Journal of Chromatography A, 1220 (2012) 101–107

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

A liquid chromatography–tandem mass spectrometry method for the detection of economically motivated adulteration in protein-containing foods

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ARTICLE INFO

Article history:
Received 20 October 2011
Received in revised form
28 November 2011
Accepted 29 November 2011
Available online 8 December 2011

Keywords:
Melamine
Economic adulteration
Mass spectrometry
Protein

ABSTRACT

A new analytical method was developed to determine the presence of six (6) compounds with the potential to be used in economic adulteration to enhance the nitrogen content in milk products and bulk proteins. Residues were extracted from the matrix with 2% formic acid, after which acetonitrile (ACN) was added to induce precipitation of the proteins. Extracts were analyzed by liquid chromatography using a ZIC-HILIC column with tandem mass spectrometry (LC–MS/MS) using electrospray ionization (ESI). Single-laboratory method validation data was collected in six matrices fortified at concentrations down to 1.0 μg/g (ppm). Average recoveries and average relative standard deviations (RSD) using spiked matrix calibration standard curves were the following: cyromazine (CY) 95.9% (7.5% RSD), dicyandiamide (DC) 98.1% (5.6% RSD), urea 102.5% (8.6% RSD), biuret (BU) 97.2% (6.6% RSD), triuret (TU) 97.7% (5.7% RSD), and amidinourea (AU) 93.4% (7.4% RSD). This method provides a rapid and effective approach to proactively combat economically motivated adulteration in protein-containing products.

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1. Introduction

There is strong evidence that in 2004 and 2007, melamine and cyanuric acid caused the pet-food associated renal failure which sickened and killed large numbers of cats and dogs [1]. In 2008, Chinese authorities discovered adulteration of milk and infant formula with melamine by several Chinese producers [2,3]. There were hundreds of thousands of victims and six confirmed deaths in China, as well as mass product recalls in many countries. Melamine is used in the manufacturing of plastics and fertilizer. It was never intended for use as a direct food additive and is not regulated as such, and while it is relatively non-toxic on its own, it is able to form strong complexes with uric acid as well as with products of melamine hydrolysis like cyanuric acid, ammeline and ammelide. These complexes then form insoluble crystals in kidneys and can lead to renal failure [4].

Compounds like melamine that contain a high percentage of nitrogen can be used to make the protein content of food appear higher than the actual value. This is because the traditional standard technique for measuring protein content in food is the Kjeldahl method. The Kjeldahl method is a quantitative determination of nitrogen content and not a direct measurement of protein, thus it is possible to artificially enhance protein concentrations by adding nitrogen-containing chemicals. A compound such as melamine, which contains 66% nitrogen, can be added as a substitute for actual protein, which contains ~10–12% nitrogen. In response to the melamine contamination outbreak, a tremendous number of methods were published for the detection of melamine and its analogues [5–7], including several published by United States Food and Drug Administration (FDA) analysts that also targeted cyanuric acid [8–10].

As a result of several FDA import alerts on melamine in protein containing products from China [11–14], and numerous analyses performed in FDA laboratories, food contaminated with melamine has been prevented from entering the United States. However, the Kjeldahl method remains the most widespread methodology worldwide for determining protein content in foods and food ingredients. As long as protein composition in foods is determined not directly but by measuring nitrogen content, economic adulteration with compounds that contain a high percentage of nitrogen will continue to be a serious concern. It is likely that unscrupulous ingredient suppliers or food producers are already looking at using other poly-nitrogenous compounds to artificially enhance the concentrations of protein detected in their products.

Any compound which contains a high percentage of nitrogen, by weight, has the potential to be used in economically motivated adulteration of protein-containing food products, but that is certainly not the only property required of an adulterant. In addition, adulterants need to be relatively odorless, colorless and tasteless to avoid negative impact upon consumer acceptance of the

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0021-9673/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.chroma.2011.11.066

fraudulent product. A food producer would be unlikely to knowingly use an acutely toxic chemical as a protein adulterant given that the ultimate goal is economic fraud, not to injure consumers; although, as we have seen with melamine, even relatively non-toxic chemicals can cause unforeseen health effects. A protein substitute would also need to be readily available commercially in large quantities. It also must result in an adulterated food product that costs less than the authentic food product to provide the economic motivation for adulteration.

The Canadian Food Inspection Agency's Food Safety Division generated a list of potential adulterants which met all of these qualifications and distributed this list to other food safety agencies. Using this list combined with intelligence information, FDA determined that the following compounds were the most likely to be used in protein adulteration: dicyandiamide (DC), urea, biuret (BU), triuret (TU), cyromazine (CY), and amidinourea (AU) (see Fig. 1).

Dicyandiamide is used in the production of melamine, as well as in fertilizers and as a fire-proofing agent. Urea is found in fertilizers, as a non-protein nitrogen source in animal feeds, as well as in the production of numerous commercial products. Biuret and triuret are non-protein nitrogen sources that may be used in certain animal feeds. Cyromazine is an insect growth regulator known to produce melamine upon metabolism. Amidinourea is used in fertilizers.

These six potential adulterants are included in the LC–MS/MS method described herein. Method performance has been validated in skim milk, skim milk powder, soy protein, wheat flour, wheat gluten, and corn gluten meal matrices at concentrations as low as 1 ppm. The focus in method development was on bulk protein food matrices. The goal is to catch the economic adulteration at its source before waiting for incorporation and dilution of the fraudulent product into diverse finished food products.

2. Materials and methods

2.1. Reagents and materials

Urea, amidinourea, dicyandiamide, biuret, triuret, and cyromazine were purchased from Sigma–Aldrich (St. Louis, MO). Guanylurea sulfate (amidinourea sulfate (AU-sulfate)) was purchased from Acros (Geel, Belgium). Melamine was purchased from Alfa Aesar (Ward Hill, MA). Acetonitrile (ACN) (CAS 75-05-8) was liquid chromatographic grade purchased from Acros. Water (H_2O) (CAS 7732-18-5) was liquid chromatographic grade purchased from Sigma–Aldrich. Formic acid (CAS 64-18-6) and ammonium formate (CAS 540-69-2) were of HPLC grade purchased from Sigma–Aldrich. Conical 50 mL polypropylene centrifuge tubes with caps were purchased from Corning (Lowell, MA). Flip-top 1.5 mL microcentrifuge tubes were purchased from Fisher Scientific (Waltham, MA). Disposable 3 mL syringes were purchased from National Scientific (Rockwood, TN) and 0.20 μm PTFE 13 mm syringe filters were purchased from Millipore (Billerica, MA). Clear glass HPLC vials with PTFE septa were purchased from Agilent (Avondale, PA). Wheat gluten and corn gluten meal were purchased from Sigma–Aldrich. Skim milk powder was a standard reference material (SRM-1549) from the National Institute for Standards and Technology (NIST) (Gaithersburg, MD). Skim milk, soy protein and wheat flour were purchased from a local organic grocery store.

2.2. Sample preparation

The extraction technique was modified from the published validated technique developed by Turnipseed et al. to detect melamine and cyanuric acid [8]. The sample(s) and matrix control portions (2.0 ± 0.02 g) were weighed into separate 50 mL polypropylene tubes with (2.0 ± 0.02 g) of water used as a method blank. Powdered milk samples were diluted to standard edible milk strength (~1:10 with water) before weighing. A calibration curve of spikes was produced by adding the appropriate amount of spiking solution to each matrix control, using a blank matrix control as the 0 point. For validation results, calibration curves were prepared of non-milk matrix controls spiked at 0.5–6.0 ppm (urea 5–60 ppm). Calibration curves for milk matrices consisted of milk matrix controls spiked at 0.8–8.0 ppm (urea at 80–800 ppm). Eighteen mL of 2% formic acid in water was added to each tube and the tubes were vigorously shaken for 60 s, ensuring the dispersion of all solid material. Wheat gluten samples must be shaken shortly after the addition of the extraction solution to avoid formation of a thick gel layer which makes the samples very difficult to homogenize. The tubes were sonicated for 30 min in a Branson Sonicator 2510 (Branson, Danbury, CT). The tubes were again vigorously shaken for 60 s. The tubes were centrifuged at 4500 rpm (~1840 × *g*) for 20 min in a ThermoFisher CR4i centrifuge with a Jouan M4 rotor from Thermo Fisher Scientific. A 50 μL portion of the supernatant from each tube was placed in separate 1.5 mL centrifuge tubes and 950 μL of acetonitrile was added to each. The 1.5 mL tubes were centrifuged at 4500 rpm (~1840 × *g*) for 10 min. Avoiding the precipitate, the supernatant was loaded into a 3 mL syringe and filtered through a 0.20 μm PTFE syringe filter into a 2 mL autosampler vial. Wheat gluten, wheat flour, corn gluten meal and soy protein samples are injected as is at this point. A 100 μL portion of any filtered milk extracts (skim milk or skim milk powder) are diluted with 500 μL of 95:5 ACN:2% formic acid in water in a 2 mL autosampler vial for injection.

2.3. Instrumental analysis

A Shimadzu Prominence UFLC XR liquid chromatography system (Shimadzu, Columbia, MD) with a SeQuant ZIC-HILIC (150 mm × 2.1 mm, 5 μm) PEEK HPLC column (EMD Chemicals/Merck, Gibbstown, NJ) was used for the LC separation. An initial flow rate of 400 μL/min of 100% mobile phase A (95:5 ACN:0.1% formic acid/10 mM ammonium formate in H_2O) for the first 5 min followed by a linear ramp to 25% mobile phase A/75% mobile phase B (50:50 ACN:0.1% formic acid/10 mM ammonium formate in H_2O) at 12.8 min, holding at 25% mobile phase A until 15.8 min, and returning to 100% mobile phase A with an increased flow rate to 600 μL/min at 16 min, remaining at that composition and flow rate until returning to initial conditions of 100% mobile phase A and 400 μL/min 24.90 min, and remaining at initial conditions until 25 min. The injection volume was 20 μL. Fig. 2 depicts the unsmoothed quantitation ion chromatograms for a 1 ppm spike in wheat gluten.

An AB Sciex 4000 QTRAP with an ESI source in positive ion mode with Analyst 1.5 software was used to control the LC and the MS (AB SciEx, Foster City, CA). The protonated molecular ions $[M+H]^+$ at *m*/*z* 85.0 for DC, 61.0 for urea, 104.1 for BU, 147.1 for TU, 167.1 for CY, 127.1 for melamine (MEL) and 103.0 for AU were the precursor ions for MS/MS; see Table 1 for a summary of MRMs. Using a syringe pump for infusion, the LC–MS was tuned by flowing a 1 ppm mixed standard of the six high nitrogen analytes and melamine at a flow rate of 10 μL/min into a T fitting combining it with 400 μL/min of mobile phase A, except for AU and MEL which were tuned with 75% mobile phase B. Source parameters such as gas flows, ion spray voltage, and source temperature were optimized in this manner, as was collision energy (CE), declustering potential (DP), exit cell potential (CXP) and entrance potential (EP). Q1 and Q3 were set at unit resolution. The curtain gas was set at 20 arbitrary units (au), the CAD gas was set at Medium, the ion spray voltage was 5000 V, the source temperature was 550 °C, gas 1 pressure was set at 50 au, gas 2 was 60 au, and the entrance potential was 10 V. The declustering potentials, collision energies and exit cell potentials for the

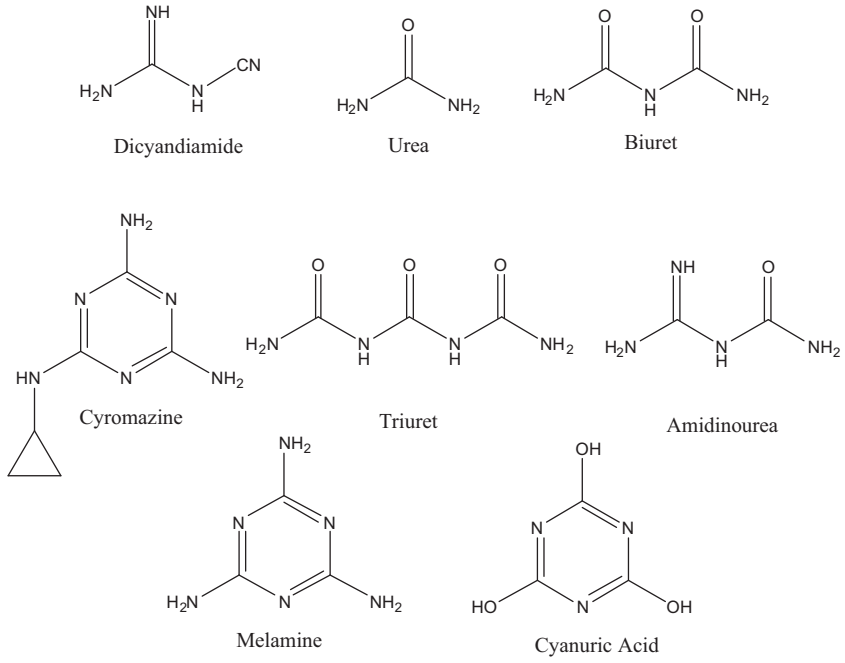

Fig. 1. Structures of high nitrogen compounds.

individual compound transitions as well as approximate analyte relative abundances and retention times (RT) are shown in Table 1. The retention times for the target compounds were determined by analyzing a mixed standard under the conditions described above using standard MRM mode (not scheduled MRM). The MS/MS data for all validation samples was collected in scheduled MRM mode with unit resolution in Q1 and Q3, a 3 ms pause between mass ranges, an MRM detection window of 60 s and a targeted scan time of 0.3 s.

2.4. Standard solutions

Individual stock solutions of approximately 1000 μg mL^{-1} of DC, BU, TU, CY, and AU and 10,000 μg mL^{-1} urea were prepared by weighing the appropriate amount of each reference standard (corrected for composition and purity) into separate 25 mL volumetric flasks and brought to volume with 0.1% formic acid in water. The milk and non-milk spiking solutions (containing CY, DC, BU, TU, and AU at 20 μg mL^{-1}) were prepared by pipetting the appropriate volume of each Individual Stock Solution into two separate 25 mL volumetric flasks. To one of the flasks an appropriate volume of Urea Stock Solution (~500 μL) was added to generate a 200 μg mL^{-1} solution of urea and the solution was brought to volume with 0.1% formic acid in water, generating the non-milk spiking solution used to spike soy protein, wheat flour, wheat gluten and corn gluten meal. To the other flask an appropriate volume of Urea Stock Solution (~5000 μL) was added to generate a 2000 μg mL^{-1} solution of urea and the solution was brought to volume with 0.1% formic acid in water, generating the milk spiking solution, used to spike skim milk and non-fat powdered milk. All standard solutions were

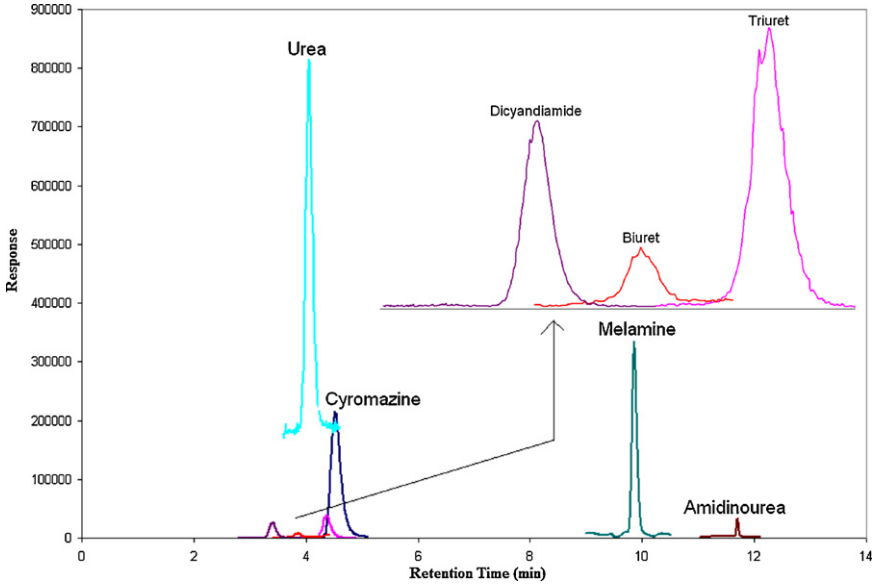

Fig. 2. Individual LC–MS/MS quantitation ion chromatograms for the potential protein adulterants in a spiked (1 ppm) wheat gluten sample.

Table 1
4000 QTRAP instrument conditions.

Analyte	Transition (m/z)	Declustering potential (V)	Collision energy (V)	Exit cell potential (V)	Approx. relative abundance (%)	Approx. RT (min)
DC	85.0 → 68.0	36	25	10	100	3.6
	85.0 → 43.1	36	25	6	25	
Urea	61.0 → 44.0	46	25	6	100	4.0
BU	104.1 → 61.0	40	16	10	100	3.9
	104.1 → 44.0	40	43	6	20	
TU	147.1 → 130.1	29	13	22	100	4.5
	147.1 → 104.1	29	13	18	60	
	147.1 → 61.1	29	23	14	20	
CY	167.1 → 85.1	66	27	14	100	4.7
	167.1 → 125	66	25	22	55	
	167.1 → 68.0	66	49	12	60	
AU	103.1 → 60.1	36	17	10	100	11.7
	103.1 → 43.1	36	37	6	25	
MEL	127.0 → 85.0	61	29	12	100	9.9
	127.0 → 68.0	61	43	10	30	

stored in a refrigerator at 4 °C. All individual stock solutions were stable for at least 10 months. All spiking and time of use standard solutions were stable for at least four months.

To generate validation data in non-milk matrices a calibration curve of spikes was made by adding the appropriate amount of non-milk spiking solution to each matrix control. Five (5) 2.0 ± 0.02 g portions of non-milk matrix controls were spiked from 0.5 μg/g (ppm) to 6.0 ppm CY, DC, BU, TU and AU (urea at 5–60 ppm). To calculate recoveries for validation, matrix controls were fortified pre-extraction by spiking with the appropriate amount of non-milk spiking solution to generate spikes at 1 ppm and 5 ppm for CY, DC, BU, TU and AU and 20 and 50 ppm for urea and quantitated using the matrix-matched calibration curve. To generate validation data in milk matrices for CY, DC, BU, TU, AU, and urea, a calibration curve of spikes was made by adding the appropriate amount of milk spiking solution to each matrix control. 2.0 ± 0.02 g of milk matrix controls (powdered milk weighed after reconstituting with water (1:10 dilution)) were spiked at 0.8–8.0 ppm (urea at 80–800 ppm). To calculate recoveries for validation, matrix controls were fortified pre-extraction by spiking with the appropriate amount of milk spiking solution to generate spikes at 1 ppm and 5 ppm for CY, DC, BU, TU, and AU and 200 and 500 ppm for urea and quantitated using the extracted matrix calibration standard curves.

3. Results and discussion

Samples in each matrix were extracted in triplicate on two separate days at two concentrations, a low and a high spike, leading to a total of six spiked replicates in each matrix at each concentration. The low spike was 1 ppm and the high spike was 5 ppm for CY, DC, BU, TU, and AU in all matrices. The low spike for urea was 20 ppm in wheat gluten, wheat flour, soy protein and corn gluten meal and the high spike was 50 ppm. For both skim and powdered milk, 200 ppm was the low spike and 500 ppm was the high spike for urea. The higher spike concentrations for urea are because urea occurs naturally in milk [15].

The HPLC system was equilibrated for 30 min under initial mobile phase conditions before the initial injection of the day. Based on a visual examination of the validation curves and analysis of the residuals it was determined that a linear regression gave the best fit for all analytes. The retention times of adulterants in all matrix control spikes and any samples were within ±5% of the average of the retention times of the calibration standards. MS/MS product ion ratios for compounds with three (3) monitored transitions (CY, TU) were required to be within ±20% of the average for the calibration standards, ion ratios for compounds with two (2) monitored transitions (DC, BU, AU) needed to be within ±10%. All quantitation ions had signal to noise ratios greater than 10:1, confirmatory ions greater than 3:1. The linear matrix calibration curves had an R^2 value of 0.99 or greater for all seven compounds in all matrices. The recoveries for the method validation ranged from 76.5 to 110.9% and the precision ranged from 1.7 to 15.1% RSD. The validation results are depicted in Table 2. Since the method utilizes a calibration curve made of pre-fortified matrix standards which are subsequently extracted, the reported recoveries are not equivalent to extraction efficiencies.

The method limits of detection (LOD) and limits of quantitation (LOQ) were determined by extracting blank skim milk and wheat flour samples, post-extraction fortifying the extracts with known standard concentrations, and determining the concentration where the signal to noise ratio of the quantitative MS/MS transition for each analyte was greater than 3:1 (LOD) and where the signal to noise ratio for the quantitative MS/MS transition for each analyte was greater than 10:1 (LOQ). Given the high naturally incurred concentrations of urea in all food matrices in the study, the LOD and LOQ for urea were determined using a blank matrix. The limits for the method are depicted in Table 3; they are higher for all analytes in skim milk due to the additional 1:6 dilution required in milk matrices to bring urea within the linear range of the instrument. While the matrices were not pre-fortified and extracted at these levels, the detectable limits are all orders of magnitude below what would be required to provide economic motivation for adulteration of bulk food products.

Matrix effect results were measured by creating a 5-point calibration curve in solvent and comparing the responses to a curve prepared by post-extraction fortifying matrix extracts. The response at each concentration in a matrix extract was divided by the response of the equivalent concentration in solvent and expressed as a percentage. The results for each point on the 5-point calibration curve of each matrix were averaged together and are summarized in Table 4. Matrix effects for urea were not measured, as it was impossible to find a matrix without naturally incurred urea. Matrix suppression is seen to varying degrees in all non-milk matrices; however matrix effects are minimal in milk matrices. Given that milk is a liquid matrix consisting mostly of water and that there is an additional dilution before analysis, this was not surprising. The use of matrix-matched calibration standards alleviates concerns of matrix suppression impacting the quality of the analytical results.

Table 2
Method performance as calculated by use of a five point extracted matrix calibration standard curve ($n = 6$ unless otherwise noted).

Matrix	Spike conc. (ppm)	Cyromazine	Triuret	Biuret	Dicyandiamide	Amidinourea	Urea spike conc. (ppm)	Urea
		% Recovery, % RSD	% Recovery, % RSD	% Recovery, % RSD	% Recovery, % RSD	% Recovery, % RSD		% Recovery, % RSD
Wheat gluten	1	95.2, 7.8	87.9, 7.8	96.7, 8.4	93.5, 7.1	97.7, 7.4	20	94.7, 15.1
	5	100.3, 6.0	101.7, 8.3	103.2, 8.8	107.0, 9.5	104.1, 10.5	50	103.7, 8.1
Soy protein	1	81.8, 5.6	91.5, 2.5	81.8, 2.5	81.8, 4.3	84.8, 5.1	20	102.9, 5.8
	5	100.1, 5.9	99.0, 3.1	99.1, 2.5	98.3, 4.8	102.0, 6.9	50	100.4, 3.4
Skim milk	1	79.5, 13.4	76.5, 6.7	82.5, 9.0	82.4, 6.9	79.6, 10.9	200	92.8, 12.0
	5	100.3, 5.8	103.4, 4.7	101.9, 5.4	100.6, 5.1	95.9, 2.4	500	103.6, 6.3
Skim milk powder SRM	1 ($n = 5$)	82.4, 13.5	102.1, 5.9	97.4, 12.4	93.5, 5.9	88.1, 7.2	200	110.9. 11.7
	5	89.3, 10.9	102.9, 9.2	106.2, 5.9	101.2, 5.3	96.6, 9.4	500	104.3, 6.8
Wheat flour	1	105.2, 7.5	100.9, 7.8	94.5, 9.8	109.6, 6.9	85.6, 9.6	20	100.9, 10.4
	5	109.7, 2.4	106.3, 4.0	102.8, 4.8	105.7, 1.7	102.2, 5.8	50	108.4, 6.4
Corn gluten meal	1	104.7, 5.9	96.8, 4.4	97.3, 4.7	101.2, 5.0	86.0, 7.1	20	106.9, 13.5
	5	102.4, 4.9	103.2, 4.4	102.2, 4.8	101.7, 4.7	98.1, 6.4	50	100.8, 3.6

Table 3
Method limits of detection (LOD) and limits of quantitation (LOQ), determined using wheat flour and skim milk extracts.

Analyte	Wheat flour		Skim milk	
	LOD (ppb)	LOQ (ppb)	LOD (ppb)	LOQ (ppb)
Cyromazine	5.4	18	60	180
Triuret	5.4	18	60	180
Biuret	18	54	80	240
Dicyandiamide	5.4	18	20	60
Melamine	54	162	160	480
Amidinourea	18	54	80	240
Urea	1.44 ppm	4.32 ppm	9.60 ppm	28.80 ppm

Initial focus was on the development of a method capable of detecting concentrations as low as 1 ppm for each adulterant. However, varying natural concentrations of urea necessitated the use of increased spiking concentrations to ensure accurate and reproducible recoveries at concentrations of significance for urea adulteration. This issue was only a factor with urea as none of the other target compounds were detected in any of the control samples. Skim and powdered milk samples, in particular, are known to have extremely high natural concentrations of urea ranging from 100 to 160 ppm [15] and required an additional dilution to avoid signal saturation. The literature indicates that 100 ppm of urea would be required to replace the nitrogen content of 1% of the protein in milk [16], so even at low levels of economically motivated adulteration, urea would still be detectable above the natural concentrations. Due to its low molecular weight, urea only forms 1 structurally significant ion by LC–MS/MS. This is not sufficient for confirmation of identity, particularly since urea's lone transition is the rather non-specific loss of ammonia ($-NH_3$). The identity of urea can be confirmed rather easily by GC–MS screening with TMS derivatization via LIB 4423 (see TMS-derivatized urea confirmatory mass spectrum in Fig. 3) [17]. This TMS derivatization approach, which was developed for melamine and cyanuric acid, was explored for all of the target compounds. However, only urea was detectable at the concentration of the low LC–MS/MS spikes. Both CY and MEL were detectable at 2.5 ppm. DC, BU, TU and AU were never detected by the TMS derivatization method despite attempts with spiking concentrations up to 10 ppm. It is possible that the analytes break down under the conditions required for this derivatization method and that an alternate approach for derivatization may be more broadly applicable to the group.

Early in the method development process, a gradual decrease in sensitivity was observed in the low mass ($m/z < 100$) region after 10 or more injections, particularly after the injection of milk extracts. It was observed that the sensitivity returned immediately to previous levels after the polarity of the system was switched to negative ion ESI mode. To prevent loss of system sensitivity, after approximately 5 matrix injections, a blank sample was run using an identical LC method with the mass spectrometer in negative ion ESI mode. This approach allowed for the analysis of several hundred samples with no decrease in sensitivity and without any venting and cleaning of the high vacuum MS system. It is possible that contaminants which do not form positive ions under the conditions of the method and are deposited on lenses are ionized when switching to negative ion mode, thereby allowing their passage and removal from the system. The source and curtain plate were thoroughly cleaned approximately once every two weeks.

The initial method validation runs for wheat flour, 1 ppm wheat gluten and one sample of the 1 ppm powdered milk spike samples on the Shimadzu UFLC were lost due to an instrument communication error with the mass spectrometer. The wheat flour and the 1 ppm wheat gluten spikes were re-extracted and the data was collected using an Agilent 1100 HPLC system and an identical AB Sciex 4000 QTrap. Aside from slightly higher carryover on the Agilent system for AU, the performance was similar to the Shimadzu UFLC which was used for all other validation runs.

At one point during the method development process, the peaks for all target compounds became extremely broad with no accompanying increase in back pressure. It appeared the column needed to be replaced. However, after running the manufacturer recommended clean-up of 30 column volumes of water, 30 column volumes of 0.5 M NaCl in water, 30 column volumes of water, and then allowing the column to equilibrate with the initial LC mobile phase for the method, the analyte peak shapes were once again acceptable. Band broadening was also seen during initial attempts to dilute milk samples with 95:5 ACN:H_2O as opposed to 95:5 ACN:2% formic acid in H_2O; this effect was most pronounced for CY, DC, BU, and TU.

Table 4
Matrix effects measurements for the matrices validated in the study.

Matrix	CY	TU	BU	DC	MEL	AU
Wheat gluten	93%	86%	58%	40%	67%	65%
Soy protein	86%	82%	86%	92%	80%	45%
Skim milk	101%	102%	102%	104%	102%	105%
Skim milk powder	102%	106%	104%	107%	106%	108%
Wheat flour	102%	98%	96%	95%	92%	69%
Corn gluten meal	92%	92%	78%	86%	58%	78%

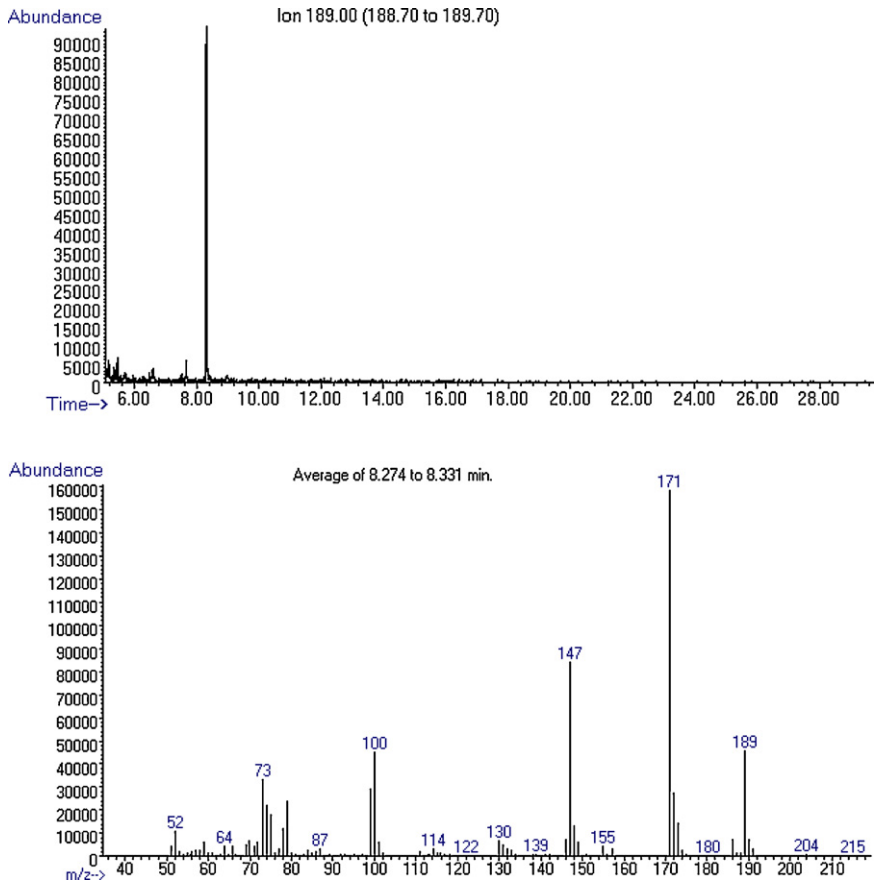

Fig. 3. Extracted ion chromatogram (m/z 189) and full scan GC–MS spectra for TMS-derivatized urea solution at 1 ppm (10 ppm spike equivalent) using method published by Litzau and coworkers [17].

The ZIC-HILIC column operates at lower pressures than a conventional C_{18} HPLC column under the method conditions specified. However, the ZIC-HILIC column requires more time to equilibrate than a conventional HPLC column. The mobile phase ramp begins at 5% aqueous and ramps to 38.75% aqueous to elute AU and for the column wash. Despite this relatively small increase in aqueous content it requires nearly 9 min with a 50% increase in flow rate to ensure consistent chromatographic performance for the entire batch, a significantly longer time than would be required to re-equilibrate a conventional reverse phase HPLC column.

The extraction technique was modified from the published validated technique developed by Turnipseed et al. to detect melamine and cyanuric acid [8]. Given that the extraction is a rapid and simple protein precipitation, it allows for the future expansion to include other small molecule protein adulterants without significant modification of the method.

4. Conclusion

The method described herein allows for the rapid extraction and detection of six (6) compounds with the potential to be used to artificially enhance the nitrogen content of food products. Concentrations were determined using calibration curves consisting of spiked matrix-matched control samples. Single-lab method validation has been completed for six matrices. This method provides a rapid and effective approach to proactively combat economically motivated adulteration in protein-containing food products.

Acknowledgments

We would like to thank Dr. Jian Wang from the Canadian Food Inspection Agency for helpful discussions, as well as providing the Triuret standard. We would also like to thank Alex Krynitsky from CFSAN, Michael Smoker from the Office of Regulatory Affairs KAN-DO and Jack Lohne from the Animal Drugs Research Center in Denver for technical advice.

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