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Development of a Liquid Chromatography−Tandem Mass Spectrometry Method Using Capillary Liquid Chromatography and Nanoelectrospray Ionization−Quadrupole Time-of-Flight Hybrid Mass Spectrometer for the Detection of Milk Allergens

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Liquid chromatography−tandem mass spectrometry (LC-MS/MS) analysis of the tryptic digest of a cleaned-up food matrix extract was used for the detection of milk allergens. The emphasis of this study was on casein, which is the most abundant milk protein and is also considered the most allergenic. A sample cleanup method was developed using an ion exchange column and centriprep device. Cookies spiked with milk powder from 0 to 1250 ppm were extracted, cleaned up, and either digested directly by trypsin or further cleaned up by gel electrophoresis before digestion. The peptide mixture was analyzed on a capillary LC−quadrupole time-of-flight system. Two marker peptides from αS1-casein were identified and used for prescreening. The MS/MS data from the mass spectrometry system were processed with Masslynx v4.0 and submitted for database search using either ProteinLynx Global Server or Mascot for protein identification. The LC-MS/MS method, using casein enzyme-linked immunosorbent assay as a reference, was tested on the cookie matrix and was extended to other sample matrices. There were good agreements between the two. This LC-MS/MS method provides a valuable confirmatory method for the presence of casein. It also allows the simultaneous detection of other milk allergens.

KEYWORDS: Milk allergen; analysis; casein; confirmation; LC-MS/MS; proteins

INTRODUCTION

Milk allergy is one of the most common food allergies in young children from birth to 1 year old. Fortunately, 85% of these children will outgrow their allergy by the age of 3. There is no treatment for the allergy, and the only way to control the symptoms is avoidance. About 3% of milk is protein, which is subdivided into two fractions—casein and whey. The caseins are found in the micelles, giving milk its cloudy appearance, and account for 80% of the milk protein. The whey fraction accounts for the other 20% and contains the two major components β-lactoglobulin (8%) and α-lactalbumin (4%). Studies (*1*) using sera from children with milk hypersensitivity have shown that caseins are the most allergenic proteins. However, in some cases, these children also show hypersensitivity toward β-lactoglobulin and α-lactalbumin. More recent studies (*2*) have shown that most milk proteins, even proteins present at low concentrations, are potential allergens.

Currently, milk allergen analyses are generally based on immunoassay methods such as enzyme-linked immunosorbent assay (ELISA) (*3*). There are commercial test kits available for casein, β-lactoglobulin, or total milk protein (casein and β-lactoglobulin) determination (*4*). Interlaboratory studies were performed to evaluate the reliability and reproducibility of these kits (*5*). Antibody cross-reactivities have been reported for some milk proteins (6). Confirmatory tests are required to corroborate ELISA detection results and improve the detection specificity of undeclared milk allergens.

For the last several decades, mass spectrometry has become the dominant technology for the identification of peptides and proteins. The primary current approaches used for protein identification are the top-down (*7*−*10*) and bottom-up (*11*) sequencing. The top-down sequencing involves introducing the intact protein into the gas phase. The protein is identified by measuring either the protein molecular weight or the fragmentation of the protein using various techniques (*12*). The bottom-up approach is more popular. The sample is usually digested with an enzyme such as trypsin followed by accurate sequence analysis of the tandem mass spectrometry (MS/MS) spectra of the proteolytic fragments for protein identification using an algorithm for database searching based on amino acid sequence assignments. Application of this approach to the detection of allergen proteins has been reported (*13*, *14*). This paper shows

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the application of using capillary high-performance liquid chromatography interfaced to a hybrid quadrupole time-of-flight (QTOF) mass spectrometry to analyze a tryptic digest from a cleaned-up food matrix for allergen proteins in milk.

MATERIALS AND METHODS

Food Matrices. Commercial food matrices such as chocolates, cookies, baby foods, frozen desserts, sausages, and ground meats were selected from the local retail market. Food matrices were tested by ELISA in duplicate. Samples below 2.5 ppm were considered negatives. Negative cookie matrices were spiked in duplicate at 0, 1.25, 12.5, 125, or 1250 ppm levels with powdered skim milk (μg of powdered milk per g of commodity). Negative chicken hot dog samples were spiked at various concentrations (0, 1, 3, 5, 10, 30, or 50 ppm) to determine the limit of detection (LOD_{50}) ($n = 4-6$). These food matrices were ground using a mortar and pestle.

Extraction and Cleanup. The extraction conditions were optimized to allow a rapid semipurification of α-casein. Briefly, 5 g of sample was extracted using 125 mL of extraction buffer (20 mM Tris and 0.1% Tween). The mixture was agitated at room temperature for 1 h and centrifuged for 30 min at 4800*g* (6800 rpm) at 4 °C using a Sorvall GSA rotor and RC5C centrifuge (DuPont Co., Wilmington, DE). The supernatant was filtered through both no. 2 Whatman paper (Whatman International Ltd., Maidstone, England) and an Acrodisc 25 mm syringe filter with a 1.2 μM Versapor membrane (Pall Corporation, Ann Arbor, MI). In a disposable Econo-Pac column (Bio-Rad Laboratories, Mississauga, Canada), 45 mL of the filtrate was loaded on 2 mL of DEAE Sepharose Fast Flow resin (Amersham Bioscience, Uppsala, Sweden) equilibrated with 10 mL of extraction buffer. The anion exchange column was washed twice with 20 mL of 20 mM Tris, pH 7.2, followed by 20 mL of 20 mM Tris, pH 7.2, containing 150 mM NaCl. The sample was eluted using 12 mL of 20 mM Tris, pH 7.2, containing 400 mM NaCl and stored at 4 °C. The next day, the eluate was desalted three times in a Centriprep YM-10 device (Millipore, Nepean, Canada) at 3000*g* at 4 °C for 60 min using 10 mL of 20 mM Tris, pH 7.2, and concentrated to a final volume of 2 mL. The cleaned-up extract was stored at −20 °C.

ELISA Methodology. ELISA was performed according to the manufacturer's recommendations using the VERATOX kit for milk allergen (Neogen, Lansing, MI). Overange samples were diluted after extraction in the provided buffer to allow appropriate quantification.

Gel Electrophoresis of Cookie Extract. Aliquots from the cleaned-up extract were mixed with equal volumes of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) containing freshly added β-mercaptoethanol (5% v/v, Sigma Aldrich), and 20 μL was loaded on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gels. A 12% polyacrylamide (acrylamide:bisacrylamide = 37:1) slab separating gel with 5% polyacrylamide (acrylamide:bisacrylamide = 100:1) stacking gel was used. The samples were electrophoresed through the gel at 125 V for 1 h. Gels bands were stained with GelCode Blue Coomassie G-250 reagent (Pierce, Rockford, IL) and destained until the background was clear.

Digestion with Trypsin. Proteolytic digestions were carried out using a multiprobe robotic liquid delivery system−The MassPREP STATION (Waters, Milford, MA)−for the fully automated destaining, reduction, alkylation, and in gel digestion of proteins. All reagents were freshly prepared prior to digestion. Two digestion protocols, one for in gel digestion and another for in solution digestion, were explored in this work.

In Gel Digestion Protocol. The excised gel bands were cut into 1 mm × 1 mm × 1 mm pieces and placed in individual wells of a 96 well v-bottomed microtiter plate and covered with 40 μL of water. The gel pieces were destained two times with 50 mM ammonium bicarbonate in 50% acetonitrile (ACN) followed by reduction with 10 mM dithiothreitol (DTT) (Sigma Chemical Co., St. Louis, MO) in 100 mM ammonium bicarbonate at 37 °C for 30 min. The plate was allowed to cool to room temperature, and an aliquot of 55 mM iodoacetamide (IA) (Sigma Chemical Co.) in 100 mM ammonium bicarbonate was added for alkylation for 20 min. The gel pieces were washed with 100 mM ammonium bicarbonate and dehydrated with ACN. Trypsin (Sequencing Grade from Promega, Madison, WI) was added for digestion at 37 °C for 5 h. The peptides were extracted with a solution of 1% formic acid (FA)/2% ACN and either transferred to another 96 well polymerase chain reaction plate for liquid chromatography (LC)-MS/MS analysis directly or transferred to 250 μL vials for storage.

In Solution Digestion Protocol. A 10−30 μL sample of the final cleaned-up extract (the protein should be in aqueous solution with a concentration within the range of 200 fmol/μL to 5 pmol/μL) was added to the 96 well v-bottomed microtiter plate. DTT (10 mM in 50 mM ammonium bicarbonate) was added to the samples, and the plate was incubated at 37 °C for 30 min. The mixture was allowed to cool to room temperature (10 min), and 55 mM IA in 50 mM ammonium bicarbonate was added and left at room temperature for 20 min. Trypsin in 50 mM ammonium bicarbonate was added and incubated at 37 °C for 3 h. Finally, 1% FA was added to the digested mixture before analysis using LC-MS/MS.

LC. The LC system used was a capLC system (Waters) comprised of three pumps, two of which provided binary gradient operation and one configured as an auxiliary pump for sample focusing and precolumn desalting. The temperature of the sample plate on the thermostated plate holder was kept at 20 °C. A 10-port stream select module connected the capLC system to the nanospray interface of the mass spectrometer. The system was set up with a trap column OPTI-PAK 0.35 mm × 5 mm packed with Waters Symmetry300 C_{18} 5 μm (Waters) for precolumn sample cleanup and an analytical nanocolumn (75 μm i.d.) or a capillary column (0.3 mm i.d.) for peptides separation. The analytical columns used were as follows: a 100 mm × 75 μm (LC Packing, Amsterdam, NL) packed with PepMap C_{18} 100 Å, 5 μm particle size, and a 50 mm × 300 μm Jupiter (Phenomenex, Torrance, CA) Proteo C_{12} 90 Å, 4 μm particle size. A binary solvent gradient was used for the analysis of the tryptic digests. Solvent A contained 3% ACN and 0.2% FA in water. Solvent B contained 0.2% FA in ACN. Solvent C contained 1% ACN and 0.2% FA in water. The sample was injected with the 10 port stream select module at the sample load position, and solvent C was run for 3 min at a flow of 20 μL/min for sample concentration and desalting. The 10 port stream select module was then switched to the run position, and the LC conditions started with 5% B followed by a 0.8%/min gradient for 40 min. For the Jupiter column, the column was allowed to equilibrate at 35% B for 5 min and then back to 5% B for 5 min. For the PepMap column, the column stayed at 35% for 20 min and then back to 5% B for 5 min. A splitter installed upstream of the gradient flow gave a resultant flow through the analytical column of 250 nL/min with the pump programmed to deliver a flow of 5 μL/min.

Mass Spectrometry. All data were acquired using a Q-Tof 2 (Waters), hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer with the nanoflow interface option. The mass spectrometer was operated in nanoelectrospray positive ion detection mode with a spray voltage of 3.5 kV at a resolution of 10000 (FWHH). The instrument was also equipped with a nanolockspray interface, which provided the capacity to introduce a reference compound flowing through a second nanospray probe. An oscillating baffle allowed analysis of each spray independently. Data from the reference spray were used to calculate a correction factor for the mass scale calibration postrun and were applied to the analyte data to provide exact mass information. The mass spectrometer was operated using data-directed analysis (DDA) software acquiring MS survey data from *m*/*z* 375 to 1300 with the switching criteria for MS to MS/MS including ion intensity and charge state. The instrument was programmed to ignore any singly charged species and performed MS/MS on three of the most intense multiple-charged eluting species from the preceding MS scan, with 1 s integration times. Switch back to the MS mode was triggered when the base peak ion current fell below a threshold of 3 counts/s. The collision energy used to perform MS/MS was varied according to the mass and charge state of the eluting peptide.

Identification of Proteins Using Database Search. Confirmation of the protein was achieved using the survey/MS/MS data. Two software packages were used. The Masslynx-Proteinlynx Global Server v2.1 (Waters) is an integrated software package used for protein identification, which includes a database search engine. The DDA survey data acquired were processed by Masslynx to create a peak list, which

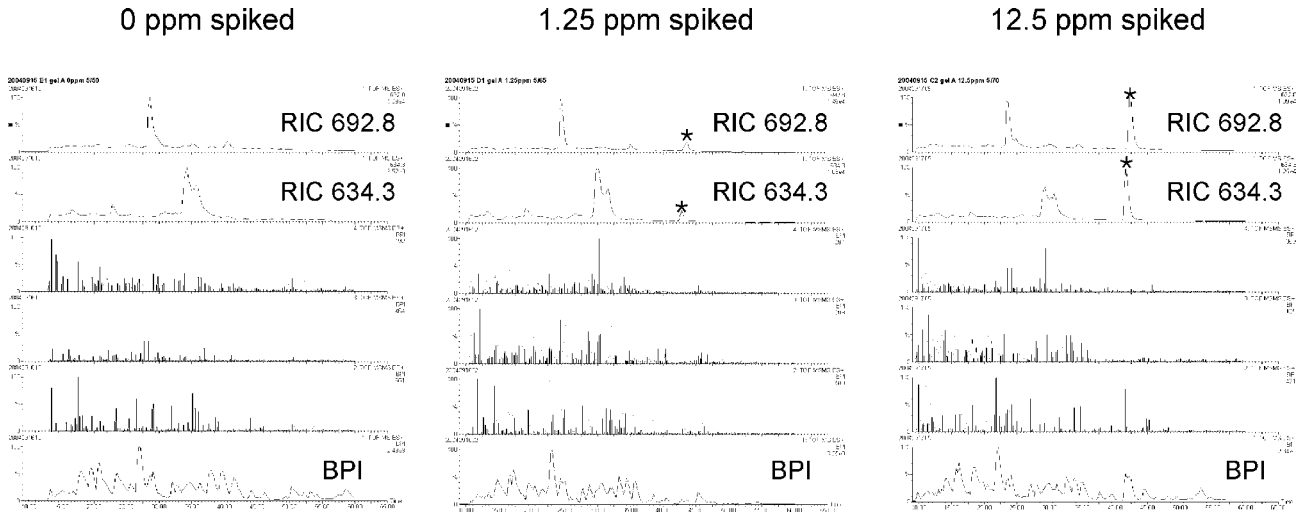

Figure 1. Typical raw data files of a cookie extract spiked with 0, 1.25, and 12.5 ppm of milk showing the BPI (bottom trace) from the TOFMS scan, the RICs of *m*/*z* 634.3 and *m*/*z* 692.9 (top traces), and three channels of MS/MS data (three middle traces) from a survey DDA acquisition. One bar in the MS/MS data scan represents one scan of a precursor ion. The peaks marked with "*" are the identified peptides from αS1-casein.

contained the MS/MS data to be searched against FASTA-formatted protein databanks such as SwissProt or NCBI nonredundant protein database. The factors contributing to the database search scores in Proteinlynx Global Server v2.1 (PLGS2) were the number of entries in the database, the mass accuracy, and the peak intensity. For the MS/MS scoring, every precursor mass in the query was compared to the mass of each theoretically determined peptide in the database, taking into account the user-defined tolerance of the precursor mass and the fragmentation characteristics. The probability for each identified protein was then calculated and given a score. The scores are reported as natural logs. So, for example, in a database with 100000 entries, the maximum score would be 11.5 and the 95% significance threshold would be 11.4. The created peak list from MassLynx was also searched against the databases using the software search algorithms MASCOT (Matrix Science, London, United Kingdom). The scoring of MASCOT incorporated a probability-based implementation of the Mowse algorithm with the formula: Mowse score $= -10 \times \log(p)$.This score was based on the probability (p) that a peptide identified from the experimental fragment matched a peptide in a protein database. A random match will have a high probability (high p), therefore, a low Mowse score whereas a valid match will have a low probability and hence a high Mowse score. MASCOT ranks the quality of the peptide matches and sums the scores of detected peptides to calculate a total protein score.

RESULTS AND DISCUSSION

In Solution Digestion. Cookie samples are spiked with powdered milk and cleaned up as described in the Materials and Methods. The concentrated and desalted extracts are digested with trypsin. The digested protein mixtures are then analyzed using LC-MS/MS. A typical representation of the LC-MS/MS raw data from the survey data for the 0, 1.25, and 12.50 ppm milk-spiked cookie extract is shown in **Figure 1**. It is possible to detect the presence of milk in a sample using the reconstructed ion chromatograms (RIC) of *m*/*z* 634.2 and *m*/*z* 692.8 as shown in the top two boxes of **Figure 1** where milk protein as low as 1.25 ppm is detected in the spiked cookie extract. Further concentration (10×) of the cleaned-up sample was required to detect the 1.25 ppm samples. The three boxes below show the three channels of MS/MS data from the run. These are base peak intensity chromatograms (BPI) of product ions from different precursor ions. The bottom boxes show the BPI from the survey MS scans. These peptides (with parent ions *m*/*z* 634.2 and *m*/*z* 692.8) elute later in the gradient run, away from most of the peptides in the sample matrix. These

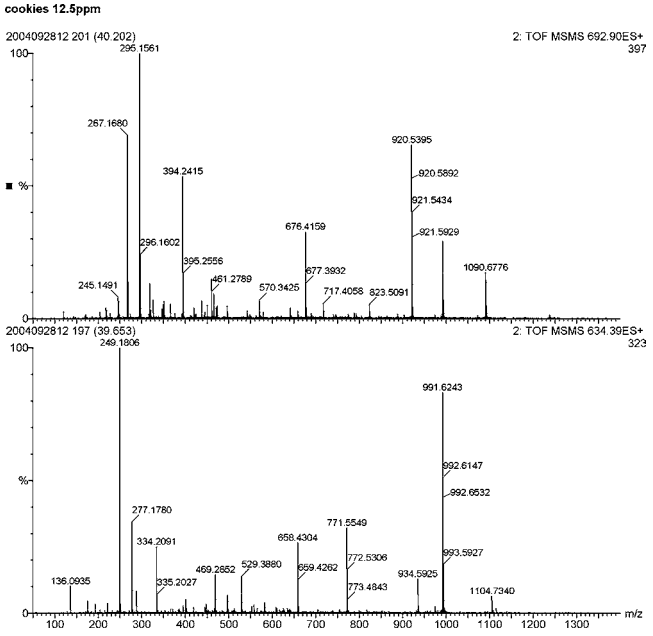

Figure 2. MS/MS spectra of the doubly charged ions *m*/*z* 634.3 and *m*/*z* 692.8 observed in a 12.5 ppm milk-spiked cookie extract.

are doubly charged ions, and their corresponding MS/MS spectra are shown in **Figure 2**.

When these data are submitted to a database search, using either Masslynx-Proteinlynx Global Server 2.1 or MASCOT 2.0 (shown in **Figure 3**), the milk protein αS1-casein (Bos taurus) is identified with two of the peptide matches being YLGYLEQLLR (*m*/*z* 634.2, charge +2) and FFVAPFPEVFGK (*m*/*z* 692.8, charge +2). **Figure 4** shows the sequence of the αS1-casein with the two marker peptides highlighted in bold. The digestion of αS1-casein using trypsin theoretically produces about 15 peptides with masses above 500 Da using the PeptideMass Peptide Characterization Software (www.expasy-.org). We noticed that these two peptides show up consistently in samples containing traces of milk. Their signals vary in intensity according to the sample concentration. The presence of αS1-casein is also confirmed by database search results in **Table 1**. With mass correction using the nanolockspray option [using the doubly charged ion *m*/*z* 785.8428 from Glu[1]-

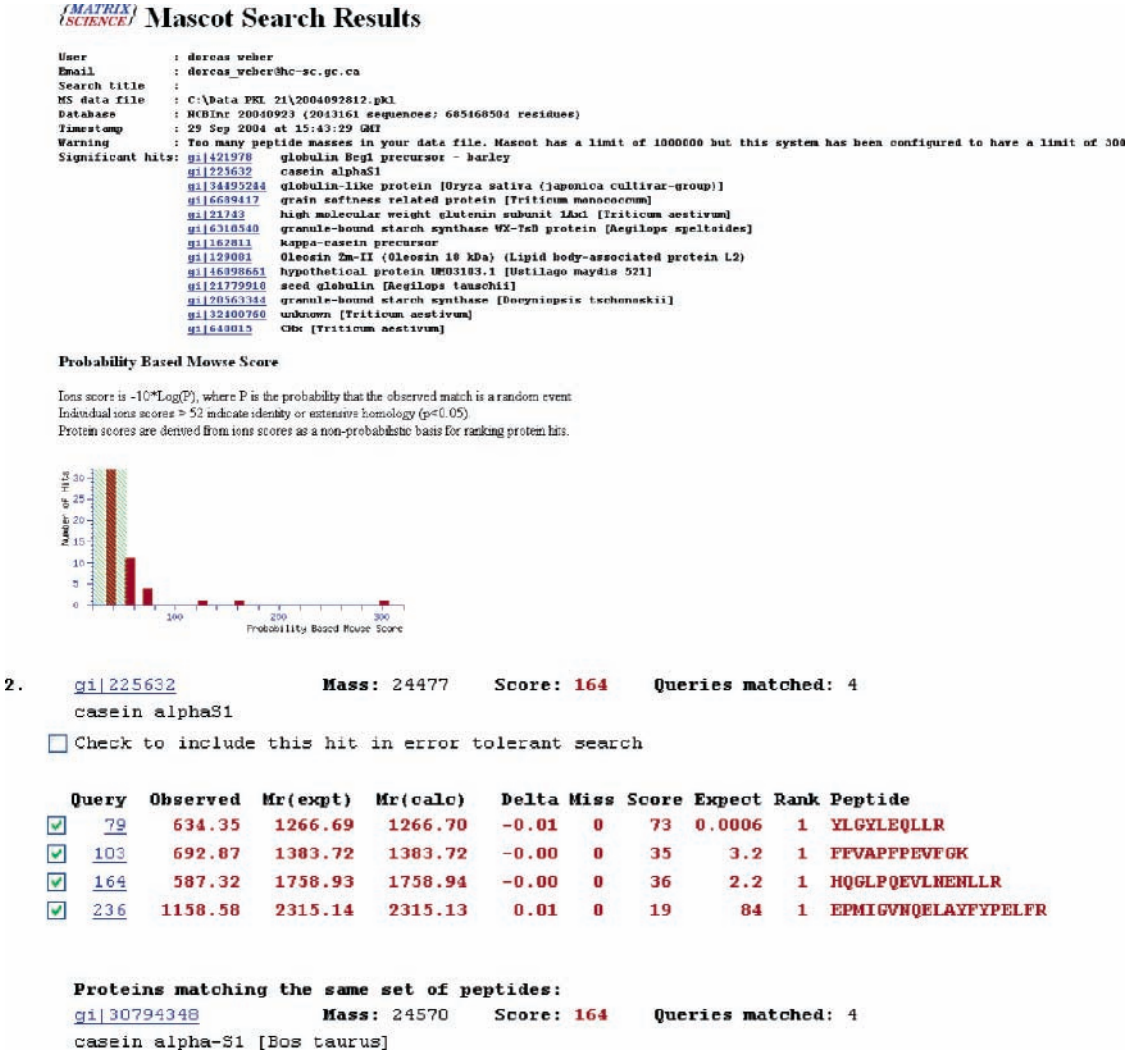

Figure 3. Screen capture of portions of a typical LC-MS/MS data search report of the 12.5 ppm milk-spiked cookie extract using Mascot (Matrix Science). The top shows a list of significant hits. The probability-based Mowse Score is illustrated by the bar chart. Below that is information on individually identified proteins including the mass, the error, and the sequence of the matched peptide.

```
1   MKLLILTCLV AVALARPKHP IKHQGLPQEV
31  LNENLLRFFV APFPEVFGKE KVNELSKDIG
61  SESTEDQAME DIKQMEAESI SSSEEIVPNS
91  VEQKHIQKED VPSERYLGYL EQLLRLKKYK
121 VPQLEIVPNS AEERLHSMKE GIHAQQKEPM
161 IGVNQELAYF YPELFRQFYQ LDAYPSGAWY
181 YVPLGTQYTD APSFSDIPNP IGSENSEKTT
191 MPLW
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Figure 4. Sequence of αS1-casein (Bos taurus) showing the two marker peptides highlighted in bold.

Fibrinopeptide B (Sigma) infused at 25−50 fmol/min], the mass accuracy was 5−10 ppm for the database-matched peptides. Although when the laboratory temperature fluctuations were held within 0.5 °C, this mass accuracy was also achieved without the use of the lockspray.

In Gel Digestion. The cleaned-up extracts from spiked cookie samples were further processed by one-dimensional (1D) SDS−PAGE gel electrophoresis to isolate the casein band from other coextracted proteins from the matrix. A representative gel is shown in **Figure 5**, which included a lane corresponding to 5 μg of milk powder and another one with 1 μg of α-casein, which served as a reference for excising the bands that contain the sought for protein. The effect of the gel cleanup on the BPI chromatogram and RIC is shown in **Figure 6b**. The in gel-digested BPI chromatogram and RICs are considerably cleaner than the cookie extracts digested directly, particularly in the RICs. This is not surprising since SDS electrophoresis gel separates the coextracted proteins in the matrix by their molecular weight and only those in the molecular weight range of casein are digested and analyzed. The drawbacks of this extra step are the longer time required for in gel digestion (5 vs 3 h or less for in solution digestion) as well as the extra time required to run the gel, stain, identify the bands, and excise the appropriate bands for in gel digestion. With these added steps, the sample process time can easily be doubled. When we compare the Mascot database result from both sample workups (**Table 1**), we find that similar levels of detection are achieved by both methods. The score is slightly lower for the in gel protocol because the extra cleanup step in the gel results in analyte loss. However, the gel method is sensitive enough for regular food surveillance for undeclared milk allergen in food at the current level of concern of about 30 ppm (*15*).

Identification of Milk Protein. Using the methods developed in this work, three stages are used to confirm the presence of milk allergens using mass spectrometry. Once validated, they could be used to set a detection threshold. First, RIC traces give a quick prescreening for the presence of casein in the sample. The result is similar to that of high-resolution LC-MS analysis where the retention of the peak at the specific mass indicates

Table 1. Comparison of Database Search Results from Cookie Extracts ($n = 2$) Spiked with Milk with and without Gel Electrophoresis Cleanup

		solution digest			gel digest		
milk spiked (ppm)	αS1-casein	Mascot score	no. of peptides matched	% protein coverage	Mascot score	no. of peptides matched	% protein coverage
1250	detected	721	10	43	263	6	35
125	detected	391	7	38	133	4	17
12.5	detected	185	4	17	164	6	29
1.25	detected	100	2	10	43	2	10
0	not detected						

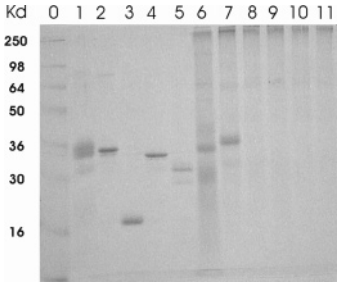

Figure 5. SDS–PAGE separation of casein standard and milk-spiked cookies: 0, MW Seeblue molecular weight marker; 1, 5 μg of milk powder; 2, 1 μg of α-casein; 3, 1 μg of β-lactoglobulin; 4, 1 μg of β-casein; 5, 1 μg of k-casein; 6, 1250 ppm milk*; 7, 1250 ppm milk**; 8, 125 ppm milk**; 9, 12.5 ppm milk**; 10, 1.25 ppm milk**; and 11, 0 ppm milk** (*, 150 mM NaCl fraction; **, 400 mM NaCl fraction).

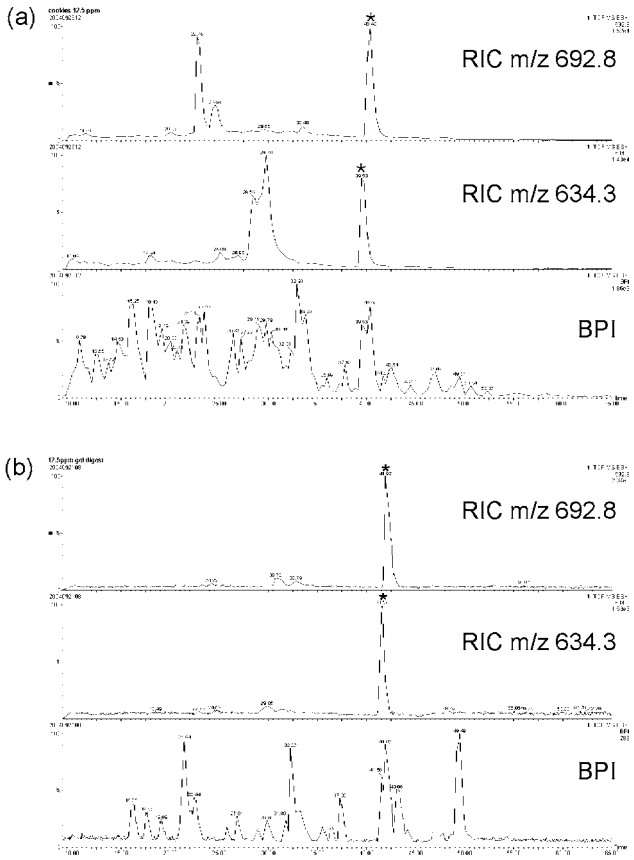

Figure 6. Comparison of the BPI chromatogram and RIC traces for cookie extract from in solution digestion (**a**) and in gel digestion (**b**) of the casein band after the extract is cleaned up further using 1D SDS gel electrophoresis.

the presence of the compound. RICs of *m*/*z* 634.3 and *m*/*z* 692.8 from the BPI chromatogram of the survey data could be used as markers to indicate the presence of milk protein in the sample.

Table 2. Mascot Database Search Results for a 1250 ppm Milk-Spiked Cookie Extract from in Solution Digestion[a]

protein no.	protein name	MW (amu)	score	peptides matched
*gi|30794348	casein α-S1 (Bos taurus)	24528	721	10
gi|27806963	casein α-S2 (Bos taurus)	26018	412	9
gi|3776017	α-S1-casein (Bubalus bubalis)	24326	410	8
gi|229460	lactoglobulin β	18367	394	6
gi|72079	β-lactoglobulin—water buffalo	18267	392	6
gi|162811	κ-casein precursor	21225	259	5
gi|223780	lactoglobulin β	18177	167	5
*gi|115660	β-casein precursor	25107	152	4
gi|421978	globulin Beg1 precursor—barley	72252	137	4
gi|248147	β-casein A2 variant	5115	129	4
gi|3703061	β-lactoglobulin variant D	5188	94	2
gi|162797	β-casein precursor	25088	77	4
gi|13661026	β-casein B (Bos taurus)	16453	64	3

[a] The proteins marked with "*" are the only ones identified from a gel digest from the same spiking experiment.

The presence of both markers in a food commodity would correlate only with the presence of milk allergens. Second, it is possible to use the characteristic MS/MS spectra of these two casein peptides with parent ions of *m*/*z* 634.2 and *m*/*z* 692.8 as a "fingerprint" to increase the confidence in the identity of the detected markers. This is particularly useful with spectra from samples of high protein contents where the markers' signal might be difficult to discriminate from the matrix background. Finally, the MS/MS spectra also provide the basis for the probabilistic calculation used by Mascot and other MS interpretation programs. The Mascot protein score is derived from the sum of ion score in a MS/MS search increasing the confidence level on the identity of the protein detected in a sample. As illustrated in **Figure 3**, using a preselected significance threshold of $p < 0.05$, the master result page for the peptide mass fingerprint search reports that scores greater than 52 are significant. This approach allows the detection of samples at 12.5 ppm. Again, the intensity and hence the quality of the spectra directly affect the ion score. Furthermore, the MS/MS database search provides other data useful in sample analysis. These include hits for other αS-casein peptides (total 10 peptides found), other milk allergens such as whey proteins, and the presence of other allergens (e.g., glutenin). An interesting advantage of in solution digestion is that more coextracted proteins are analyzed through MS/MS, which allow other milk allergens to be detected. This is illustrated in **Table 2** with the Mascot database search result from a 1250 ppm milk-spiked cookie extract using in solution digestion. Similar samples analyzed from the in gel digestion only show casein αS1 and the β-casein precursor. The pros and cons of using in solution digestion vs in gel digestion are summarized in **Table 3**.

Application of the Method to Other Food Matrices. Food samples tested positive ($n = 17$) and negative ($n = 10$) with casein ELISA were subjected to the cleanup and digestion of

Table 3. Pros and Cons of in Solution Digestion and In Gel Digestion

	in solution digestion		in gel digestion	
	pros	cons	pros	cons
sample process time	faster (5 h)			longer (>10 h)
sensitivity	better, less sample loss			lower, more steps lead to more sample loss
selectivity	capable of identifying multiple proteins simultaneously	too many interfering protein from matrix	selective for sought for protein	may miss other interesting protein in the sample

Table 4. Comparison of Results from Casein ELISA and LC-MS/MS for Different Food Matrices

		LC-MS/MS[b]				
	ELISA[a]	RIC	Mascot		PLGS2	
food matrices	μg/mL	*m*/*z* 634.3 and 692.8	score for casein	no. of peptides matched	score for casein	no. of peptides matched
orange sherbet	14.41	detected	373	11	14.7	6
lacto-free ice cream	442.37	detected	425	9	14.7	6
Enfalac regular	1374.93	detected	512	8	14.7	7
oatmeal cereal	879	detected	629	14	14.7	8
cookies arrowroot	0.93	detected	101	3	13.7	2

[a] Samples were tested in duplicate by ELISA. [b] Representative LC-MS/MS values. Samples were analyzed in triplicate.

the extract using the in solution digestion protocol, followed by LC-MS/MS analysis developed in this paper. The relative specificity was 100% using either the presence of casein peptide markers (*m*/*z* 634.3 or *m*/*z* 692.8) or a significant mascot score ($p < 0.05$). The sensitivities of ELISA and LC-MS/MS were comparable. Representative results are shown in **Table 4**. The LOD_{50} was determined to be 5 ppm using spiked hot dog sausage (data not shown). While both casein peptide markers were present in all spiked samples, the *m*/*z* 692.8 was absent in 33% of the positives food matrices tested while the peptide marker *m*/*z* 634.3 was always present (data not shown). For the 5 ppm spiked samples, an average Mascot score of 67 $\pm$ 23 ($n = 4$) was obtained. The Mascot score reflects the relative quantity of the protein present (digestion of more protein produce more peptides, which in turn produces better quality spectra enabling a better match or a higher score) but cannot be used as a quantitative measure. The amplitude of the RIC is also not used for quantification since the MS acquisition mode used was DDA. In this mode, the mass spectrometer was running in TOFMS mode until encountering multiple charged ions upon which it will switch to MS/MS mode. The RIC is derived from the TOFMS channel; hence, the peak intensities of *m*/*z* 692.8 and *m*/*z* 634.3 do not reflect the true amount present since the MS has switched over to acquire MS/MS data once these ions were detected. In this case, we sacrifice the quantification data but we gain in specificity—The MS/MS spectra confirm the presence of the allergen. In addition, several factors such as the food matrices themselves, the variation in the different extraction, purification, and digestion steps, and the inherent dead time properties of the time to digital converter used also limit the application of this approach in a quantification mode.

In conclusion, we have shown that proteolytic digestion followed by LC-MS/MS can be used for the analysis of allergenic proteins in food matrices. The method can be used to confirm or corroborate other methods such as ELISA. We have illustrated also the capability of the MS/MS database search to provide reliable confirmation of the presence or absence of milk allergenic proteins. In addition, data on the presence of specific casein or whey allergen components could be a valuable tool to understand consumer complaints.

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