization is especially helpful when investigating results at or near the detection limit, as in the case of a sample contaminated with 0.2% melamine (Figure 5). While this sample triggered a "fail" result for both SIMCA and Adulterant Screen, indicating a need for further analysis, the Adulterant Screen result strongly indicates that melamine may be present, giving significantly more information to guide the next step to take.

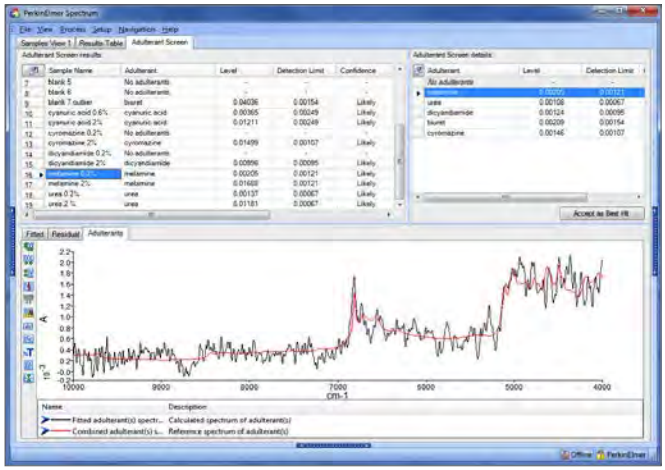


Figure 5. Estimated (black trace) and library (red trace) adulterant spectra for a sample contaminated with 0.2% melamine.

Comparison with SIMCA: Synthetically Spiked Samples

The sensitivity of SIMCA and Adulterant Screen was further investigated using synthetically spiked spectra created by adding between 0.1% and 2.0% of each adulterant spectrum to one of the blank spectra. The spectra so produced are not equivalent to spectra of physically spiked samples, since the effective pathlength for the adulterant is dependent on the measurement matrix (i.e. either the adulterant itself or the milk powder), but do allow a comparison of the relative sensitivities of the two methods.

Table 2. Performance of Adulterant Screen compared with SIMCA (at the indicated confidence level) for synthetically spiked samples.

Adulterant	Lowest Detected Concentration (Synthetic Spiking) SIMCA (99%)	SIMCA (95%)	Adulterant Screen
Biuret	>2%	>2%	0.2%
Cyanuric acid	>2%	>2%	0.3%
Cyromazine	0.6%	0.5%	0.3%
Dicyandiamide	1.4%	1.2%	0.3%
Melamine	0.7%	0.6%	0.3%
Urea	1.7%	1.4%	0.3%

Clearly, Adulterant Screen offers significantly better performance. This is because while SIMCA considers only the magnitude of the residuals, Adulterant Screen is actively searching for structure corresponding to the spectra of identified adulterant threats.

While the difference in performance on the physically spiked samples was not as dramatic, Adulterant Screen consistently outperformed SIMCA for the detection of contaminated samples.

Integration Within a Complete Workflow

Pass/fail criteria, considering both level (concentration) and confidence, can be set, enabling Adulterant Screen to be used as part of a routine incoming-material test. The process can be incorporated as part of a Spectrum Touch™ App, enabling simple, reliable operation by non-specialist users. The DairyGuard Milk Powder Analyzer system includes the Spectrum Touch App (Figure 6) containing SIMCA and Adulterant Screen analyses as well as example quantitative methods, which can be used as a starting point for developing your own apps.

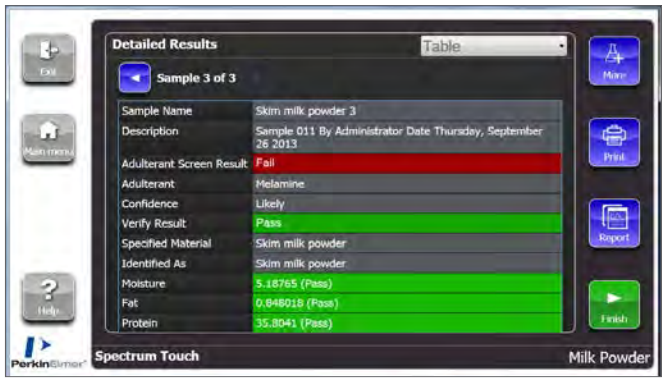


Figure 6. Results screen for the DairyGuard Touch App, showing a sample that has passed the Certificate of Analysis (COA confirmation tests and the SIMCA non-targeted screen, but failed on the more sensitive Adulterant Screen due to a low concentration of melamine.

Summary

Adulteration of food and food ingredients for economic gain is an old practice and, sadly, one that is unlikely to be eliminated in the near future. This problem needs to be tackled with all the analytical techniques at our disposal: NIR spectroscopy clearly has a role to play, given its ubiquity in raw materials testing. The unique Adulterant Screen algorithm from PerkinElmer retains the strengths of non-targeted chemometric methods like SIMCA, but obtains greater sensitivity by utilizing a library of spectra of potential adulterants. The method can be easily adapted to screen new products or for new adulterants, without having to prepare mixture samples for calibration. The use of an adulterant library also allows much richer diagnostic information to be produced, giving much greater confidence in the results and informing the next analytical steps to take for a suspect sample. The PerkinElmer DairyGuard Milk Powder Analyzer is a complete solution pre-configured for Adulterant Screen analysis of milk powders (along with the standard quantitative analyses), with all the features integrated into a simple, touchscreen-based interface.

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APPLICATION BRIEF

ICP - Mass Spectrometry

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The Elemental Analysis of Milk Powder with NexION 300/350 ICP-MS

Introduction

The elemental capabilities and dynamic range of inductively coupled plasma mass spectrometry (ICP-MS) makes it ideally suited for the analysis of food materials. The ultratrace detection limits of ICP-MS permit the determination of low-level contaminants, such as Pb, As, Se, and Hg, while the macro-level nutritional elements, such as Ca, Mg, K, and Na, can be quantified using the extended dynamic range capability of ICP-MS which provides the ability to measure concentrations over nine orders of magnitude. However, there are still a number of challenges to overcome, including complex sample matrices, high levels of dissolved solids, and interferences. With the proper ICP-MS instrumental conditions and design, all of these issues can be overcome, allowing for the successful analysis of food samples, as described elsewhere[1]. This work will focus on the analysis of non-fat milk powder, an important food staple, especially in the developing world.

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Experimental

Sample Preparation

NIST® 1549 Non-Fat Milk Powder was used for this analysis. Approximately 0.5-0.6 g was digested in duplicate with 5 mL of nitric acid (Fisher Scientific™, Optima grade) and 2 mL of hydrogen peroxide (Fisher Scientific™, Optima grade) in pre-cleaned PTFE microwave sample vessels. The digestion program consisted of 30 min of heating and 15 min of cooling, as shown in Table 1. All samples were completely dissolved, resulting in clear solutions that were diluted to a final volume of 50 mL with deionized water. No further sample dilutions were necessary. Gold was added to all solutions at a final concentration of 200 µg/L to stabilize mercury. Preparation blanks, consisting of the acid mixture, were taken through the same microwave digestion program as the samples.

Table 1. Microwave Digestion Program.

Step	Power (W)	Ramp (min)	Hold (min)
1	500	1	4
2	1000	5	5
3	1400	5	10
4 (cooling)	0	—	15

Instrumental Conditions

All data in this study were generated under normal operating conditions on a PerkinElmer NexION® 300X/350X ICP-MS using an autosampler. The instrumental operating conditions are shown in Table 2.

Table 2. ICP-MS Instrumental Operating Conditions for this Application.

Parameter	Value
Nebulizer	Glass concentric
Spray chamber	Glass cyclonic
Cones	Nickel
Plasma gas flow	18.0 L/min
Auxiliary gas flow	1.2 L/min
Nebulizer gas flow	0.98 L/min
Sample uptake rate	300 µL/min
RF power	1600 W
Total integration time	0.5 (1.5 seconds for As, Se, Hg)
Replicates per sample	3
Universal Cell Technology™*	Collision mode

*PerkinElmer, Inc.

Calibration

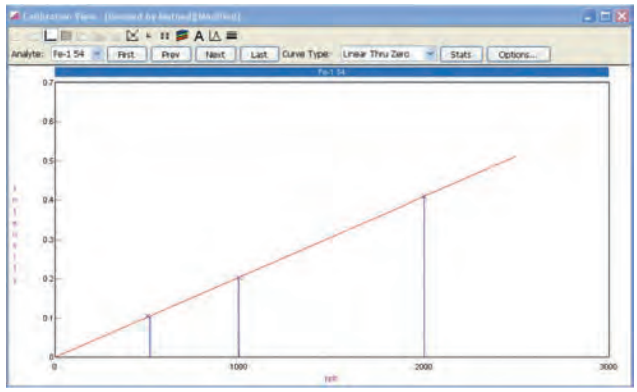
Multielement calibration standards, representing all the analytes in the SRM, were made up from PerkinElmer Pure single and multielement standards and diluted into 10% HNO_3. Gold was added to all solutions at a final concentration of 200 µg/L to stabilize mercury. Calibration standard ranges were based on whether the analyte was expected to be a high-level nutritional element like potassium (K) or sodium (Na), low/medium-level essential element like manganese (Mn) or iron (Fe), or trace/ultratrace contaminant such as lead (Pb) or mercury (Hg).

Depending on the certificate value of the analytes, five different calibration ranges were made up to cover the complete range of elements being determined:

- High-level nutritional analytes: 0-300 ppm
- Medium-level essential analytes: 0-20 ppm
- Low-level essential analytes: 0-2 ppm
- Trace-level contaminants: 0-200 ppb
- Ultratrace-level contaminants: 0-20 ppb

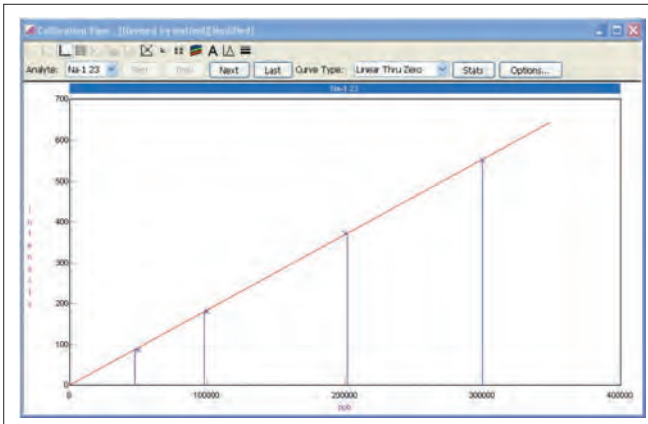
Figures 1 to 5 show representative calibration curves for each range.

In addition to the analyte elements used for the multielement calibration, the standards, blanks, and samples were also spiked on-line using a mixing tee with a solution of ^{6}Li, Sc, Ge, In, and Tb for internal standardization across the full mass range. Acetic acid was added to the internal standard solution to compensate for residual carbon leftover from the sample digestion.



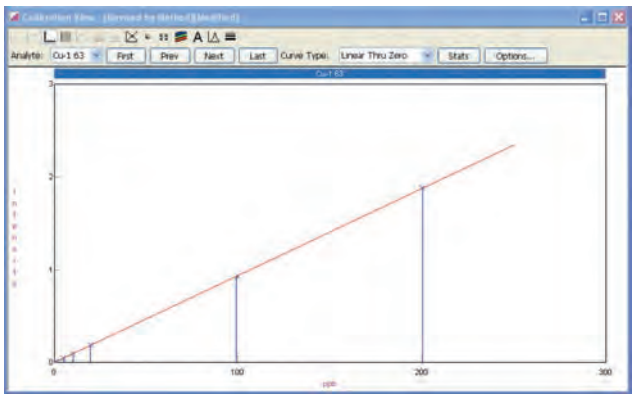
^{54}Fe Correlation Coefficient = 0.99997.

Figure 1. Calibration curves for ^{54}Fe (0-2 ppm).



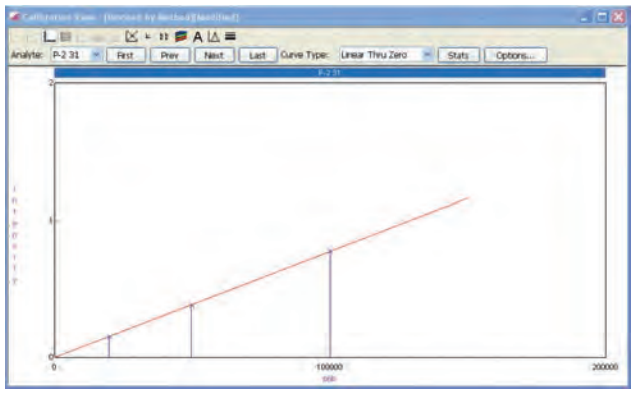
^{23}Na Correlation Coefficient = 0.99996.

Figure 2. Calibration curve for ^{23}Na (0-300 ppm).



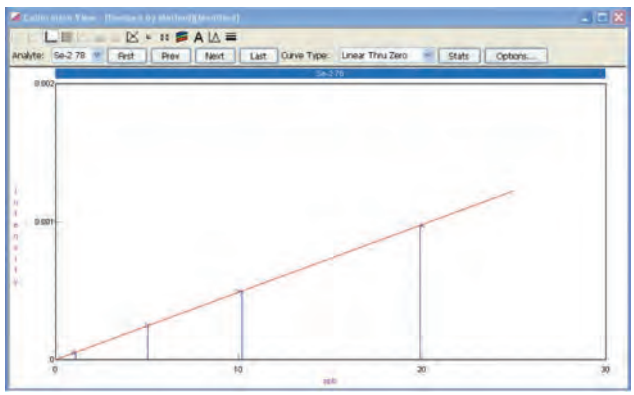
^{63}Cu Correlation Coefficient = 0.99999.

Figure 3. Calibration curve for ^{63}Cu (0-200 ppb).



^{31}P Correlation Coefficient = 0.99999.

Figure 4. Calibration curve for ^{31}P (0-100 ppm).



^{78}Se Correlation Coefficient = 0.99995.

Figure 5. Calibration curve for ^{78}Se (0-20 ppb).

Results

Quantitative results for two sample preparations of the NIST® 1549 Milk Powder are shown in Table 3. All elements in every sample were determined with Universal Cell operating in Collision mode using helium as the cell gas. Figures in parentheses ( ) in the Reference Value column are not certified values but are included for information purposes only. The data show very good agreement with the certified values, especially for the elements that suffer from known spectral interferences. The elements that are outside the specified limits are mostly the ones that are well recognized as environmental contaminants, which have most likely been impacted by the sample preparation procedure.

Table 3. Analysis of NIST® 1549 Milk Powder using the NexION 300X ICP-MS.

Element	Mass (amu)	Reference Value (mg/kg)	Experimental Value (mg/kg)
B	11	–	2.1
Na	23	4970 ±100	4700
Mg	26	1200 ±30	1170
Al	27	(2)	0.7
P	31	10600 ±200	10500
S	34	3510 ±50	3290
K	39	16900 ±300	16500
Ca	44	13000 ±500	12800
V	51	–	0.003
Cr	52	0.0026 ±0.0007	<0.003
Fe	54	1.78 ±0.10	1.98
Mn	55	0.26 ±0.06	0.26
Co	59	(0.0041)	0.005
Ni	60	–	0.013
Cu	63	0.7 ±0.1	0.6
Zn	66	46.1 ±2.2	46.7
As	75	(0.0019)	<0.006
Se	78	0.11 ±0.01	0.17
Sr	88	–	3.7
Mo	98	(0.34)	0.37
Cd	111	0.0005 ±0.0002	<0.002
Sn	118	–	<0.002
Sb	121	(0.00027)	<0.001
Ba	137	–	0.83
Hg	202	0.0003 ±0.0002	<0.0007
Pb	208	0.019 ±0.003	0.019
Tl	205	–	<0.0001
Th	232	–	<0.00008
U	238	–	<0.00002

Conclusion

This work has demonstrated the ability of PerkinElmer's NexION 300X/350X ICP-MS to effectively measure macro-level nutritional elements in the same analysis run as lower-level elements, without having to dilute the samples. The agreement between experimental and certified results for NIST® 1549 Milk Powder demonstrates the accuracy of the analysis. Instrument design characteristics eliminate deposition on the ion optics, leading to long-term stability in high-matrix samples, while permitting trace levels to be accurately measured.

References

1. "The Determination of Toxic, Essential, and Nutritional Elements in Food Matrices Using the NexION 300/350 ICP-MS", PerkinElmer Application Note.

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APPLICATION NOTE

LC/Mass Spectrometry

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LC/MS Study of Casein Proteins in Milk

Introduction

We have developed a method for extracting and measuring the masses of proteins in milk by using liquid chromatography coupled to electrospray time-of-flight mass spectrometry (LC-TOF MS). This method was used to look at the patterns and masses of the casein proteins in cow milk.

Methods

Proteins were extracted from milk by the addition of a two-fold volume of an aqueous denaturing solution containing 8 M urea, 165 mM TRIS, 44 mM sodium citrate and 0.3% (v/v) mercaptoethanol, followed by filtration[1]. This method extracted fat soluble casein proteins in addition to whey proteins.

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Following extraction, the proteins were separated using a fast gradient HPLC method and detected with an AxION® 2 TOF mass spectrometer, with ion optics tuned to optimize transmission of the mass range m/z 1000-2000.

Spectra in the acquired datasets were averaged for selected time ranges and deconvoluted using a proprietary algorithm to create zero charge spectra which show both the accurate mass and intensity ratios of the proteins eluting at that time. The high resolution and high mass accuracy of TOF MS allows for measurement of a protein average mass to within 1 Da of the calculated mass.

LC conditions:

Pump:	PerkinElmer Flexar™ FX-15 pump
Flow:	0.4 mL/min
Mobile phase A:	Water + 0.1% formic acid + 0.01% trifluoroacetic acid
Mobile phase B:	Acetonitrile + 0.1% formic acid+ 0.01% trifluoroacetic acid
Gradient conditions:	25-43% B over 6 min
Injection volume:	2 µL of 20x diluted sample
Column used:	Phenomenex wide pore XP-C8 2 x100 mm, heated to 50 °C

MS conditions:

Mass spectrometer:	PerkinElmer AxION 2 TOF MS
Ionization source:	PerkinElmer Ultraspray™ 2 (Dual ESI source)
Ionization mode:	Positive
Spectral acquisition rate:	3 spectra/sec

Results

Milk contains lactoglobulin, lactoferrin and casein proteins at high concentrations. Caseins are relatively hydrophobic phosphoproteins, which comprise 80% of the cow milk proteins; there are several related caseins, which are phosphorylated to different degrees. The alpha and beta caseins are insoluble in water and are bound together with the more soluble kappa-casein to form micelles.

The caseins are extracted from milk with a denaturing 8 M urea protocol, which breaks apart the micelles and solubilizes the proteins. Chromatography of the proteins in the urea extract separates the various phosphorylated variants of alpha, beta and kappa forms of casein (Figure 1). Other non-casein proteins from the milk are also observed. Peak identification is based on the protein masses in the deconvoluted spectra.

Alpha S1-Casein

Alpha S1-casein is a heavily phosphorylated protein which causes the most common milk allergic reaction[2]. Detection of this protein is of interest in food allergy studies.

A deconvoluted spectrum from the averaged alpha S1-casein elution time-range spectra clearly shows the presence of two major different forms of the protein (Figure 2), with 8 and 9 phosphorylated serine residues.

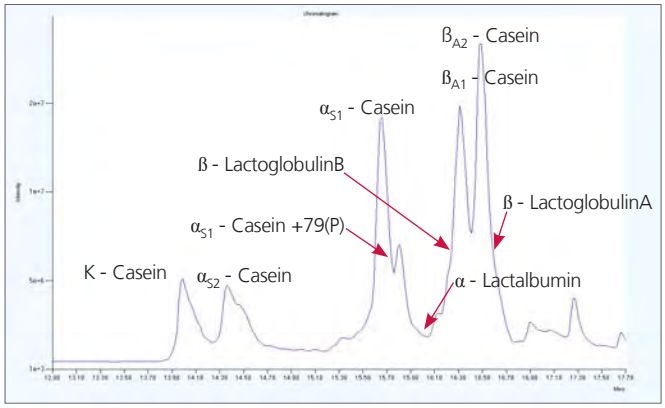

Figure 1. Total ion current trace showing the profile of the proteins in the separation of urea-extracted US supermarket cow's milk.

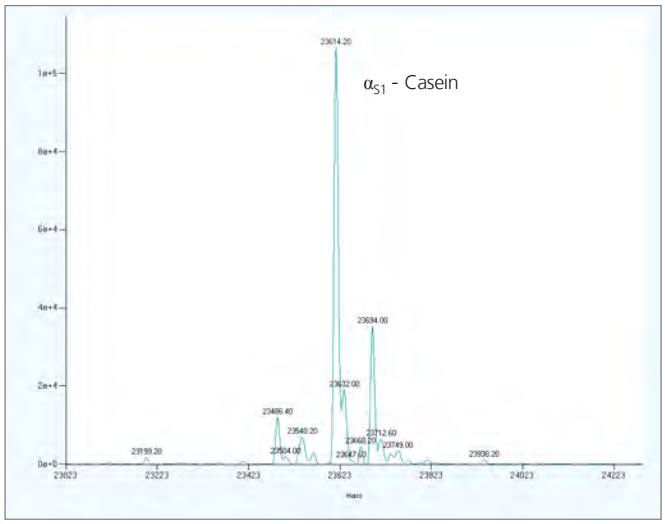

Figure 2. Deconvoluted spectrum of the averaged alpha-S1-casein elution region showing two major highly phosphorylated forms of the protein (23,614 Da) and (23,694 Da), differing by one phosphorylation site (80 Da).

A1 and A2 Beta-Casein

Cows, depending on the breed, have genetic variants resulting in the production of different sequences of beta-casein. Older breeds of African and Indian cows and water buffalo have the A2 beta-casein genetic allele and produce only this protein. Some cows, such as Holsteins and Friesians, have a genetic mutation which causes them to produce a variant protein, A1 beta-casein. These breeds carry A1 and A2 alleles in equal distribution, while Jersey and Guernsey cows have higher ratios of the A2 allele. Consequently, milk from most European herds contains a mix of A1 and A2 beta-caseins.

The presence of A1 beta-casein in bovine milk has been linked to health concerns. The A1 variant of beta-casein differs from the A2 variant by one amino acid at position 67, having Ile-His67 in A1 and Ile-Pro67 in A2. This difference of 40 Da is easily detected. The A1 form cleaves at the Ile-His peptide bond during human digestion, to produce the bioactive peptide beta-casomorphin-7. This peptide has been suggested as a potential disease risk factor[3]. Thus measurement of A1 and A2 beta-casein levels in the milk supply is of interest.

In the U.S. milk supply, the breeds of cows, predominantly Holstein, provide milk with A1 and A2 beta-casein proteins in approximately equal ratios. Peaks for the two forms of A1 and A2 beta-casein are shown in the elution profile of supermarket milk (Figure 1). The deconvoluted spectra from the two peaks in this milk sample show the A1 form at a slightly higher level than the A2 form (Figure 3).

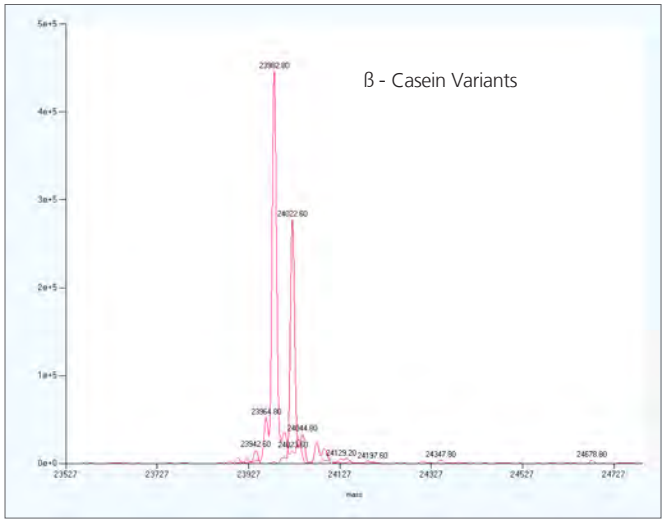

Figure 3. Overlay of the deconvoluted spectra of the two beta-casein peaks showing that this milk sample contains both of A1 (23,982 Da) and A2 (24,022 Da) variants. The variants are due to a His/Pro substitution differing by 40 Da. Both beta-caseins are multiply phosphorylated.

Conclusions

A fast and simple sample preparation, combined with a rapid reversed-phase LC/MS analysis, was used to investigate the presence of different caseins and phosphorylation levels of caseins in different milk samples.

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APPLICATION NOTE

LC/Mass Spectrometry

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Milk Adulteration: Detecting Species-Specific Proteins by LC/MS

Introduction

The adulteration of milk via the addition of low molecular weight compounds, such as melamine for economically motivated fraud, is a well-known concern[1]. Methods for adulteration detection have been examined extensively using, amongst other tools, LC/MS techniques. Current methods tend to focus on small molecule adulteration; however, large molecule adulteration with other animal or plant proteins is becoming increasingly common and is not yet regulated within the dairy industry.

One of the simplest forms of milk adulteration is to dilute more expensive milks from species, such as sheep and buffalo, with less expensive cow milk. We have developed a method for measuring the addition of bovine milk to more expensive milks (goat, sheep, buffalo and camel) by using liquid chromatography coupled to electrospray time-of-flight mass spectrometry (LC-TOF MS) to detect species-specific marker proteins in the milk.

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Methods

Milk samples from different animals were spiked with varying levels of bovine milk and homogenized with a vortex mixer for 30 seconds. Proteins were then extracted from the samples for LC/MS analysis.

Whey proteins in milk were purified by liquid/liquid extraction; the proteins remain in the aqueous layer after addition by volume of 20% of dichloromethane and 10% of an aqueous solution of 5% acetic acid[2] to the milk to separate out the lipids.

Following extraction, the proteins were separated using a fast gradient HPLC method and detected with an AxION® 2 TOF mass spectrometer, with ion optics tuned to optimize transmission of the mass range m/z 1000-2000.

Spectra in the acquired datasets were averaged for selected time ranges and deconvoluted using a proprietary algorithm to create zero charge spectra which show both the accurate mass and intensity ratios of the different proteins eluting at that time.

LC conditions:

Pump:	PerkinElmer Flexar™ FX-15 pump
Flow:	0.4 mL/min
Mobile phase A:	Water + 0.1% formic acid + 0.01% trifluoroacetic acid
Mobile phase B:	Acetonitrile + 0.1% formic acid+ 0.01% trifluoroacetic acid
Gradient conditions:	25-43% B over 6 min
Injection volume:	2 µL of 20x diluted sample
Column used:	Phenomenex wide pore XP-C8 2 x 100 mm, heated to 50 °C

MS conditions:

Mass spectrometer:	PerkinElmer AxION 2 TOF MS
Ionization source:	PerkinElmer Ultraspray™ 2 (Dual ESI source)
Ionization mode:	Positive
Spectral acquisition rate:	3 spectra/sec

Results

Markers for adulteration with cow milk

All mammalian milks contain lactoglobulin, lactoferrin and casein proteins at high concentrations. A liquid/liquid extraction protocol was selective for the water soluble proteins lactoferrin and lactoglobulins present in the whey fraction of milk.

Milk obtained from many mammalian species, including cow, buffalo and sheep, although not human or camel, contain the glycoprotein beta–lactoglobulin. Human allergies to cow milk may be related to the presence of this protein[3].

Genetic phenotypes of beta–lactoglobulin are predominantly forms A and B, where the sequences differ by two amino acids. Cows and sheep have both A and B forms, while buffalo has only one form of beta–lactoglobulin, which is very close in sequence and mass to the cow B form. The protein sequence for beta-lactoglobulin B is highly homologous for all species that produce this protein in milk, while the sequence of beta-lactoglobulin A varies with species and has additional amino acid substitutions

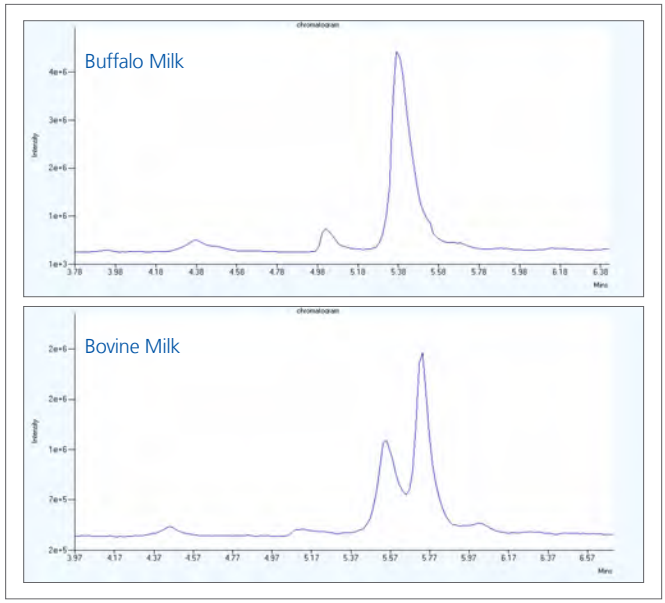

Figure 1. LC/MS analysis showing the lactoglobulin elution profiles for different milks. Buffalo milk has one form of beta-lactoglobulin, where bovine milk has two forms – beta-lactoglobulin A and B – which elute at different times in the chromatogram.

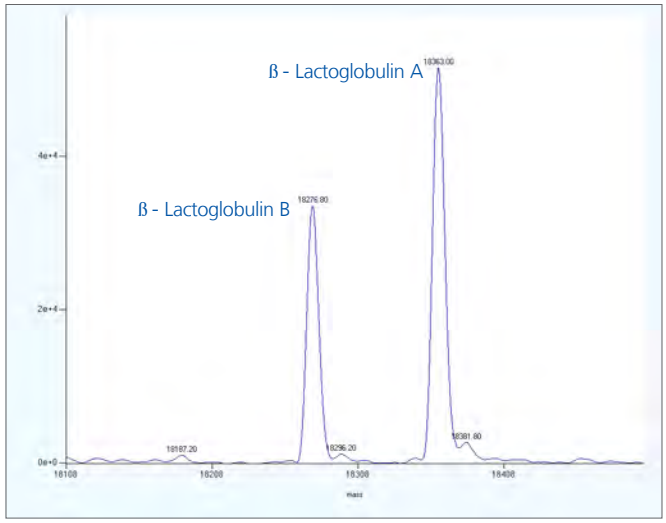

Figure 2. Deconvoluted spectrum of the beta-lactoglobulins from pure cow milk after averaging the spectra in the time range where both beta-lactoglobulin A and B elute. The B form has an average mass of 18276.8 Da and the A form of 18,363.0 Da.

due to genetic variants. The amino acid sequence differences of the A and B forms allow them to be separated by chromatography (Figure 1).

A deconvoluted spectrum for the averaged spectra of the two forms shows the intensity ratio and masses of bovine beta-lactoglobulin A and B forms (Figure 2). The high resolution and high mass accuracy of TOF MS enables measurement of a protein average mass to within 1 Da of the calculated mass. The theoretical average masses of bovine lactoglobulin A and B forms are calculated as 18,363.3 Da and 18,277.2 Da respectively for the forms in which four of the cysteine residues are in the oxidized form and one in the reduced form[4]. The experimentally determined lactoglobulin masses from bovine milk were within 0.4 Da of the theoretical masses.

Measurement of the proteins in the buffalo milks spiked with cow milk provides unambiguous confirmation of the presence of bovine lactoglobulin A (Figure 3) down to low levels resulting from 5% adulteration.

Thus beta–lactoglobulin A is a marker for the presence of bovine milk, even in the presence of lactoglobulin B, and the specific mass of this marker protein is linked to the breed of cow[5]. Similar results were obtained for other milks, such as sheep (Figure 4) and camel (Figure 5).

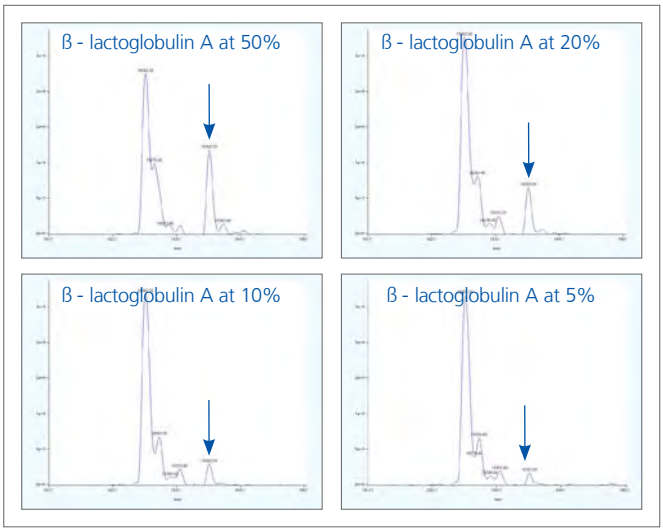

Figure 3. Adulteration levels down to 5% of cow milk in buffalo milk are detected in the deconvoluted spectrum of the averaged lactoglobulin elution region using β-lactoglobulin A as the marker protein.

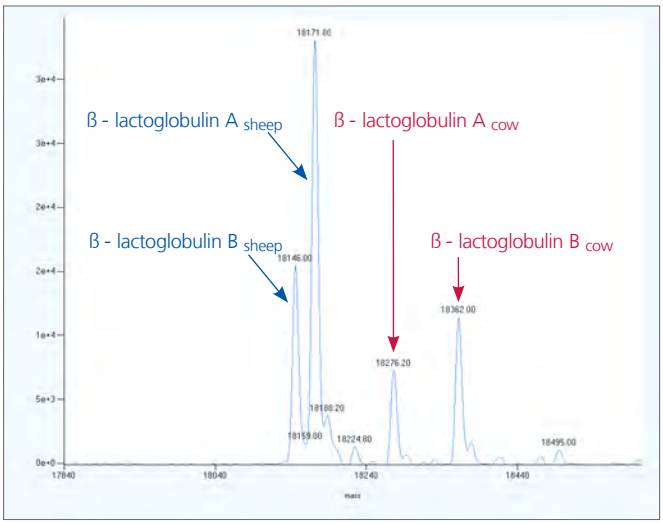

Figure 4. Adulteration of sheep milk by cow milk is detected by the different masses of both the A and B forms of lactoglobulin for each species.

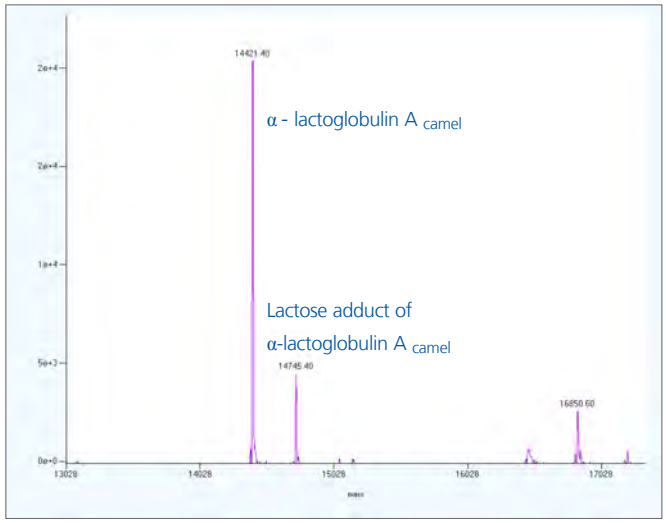

Figure 5. Camel milk contains no lactoglobulin B; only lactoglobulin A and the lactosylated form of the protein are detected.

Conclusions

A fast and simple sample preparation, combined with a rapid reversed-phase LC/MS analysis, provided information on the masses and levels of a number of different proteins in the milk of different species and breeds.

A protein of a specific mass only found in bovine milk was used as a marker protein for the detection of adulteration of expensive milks by less expensive bovine milk. Low levels of 5% adulteration were detected. This detection method was successfully applied to buffalo, goat, camel and sheep milk.

Acknowledgements

The authors would like to thank Thomas Olson at The Turkey Creek Co., Texarkana, Arkansas for providing some of the milk samples.

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APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

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Analysis of Quaternary Ammonium Compounds (QACs) as Possible Disinfectant Residues in Milk by LC-TOF

Introduction

Quaternary ammonium compounds (QACs) have the basic structure NR_4^+. Those possessing R groups with long alkyl chains are known to be especially effective as antimicrobial agents and particularly useful for the disinfection of containers and surfaces. This is particularly relevant in the milk industry, as QACs are typically used to disinfect the insides of tanks used for transporting milk from farms to processing plants. If significant QAC residues are left behind after tank disinfection, these compounds may leach into the milk and, ultimately, may get into the store-bought milk supplies at levels compromising personal health. Recent data points to nearly 12% of all monitored milk to be tainted with QACs.[1]

The primary QACs that may be found in milk are benzyldimethyldodecylammonium chloride (BAC 12), benzyldimethyltetradecylammonium chloride (BAC 14), benzyldimethylhexadecyl ammonium chloride (BAC 16) and didecyldimethylammonium chloride (DDAC). Their chemical structures and expected parent masses in solution are shown in Figure 1.

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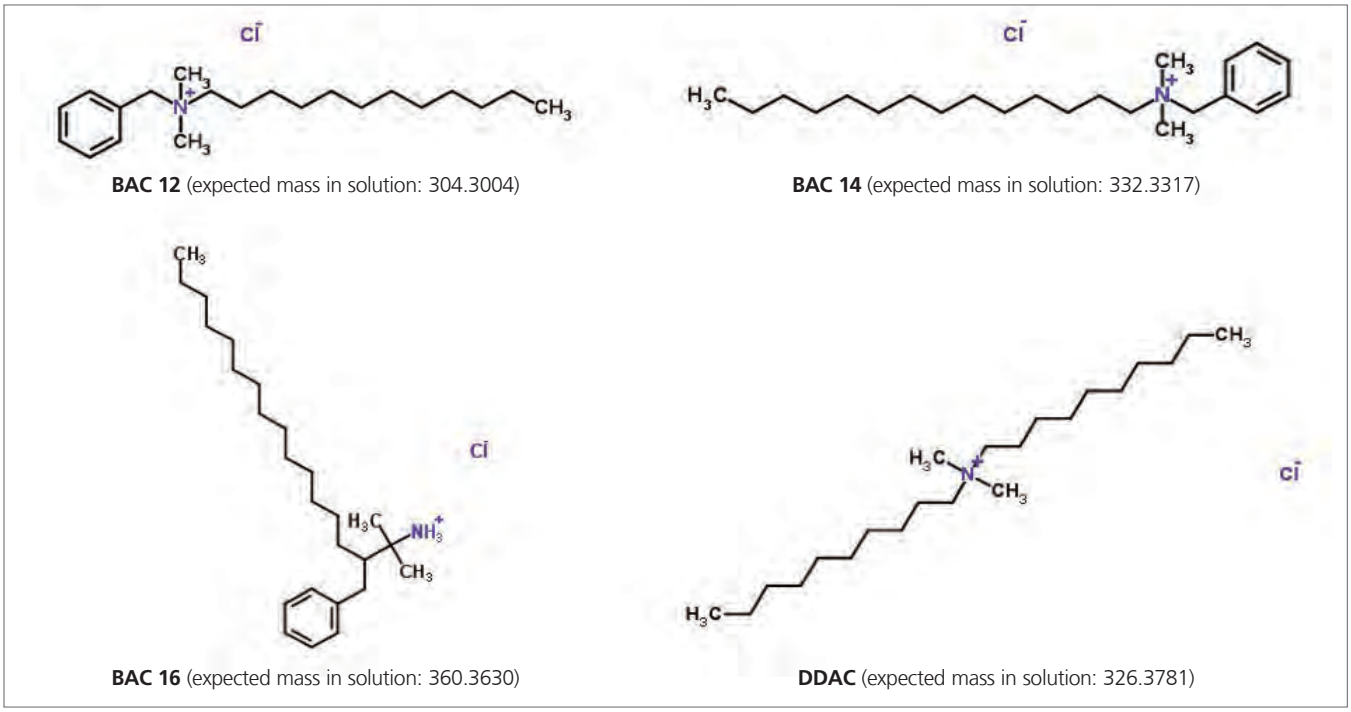

Figure 1. Chemical structures and expected masses of the four quaternary ammonium compounds (QACs) analyzed.

Regarding safety and regulations, the European Union Reference Laboratory (EURL) has taken the following position:

"Because no specific maximum limit for residues of DDAC and BAC was established under EU Regulation No. 396/2005, the general residue limit of 0.01 mg/kg applies.

In October 2012, the Standing Committee on the Food Chain and Animal Health (SCoFCAH) endorsed Guidelines on measures to be taken regarding the presence of DDAC[2] and BAC[3] in or on food and feed. It was recommended that EU Member States carry out investigations on the possible causes of BAC/DDAC contamination and to put in place a monitoring program to get an overview of the BAC and DDAC levels in all food and feed of plant and animal origin. Considering that the current default MRLs for DDAC and BAC (of 0.01 mg/kg) are not a health standard, a temporary enforcement level of 0.5 mg/kg was agreed upon. As no specific residue definition was defined, there is still uncertainty as to how residues are to be expressed. Based on the first results of the monitoring program a lower enforcement level for QACs is under discussion."[4]

Considering the above, this application note presents an LC-TOF (time-of-flight) method for the analysis of the four most common QACs that may be found in milk (a rather complex matrix), with relatively little sample preparation. This method takes advantage of the inherent mass accuracy and high resolution afforded by TOF detection for specificity and component identification. Method conditions and performance data, including linearity and repeatability, are presented.

Experimental

Hardware/Software

For all chromatographic separations, a PerkinElmer Flexar UHPLC System was used, including the Flexar FX-20 pump, FX autosampler, column oven and AxION 2 TOF MS detector.

All instrument control, analysis and data processing was performed using the PerkinElmer Chromera® CDS software platform.

Method Parameters

The HPLC method parameters are shown in Table 1.

Table 1. HPLC Method Parameters.

HPLC Conditions	
Column:	PerkinElmer Brownlee™ 2.7-µm 2.1 x 50-mm C18 (Part# N9308402)
Mobile Phase:	Solvent A: 0.1% formic acid in water Solvent B: 0.1% formic acid in acetonitrile Solvent Program:

Step	Time	Flow Rate (mL/min)	%A	%B	Curve
0 (Equil.)	4.0	0.4	70	30	1
1	1.5	0.4	50	50	1
2	2.0	0.4	0	100	1
3	0.5	0.4	0	100	1

Analysis Time:	4 min.
Flow Rate:	0.4 mL/min. (maximum pressure: ~3900 psi)
Oven Temp.:	25 °C
Detection:	Perkin Elmer AxION 2 TOF MS; positive ion mode; mass range: 100-1250 MW Ions 118.0862 and 922.0098 were used as lock mass calibrants to compensate for any mass drift during acquisition; spectral rate: 5 spectra/sec MW channels (EICs): BAC 12: 304.300; BAC 14: 332.332; BAC 16: 360.363; DDAC: 326.378
Injection Volume:	2 µL

Solvents, Standards and Samples

All solvents used were HPLC grade and filtered via 0.45-µm filters.

All QAC standards were purchased from Sigma-Aldrich (St Louis, MO USA), including the following: benzyldimethyldodecylammonium chloride (BAC 12), benzyldimethyltetradecylammonium chloride (BAC 14), benzyldimethylhexadecyl ammonium chloride (BAC 16), and didecyldimethylammonium chloride (DDAC).

A 10-ppm stock standard solution was prepared by dissolving 10 mg of each of the four QAC components into 1 liter of 50:50 acetonitrile/water. This stock solution was then used to prepare 1-ppm to 0.05-ppm standard solutions by serial dilution.

The analyzed product was a store-bought container of whole milk. Using a 1-L solvent bottle, a spiked sample was prepared by spiking 10 mg of each standard component into 1 liter of milk. Both this spiked sample and an unspiked liter of milk were then placed on a magnetic stirring plate and stirred for 30 minutes at 30 °C. Both samples were then capped and refrigerated until further use.

When ready for analysis, after allowing both samples to equilibrate to room temperature, 20 mL of each sample was transferred to a separate 50-mL polypropylene centrifuge tube. 20 mL of acetonitrile was then added to each tube, causing the proteinaceous ingredients in the milk to precipitate ("protein crash"). Both samples were then centrifuged at 8000 rpm for five minutes. After centrifugation, 2 mL of each supernatant was filtered through a 0.45 µm filter, transferred into a 2-mL sample vial and subsequently injected.

Prior to injection, all calibrants were also filtered through 0.45-µm filters, removing any small particles.

Results and Discussion

Figure 2 shows the chromatographic separation of the 0.5-ppm QAC standard, separating the four QAC compounds in under 3.5 minutes. All displayed chromatograms consist of the overlays of four EICs (extracted ion chromatograms), each corresponding to the expected mass of the individual QAC components.

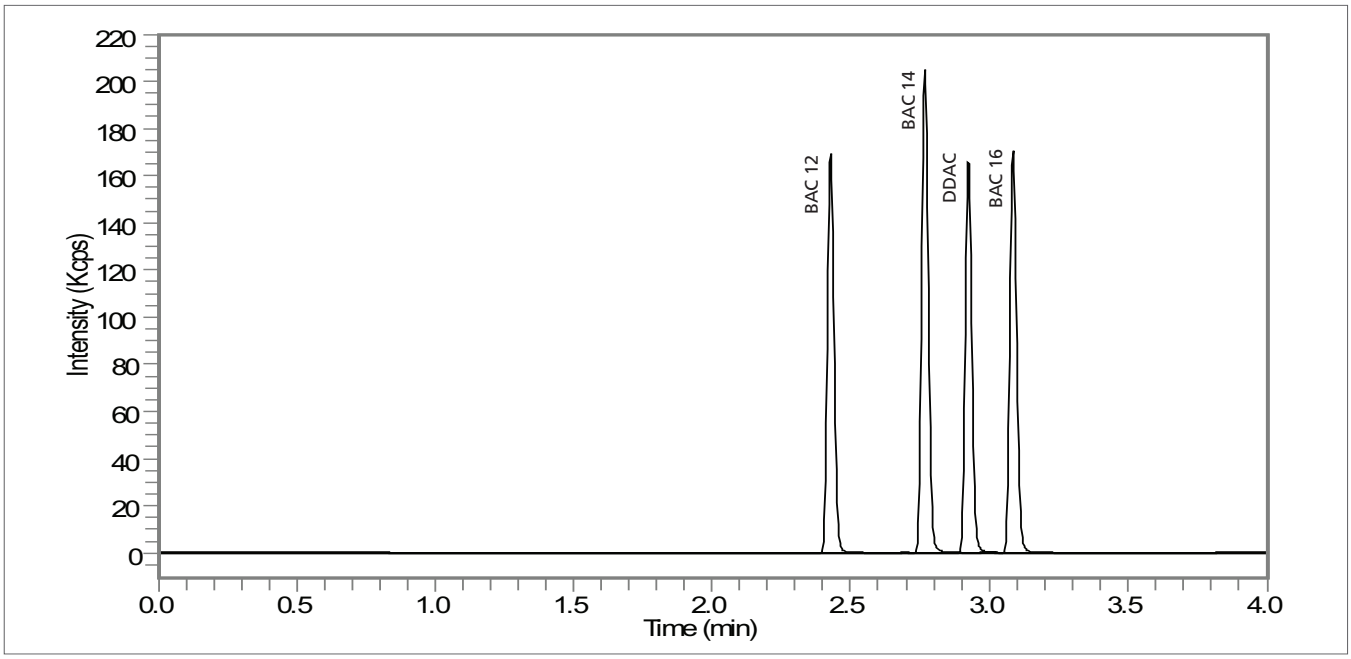

Figure 2. Chromatogram of 0.5-ppm QAC standard; EIC channels: BAC 12: 304.300; BAC 14: 332.332; BAC 16: 360.363; DDAC: 326.378

Figure 3 shows the overlay of 10 replicate injections of a QAC standard mix, demonstrating exceptional reproducibility. The retention time (RT) %RSDs ranged from 0.09 to 0.16.

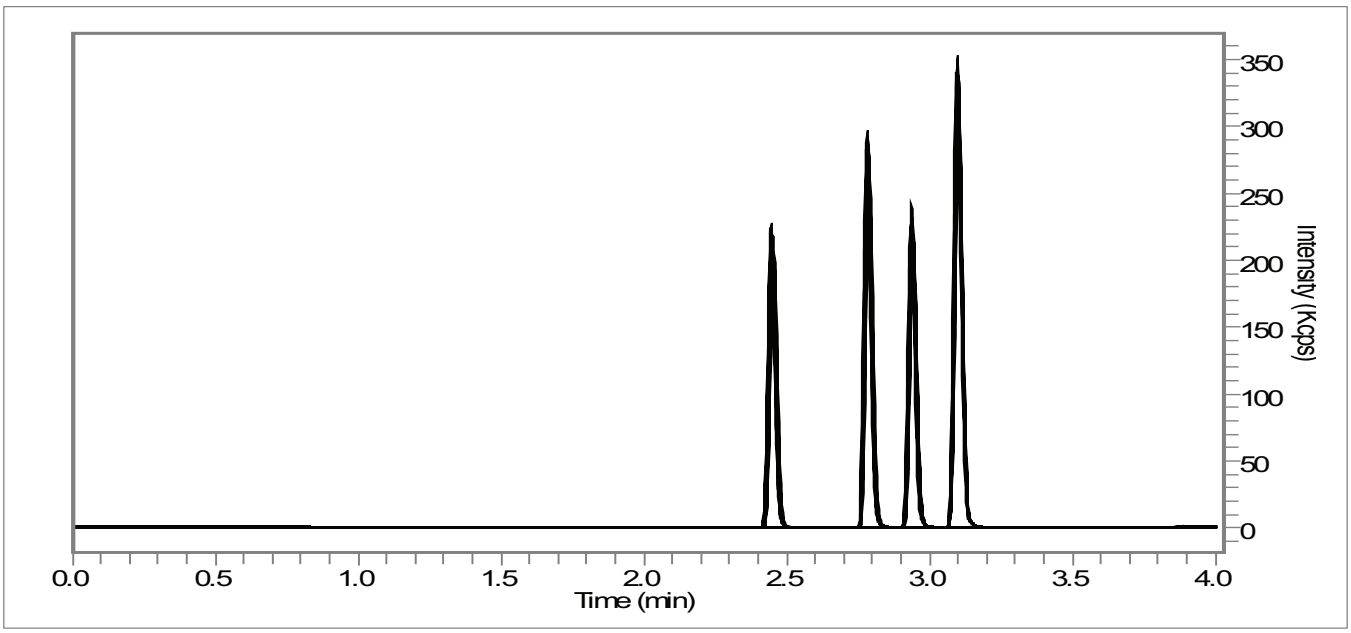

Figure 3. Overlaid chromatograms of 10 replicates of a preliminary standard mix.

Figure 4 shows the calibration plots over a concentration range of 0.05 to 1 ppm, with all four QAC components having quadratic fit coefficients > 0.99 (n = 3 at each level).

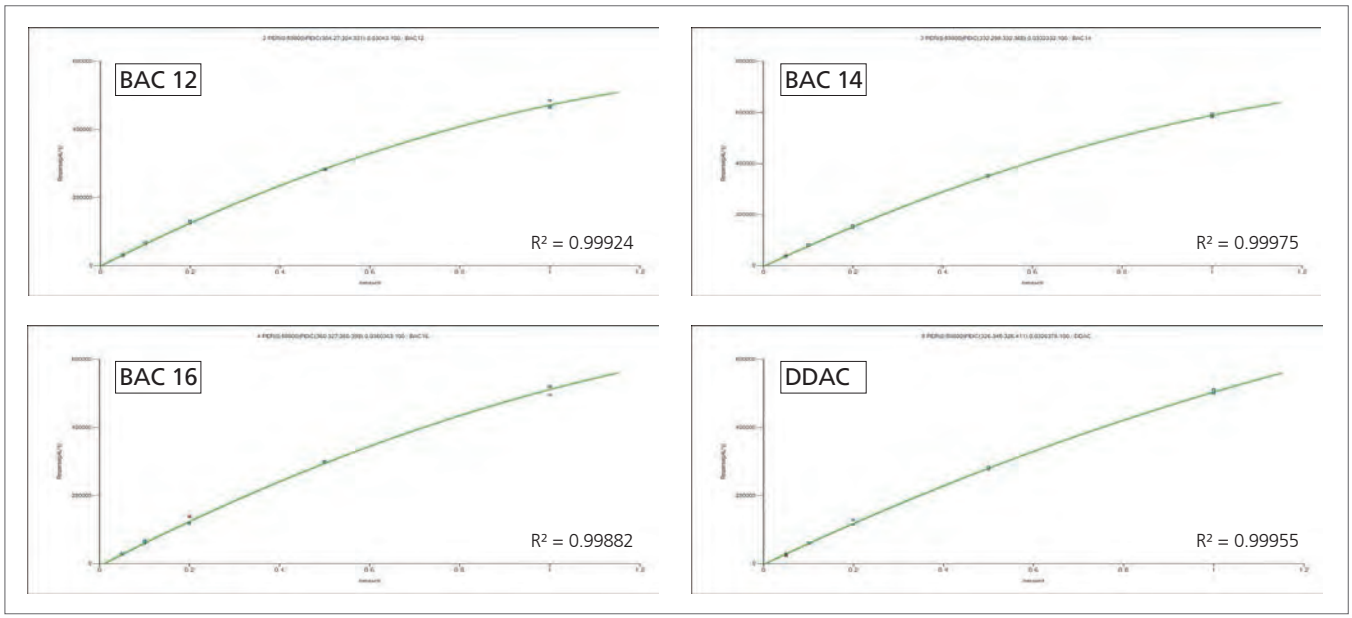

Figure 4. Plots of 5-level calibration set for all four QACs.

Figure 5 shows the averaged MS spectra for all four QAC components, highlighting the exceptional mass accuracy that was achieved using the integrated lock mass option. These were based on the expected exact masses for each component in solution.

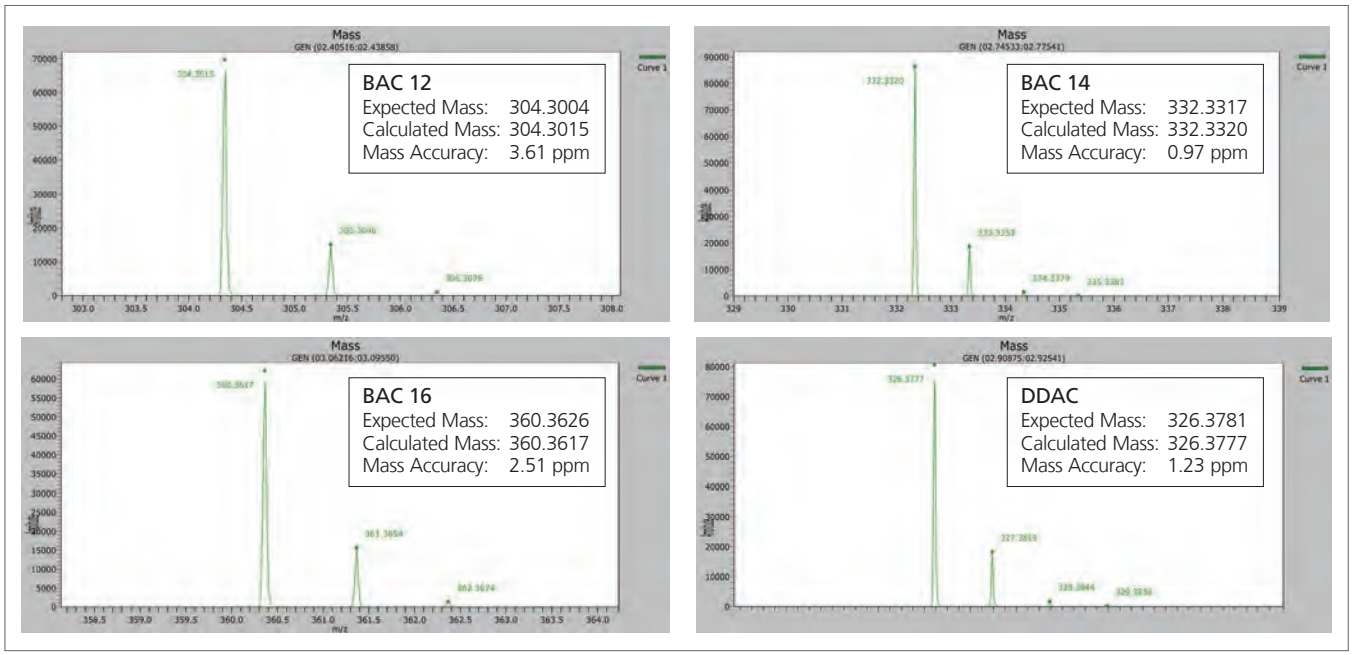

Figure 5. Averaged MS spectra showing the mass accuracy achieved for each of the four QAC components.

The identity of the QACs was further confirmed with the help of elemental composition matching via AxION EC ID software. Per example shown in Figure 6, the accurate mass and isotope information for DDAC was entered into the software and searched against a selected database, in this case, PubChem. The search resulted in an elemental composition that perfectly matched DDAC.

Following the liquid-liquid extraction procedure described earlier, an extracted sample of whole milk and the same whole milk previously spiked with of 1-ppm QAC were analyzed. The overlaid chromatograms (EICS) of both extracts are shown in Figure 7.

The quantitative results for the spiked milk extract are shown in Table 2. Considering that the liquid-liquid extraction procedure involved a 1:1 sample dilution, the normalized amounts were 0.984, 0.730, 0.852 and 0.952 ppm, for BAC 12, BAC 14, BAC 16 and DDAC, respectively. Further refinements in the extraction procedure may very well be able to improve on this recovery.

Also, in Figure 7, the expanded view of the four components shows a small amount of QACs in the unspiked milk extract. However, as shown in Figure 8, these amounts are very low and are similar to levels observed for the 70% acetonitrile (ACN) blank injections. Follow-up testing revealed this small amount of QAC background to be due to slight column carryover. As this level is far below the maximum allowable 0.5 mg/kg (ppm) regulation QAC level set in the EU (the only global region that has any regulations for this thus far), this background was considered insignificant and quite acceptable.

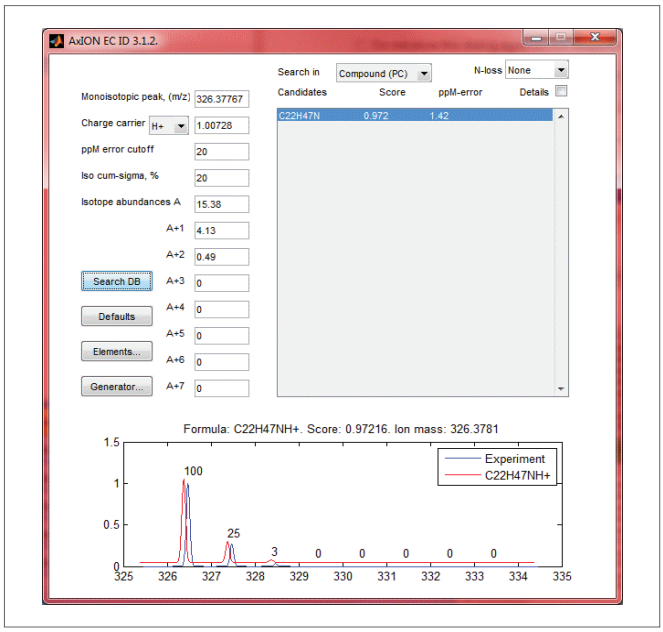

Figure 6. Example of a database search for the formula confirmation of DDAC.

Table 2. Calculated/normalized amounts for QACs in 1 ppm-spiked whole milk extract.

Component	Calculated Amount (ppm)	Normalized Amount (ppm) – considering 1:1 dilution	% Recovery
BAC 12	0.492	0.984	98%
BAC 14	0.365	0.730	73%
BAC 16	0.426	0.852	85%
DDAC	0.476	0.952	95%

* All samples were run in duplicate, using averaged values

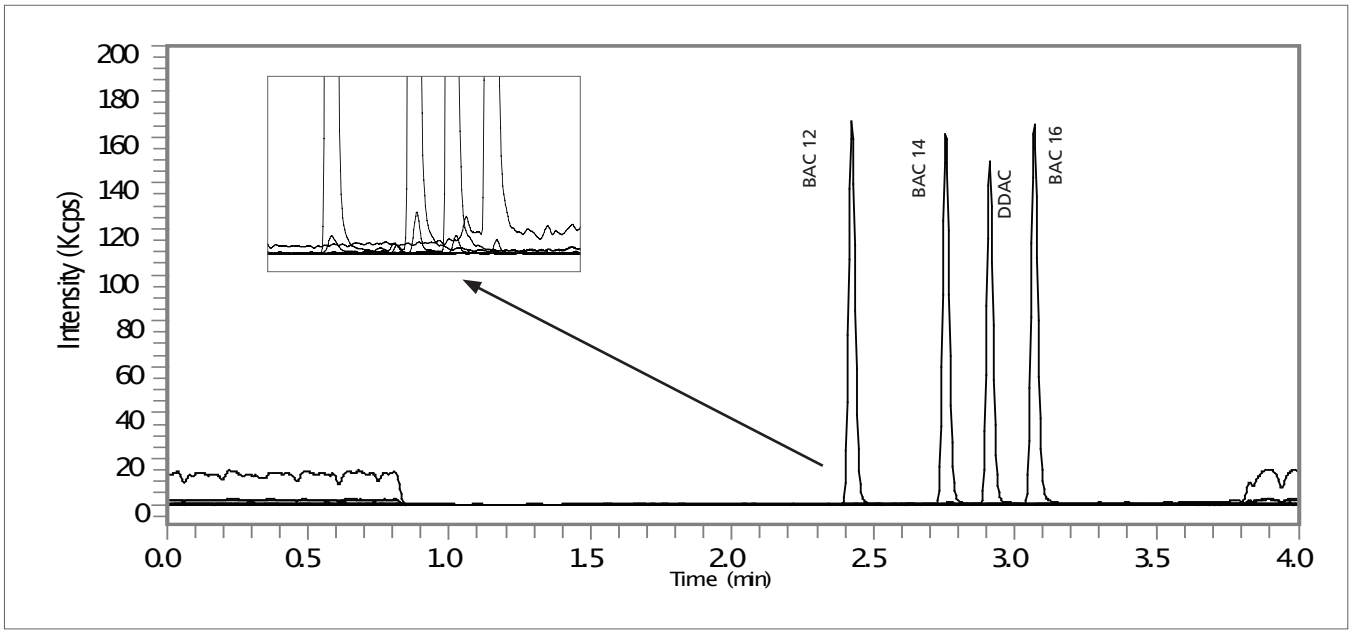

Figure 7. Chromatograms (EICs) of 1 ppm-spiked whole milk extract (four large peaks) overlaid with that of the unspiked milk extract. Due to the very low levels, the EICs of the unspiked milk extract can only be seen in the expanded view.

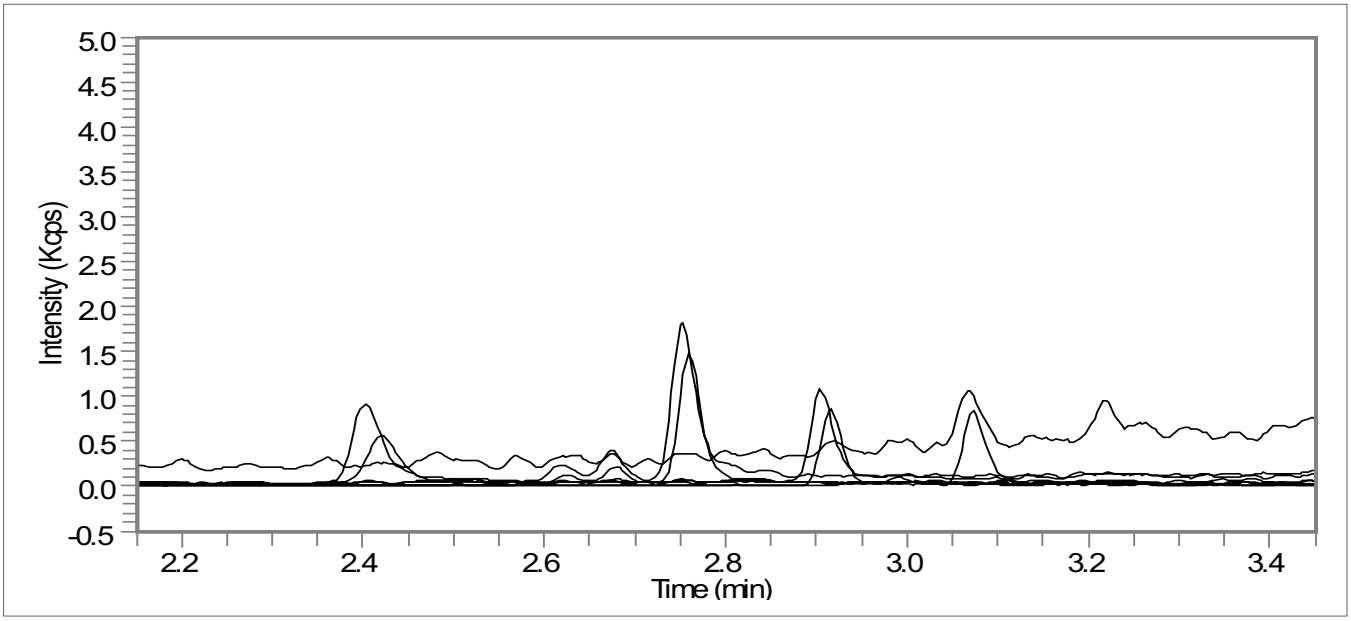

Figure 8. Chromatogram (EICs) of un-spiked milk extract overlaid with 70% ACN/water blank injection.

Conclusion

With rising health concerns and the large quantities of milk that are consumed, it is important to have reliable procedures for the monitoring of possible unhealthy contaminants in dairy products. With this in mind, this work demonstrated the fast/effective chromatographic separation for the quantitative analysis of four quaternary ammonium compounds (QACs) in milk by LC-TOF. The results exhibited exceptional reproducibility with more than adequate sensitivity for monitoring down to current regulated levels. In addition, by using a TOF detector, the combination of averaged MS spectra, mass accuracy checks and database search results allowed for the definitive identification/confirmation of the four QAC components.

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4. EU Reference Laboratory for Pesticides Requiring Single Residue Methods, CVUA Stuttgart, Schaflandstr. 3/2, 70736 Fellbach, Germany.

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APPLICATION BRIEF

Near-Infrared Spectroscopy

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Use of NIR Spectroscopy and Adulterant Screen for the Detection of Common Adulterants in Milk

Introduction

The value of milk on the open market is linked to its protein content, and standard methods for protein analysis rely on a simple nitrogen assay, with the protein concentration inferred from the nitrogen content. Consequently, the addition of chemicals rich in nitrogen, such as urea, can artificially increase the apparent protein content and thus the price demanded. Urea occurs naturally in milk and is typically present at levels of about 0.02% - 0.05%. Higher levels of urea in milk are present only in cases of adulteration. Cane sugar is another known milk adulterant used to increase its carbohydrate content and weight. This allows extra water to be added into the milk without detection from a standard lactometer test for milk quality.

NIR spectroscopy coupled with PerkinElmer's Adulterant Screen™ is shown here to be capable of detecting adulterants intentionally or accidentally added to milk.

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Method

Spectra of a variety of milk samples were collected on a PerkinElmer Frontier™ NIR spectrometer in transflectance using the NIRA II sampling accessory. The set of samples was selected in order to cover adulteration within a broad range of different types of milk, and included full fat, semi-skimmed, skimmed, lactose-free, and organic varieties. These spectra were defined in Adulterant Screen as our set of Material Spectra and represented "good samples." A spectrum of cane sugar, urea, and a spectrum of a 10% aqueous urea solution were measured as adulterants.

A full-fat milk sample was spiked with urea to give a urea concentration of 1% w/w. The spectra of the milk sample and the urea-spiked sample are shown in Figure 1.

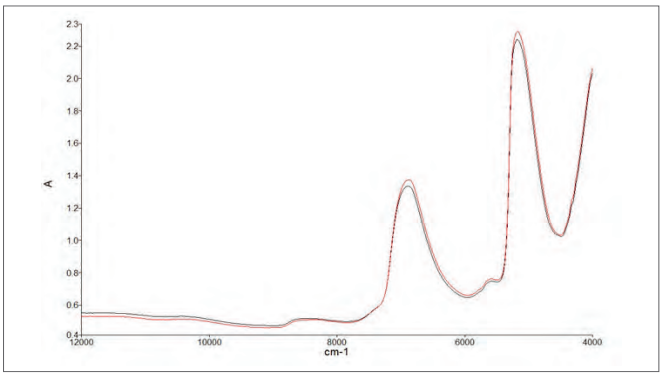

Figure 1. Spectra of whole milk (red) and milk spiked with 1% urea (black).

Although the spectra appear to be very similar, the application of a second derivative function shows that there are clear differences in the spectral region associated with urea absorptions as shown in Figure 2, thus allowing for the detection of urea in an adulterated milk sample.

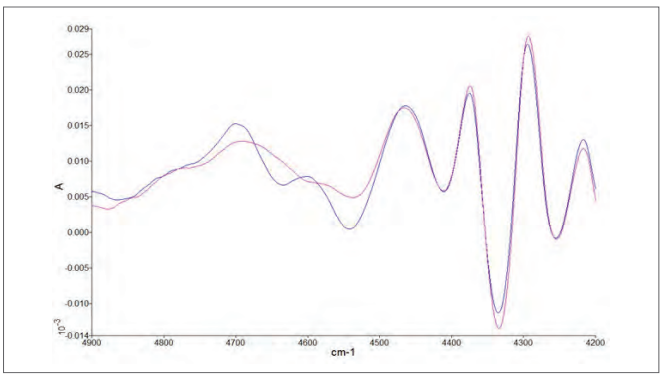

Figure 2. Second derivatives showing differences between milk (purple) and sample spiked with urea (blue).

The normal process for finding adulterants simply requires the measurement of a sample of the adulterant to provide a reference for subsequent comparison with the sample spectra. However, in the case of urea, the infrared spectrum changes significantly in the presence of water, resulting in the urea adulterant being incorrectly determined. The spectra of urea powder and urea solution (with the water subtracted) are shown in Figure 3.

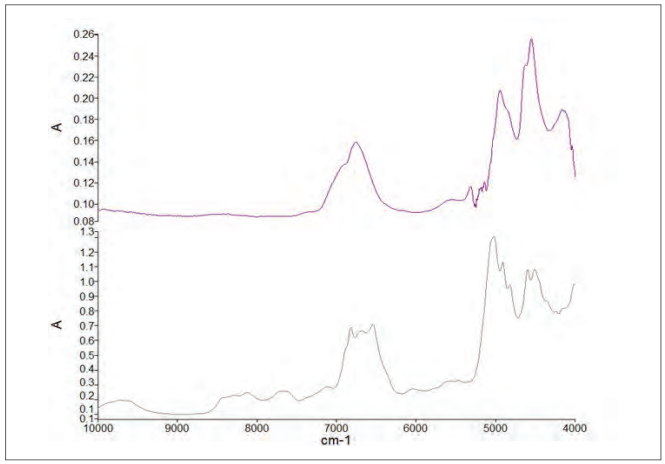

Figure 3. Spectra showing differences between 10% urea solution with water subtracted (top) and urea powder (bottom).

The urea solution spectrum with water subtracted is a more representative spectrum of urea in aqueous samples, such as milk, and should be used as the adulterant spectrum. This spectrum was then normalized to represent a 100% urea standard and added to the list of adulterants for this method. A spiked full-fat milk sample could then be checked for adulterants using Adulterant Screen. The result for the spiked sample is shown as Figure 4.

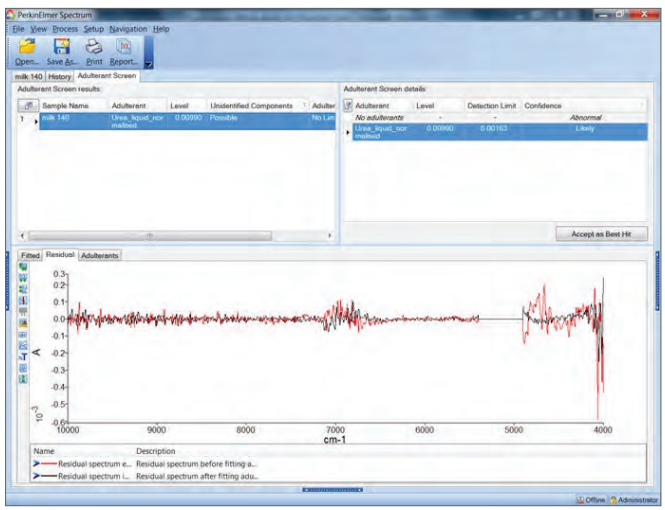

Figure 4. Adulterant Screen result for full-fat milk sample spiked with 1% urea.

The Adulterant Screen result that appears here shows the spectral residual when the unknown is tested against the model for the "good spectra" and the improvement achieved in reducing the residual when the spectrum of the adulterant (urea) is added. Adulterant Screen will also generate an estimated concentration of the adulterant and a detection limit. The estimated concentration of urea in this sample is 0.990%, very close to the known 1% concentration.

A different full-fat milk sample was spiked, but this time with sugar to give a 10% and 20% w/w of sugar. Adulterant screen was applied, and the results are shown in Table 1.

Since the cane sugar spectrum was measured in reflectance on the powder and the milk measurement is performed in transflectance on the liquid there are differences between the expected and observed levels of the adulterant. Therefore, the adulterant spectrum for sugar was normalized based on a known 10% sample. The limit of detection for cane sugar as estimated by the software is 3.5%.

Conclusion

Adulterant screen has been shown to be an effective method in detecting the adulteration of milk. Normalization of adulterant spectra may be required for some samples due to spectral changes that occur in solution. Nevertheless, NIR with Adulterant Screen is a fast and simple technique to use for the detection of adulterants. Additional adulterants can be readily added to the method by simply measuring the spectrum of the pure adulterant; thus providing a dynamic platform for adulterant screening.

Table 1. Adulterant screen results for milk spiked with sugar.

Sample Name	Adulterant	Level	Confidence	Material Fit
10% sugar	Cane sugar	0.10529	Likely	Abnormal
20% sugar	Cane sugar	0.21032	Likely	Abnormal

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