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Confirmation of the Allergenic Peanut Protein, Ara h 1, in a Model Food Matrix Using Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)

KEVIN J. SHEFCHECK* AND STEVEN M. MUSSER

Center for Food Safety and Nutrition, Food and Drug Administration, College Park, Maryland 20740

Enzymatic digestion of total protein along with liquid chromatography/tandem mass spectrometry (LC/MS/MS) was used to confirm the presence of a major peanut allergen in food. Several peptides obtained from the enzymatic digestion of the most abundant peanut allergen, Ara h 1, were identified as specific peptide biomarkers for peanut protein. Using ice cream as a model food matrix, a method was developed for the detection of the allergen peptide biomarkers. A key component of the method was the use of molecular mass cutoff filters to enrich the Ara h 1 in the protein extracts. By applying the method to ice cream samples containing various levels of peanut protein, levels as low as 10 mg/kg of Ara h 1 could routinely be detected. This method provides an unambiguous means of confirming the presence of the peanut allergen, Ara h 1, in foods and can easily be modified to detect other food allergens.

KEYWORDS: Allergenicity; analysis; confirmation; LC/MS/MS; peanut; proteins

INTRODUCTION

Food allergens are a significant worldwide public health issue. In the United States, more than 4 million people have been estimated to have serious food allergies (*1*). Of these, more than 50% (0.5−1.1% of total population) have allergies to peanut (*Arachis hypogea*) and tree nuts, making these foods one of most frequently associated food-induced causes of anaphylaxis. Ingested amounts of as little as 100 μg of peanut protein can cause allergenic reactions in extremely hypersensitive people (*2*). Consequently, people with allergies to peanuts, who are accidentally exposed through food, can experience a life-threatening anaphylactic reaction. These reactions are usually very traumatic and can result in death (*3*−*5*).

Human allergic reactions have been linked to different proteins within the peanut. These proteins are extensively glycosylated, and their polypeptide chains range in molecular masses between 10 and 70 kDa. The allergenic proteins come from a variety of peanut families, such as vicilins (*6*, *7*), conglutins (*8*), and glycinins (*9*). There are seven known peanut proteins, Ara h 1−7, which have been shown to react with human IgE and induce an immunogenic response (*6*, *8*−*12*). The two peanut allergens that are most often associated with peanut hypersensitivity are Ara h 1, a 68 kDa vicilin seed storage protein, and Ara h 2, a 17 kDa conglutin protein. These two allergens cause reactions in over 90% of peanut hypersensitive individuals (*13*). Of these, Ara h 1 is by far the most abundant and accounts for approximately 12−16% of the total protein in a peanut (*14*).

Test kits employing enzyme-linked immunosorbent assays (ELISA) are the most common techniques for detecting peanut allergens in food (*15*−*19*). The typical limit of detection for these kits is about 5 ppm of peanut protein. Although commercial test kits are widely used, they often yield inconsistent results due to a variety of factors. For example, many of these kits use polyclonal antibodies, which are not allergen specific; they can only detect a broad group of proteins, such as peanut proteins. Human IgE is not suitable for reliable allergen determination in food products, since the specificity of IgE from sensitized individuals differs considerably (*20*); thus, standardization is difficult. Also, the standards used for the test kits are not well-defined, and several food matrices can contribute interferences. Cross-reactivity with other allergenic foods can produce false positive results. These issues can contribute to unacceptably high levels of false positive results during routine testing. A confirmatory test for allergen test kits is essential based on these issues.

Confirmatory testing of proteins has not been a traditional issue of proteomics; however, it will become more important as medical diagnostics and food authenticity/safety issues become more significant. Mass spectrometry (MS) has proven to be a powerful analytical technique for protein and peptide analysis. Now used routinely for most proteomics investigations, MS has become an indispensable tool in early disease diagnosis (*21*−*27*) and biomarker discovery (*28*−*30*). MS has become popular for biomarker analysis for a number of reasons, including sensitivity and accurate molecular mass determination. Additionally, when combined with tandem MS (MS/MS) approaches, it often produces complete sequence information for peptides of interest. This information, along with peptide

* To whom correspondence should be addressed. Tel: +(301)436-2038. Fax: +(301)436-2624. E-mail: kshefche@cfsan.fda.gov.

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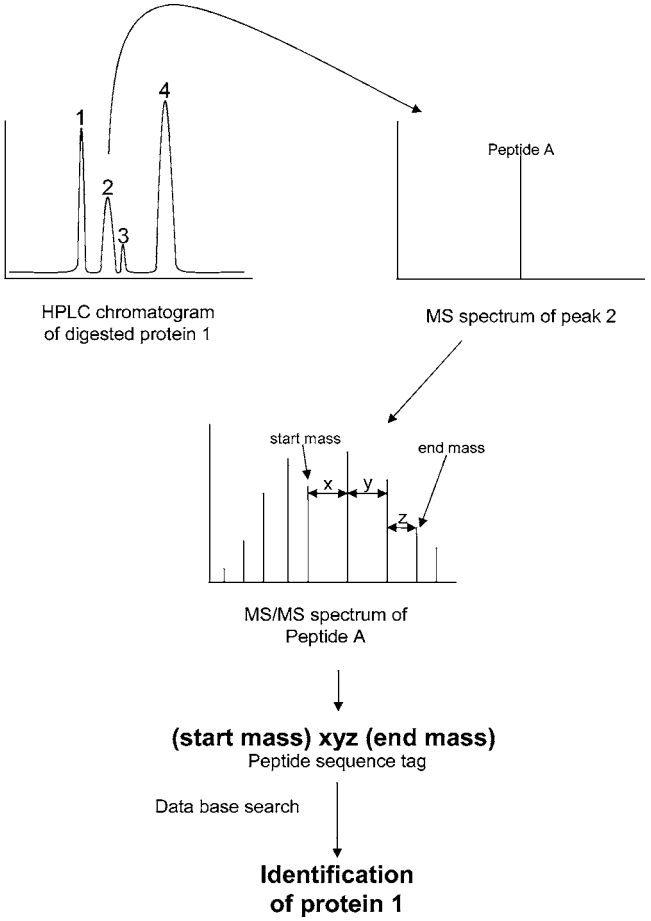

Figure 1. Illustrated protocol for protein identification using peptide sequence tag analysis.

database searching, allows for accurate, unambiguous identification of the original protein. We show here that high-performance liquid chromatography/mass spectrometry (HPLC/MS) can be used to elucidate and characterize peptide biomarkers of the peanut allergen, Ara h 1. Furthermore, we show these biomarkers can be used to identify Ara h 1 in a food matrix and that it can be used as a confirmatory test for the detection of other peanut specific allergens.

MATERIALS AND METHODS

Ice Cream Spiking with Ara h 1. Vanilla ice cream was used as a model food matrix for our spiking experiments. The ice cream was allowed to liquefy for easier mixing. Three 1 g samples of ice cream were weighed out. The Ara h 1 (TNO, Zeist, Netherlands) was solublized in 100 mM ammonium bicarbonate to give a final concentration of 10 mg/mL. The ice cream samples were spiked with differing amounts of Ara h 1: 0, 10, and 1000 μg. Each spiked sample was mixed by vortexing for 1 min. Extraction was started immediately at this point.

Extraction of Protein from Ice Cream. Each spiked ice cream sample was diluted with 1 mL of 100 mM ammonium bicarbonate. Ten milligrams of potassium oxalate was added to each sample before fat separation to destabilize casein micelles in the ice cream. Ten milliliters of hexane was added to each sample, and the samples were vortexed until they formed an emulsion. The samples were then centrifuged at 8422*g* at room temperature. The bottom layer was removed, and the hexane wash procedure was repeated on this aqueous layer. The protein mixture was acidified to ~pH 2 to precipitate the casein in the sample. The sample was centrifuged at 21 000*g* for 15 min at room temperature, and the supernatant was used for further analysis.

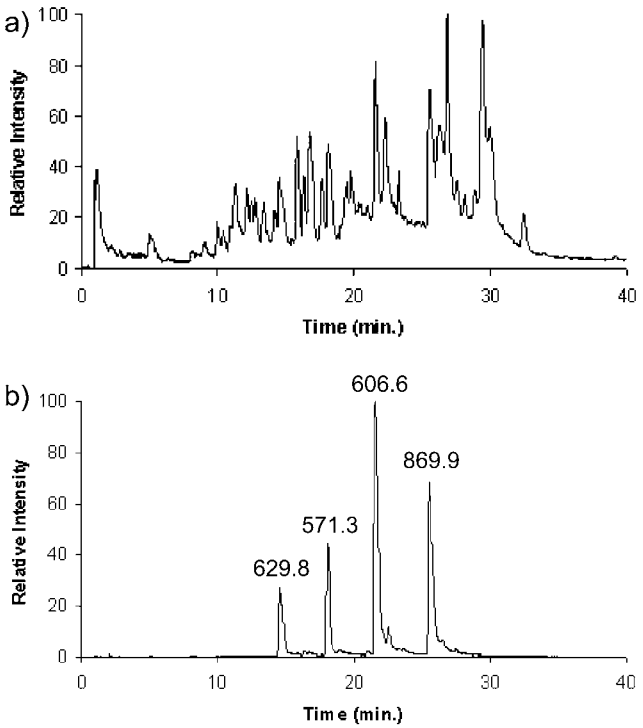

Figure 2. (**a**) Total ion chromatogram of an Ara h 1 tryptic digest. (**b**) Selected ion chromatogram of four abundant peptides (shown with *m*/*z* ratios) from Ara h 1 used as markers.

MRGRVSPLMLLLGILVLASVSATQAKSPYRKTENPCAQRCLQSCQQEPDDLKQKACE

SRCTKLEYDPRCVYDTGATNQRHPPGERTRGRQPGDYDDDRRQPRREEGGRWGP

AEPRERE REEDWRQPREDWRRPSHQQPRKIRPEGREGEQEWGTPGSEVREETSR

571.3

NNPFYFPSRRFSTRYGNQNGRIRVLQRFDQRSKQFQNLQNHRIVQIEARPNTLVLPK

629.8

HADADNILVIQQGQATVTVANGNNRK**SFNLDEGHALR**IPSGFISYILNRHDNQNLRVA

869.9

KISMPVNTPGQFEDFFPASSRDQSSYLQGFSR**NTLEAAFNAEFNEIR**RVLLEENAGG

EQEERGQRRRSTRSSDNEGVIVKVSKEHVQELTKHAKSVSKKGSEEEDITNPINLRD

GEPDLSNNFGRLFEVKPDKKNPQLQDLDMMLTCVEIKEGALMLPHFNSKAMVIVVVN

KGTGNLELVAVRKEQQQRRREQEWEEEEEDEEEEGSNREVRRYTARLKEGDVFIMP

606.6

AAHPVAINASSELHLLGFGINAENNHR**IFLAGDKDNVIDQIEK**QAKDLAFPGSGEQVEK

LIKNQRESHFVSARPQSQSPSSPEKEDQEEENQGGKGPLLSILKAFN

Figure 3. Protein sequence for Ara h 1, clone P17 precursor (*31*). Peptides used as markers are highlighted in bold.

Protein Cleanup and Digestion. Dialysis was performed using 1 kDa cutoff membranes (Mini Dialysis Kit, Amersham Pharmacia Biotech, Uppsela, Sweden). Five hundred microliters of the protein mixture was dialyzed for 4 h at room temperature into 100 mM ammonium bicarbonate, pH 8.0.

Alternative Ara h 1 Enrichment Protein Cleanup. An alternative cleanup method to remove ice cream matrix was performed by adding 1.2 mL of 10 ppm spiked sample to a 30 kDa cutoff Ultrafree 0.5 mL centrifugal filter (Millipore, Billerica, MA) or 50 kDa cutoff Ultrafree 0.5 mL centrifugal filter (Millipore). Five hundred microliters was added to the filter and spun at 9300*g* for 10 min. This was repeated until all of the sample was washed through the filter. The sample was then reconstituted into 500 μL ammonium bicarbonate, pH 8.0.

Sample Digestion. One milligram of RapiGest (Waters, Bedford, MA) and 2.5 μL of 1 M dithiothreitol (Sigma Chemical Company, St. Louis, MO) were added to each sample. RapiGest is a surfactant that hydrolyzes at low pH and is rendered insoluble. The samples were heated at 60 °C in a water bath for 30 min to facilitate reduction of the cysteines. Afterward, the samples were allowed to cool to room temperature, and 7.5 μL of 1 M iodoacetamide was added, and the

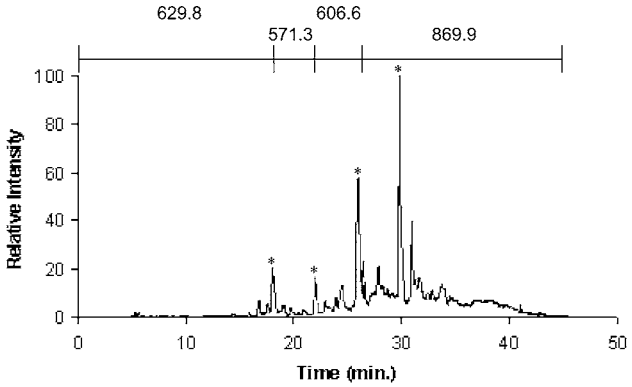

Figure 4. MS/MS RIC of the 1000 ppm spiked vanilla ice cream. The MS/MS scan of *m/z* 629.8 is between 0 and 19 min. The MS/MS scan of *m/z* 571.3 is between 19 and 23 min. The MS/MS scan of *m/z* 606.6 is between 23 and 26.5 min. The MS/MS scan of *m/z* 869.9 is between 26.5 and 50 min. Stars indicate the chromatographic peaks where the respective peptides are detected.

samples were incubated at room temperature in the dark for 30 min. Digestion was done by adding 100 μL of immobilized trypsin (Pierce, Rockford, IL) to each sample. The samples were incubated overnight at 37 °C while mixing. After digestion, the samples were removed from the immobilized trypsin by centrifugation at 2300*g* for 10 min at room tempature. The RapiGest was removed from the peptide mixture by acidifying the solution to ~pH 2 and incubating it at 37 °C for 30 min. The samples were then centrifuged at 21 000*g* for 15 min at room tempature. The peptide mixture was then removed from the insoluble RapiGest component.

Liquid Chromatography (LC)/MS and Database Searching. LC (Agilent 1100 series, Agilent Technologies, Palo Alto, CA) was performed by injecting 40 μL of the peptide mixture onto a 0.32 mm × 150 mm Symmetry300 C_{18} 5 μm particle size column (Waters) with a flow rate of 20 μL/min. A 0−50% acetonitrile with 0.5% acetic acid gradient was used for the separation. Characterization of the peptides was achieved using a Micromass Q-TOF II (Waters). Peptides were identified using peptide sequence tag analysis and database searching with PeptideSearch (http://www.mann.emblheidelberg.de/GroupPages/PageLink/peptidesearchpage.html) (EMBL-Heidelberg).

The protocol for our peptide identification is shown in **Figure 1**. After tryptic digestion of the protein of interest, the peptide mixture was separated on the HPLC column and detected by MS. The selected peptide ion was then bombarded against an inert gas (in our case argon). This fractured the peptide at the peptide bond, and a MS/MS was produced. Each peak in this spectrum was a fragment ion of the peptide ion. Because the peptide was fragmented at the peptide bond, the mass difference between two fragment ions was the mass of an amino acid. A three or four amino acid sequence can be used along with the total mass of the peptide and the mass of the fragmented peptide at the start and end of the sequence to produce a peptide sequence tag. The peptide sequence tag can be entered into a database search algorithm to identify the protein from which the peptide came.

RESULTS AND DISCUSSION

Identification of Biomarkers for Peanut Allergen Ara h 1. The total ion chromatogram of the trypsin digest from Ara h 1 is shown in **Figure 2a**. Data-dependent MS/MS followed by sequence tag identification of the peptides in this mixture identified a number of Ara h 1 specific peptides. The four most abundant peptides having the following mass/charge ratios, *m/z* 629.8, *m/z* 571.3, *m/z* 606.6, and *m/z* 869.9 (**Figure 2b**), were chosen as biomarkers for Ara h 1. These were chosen based on the intensity and reproducibility of retention time in successive HPLC/MS runs. More importantly, these peptides and their corresponding sequences were found to be unique to Ara h 1 and were not identified in any other known protein sequence. The Ara h 1 peptide sequence is shown in **Figure 3**, with the biomarker sequences highlighted.

To evaluate the utility of this MS-based technique for confirming peanut allergen in foods, Ara h 1 was spiked into ice cream at two different levels. The peptide biomarkers for Ara h 1 were analyzed in the ice cream by extracting and digesting all of the protein in the sample and then using the mass spectrometer to select for the peptides of interest. To

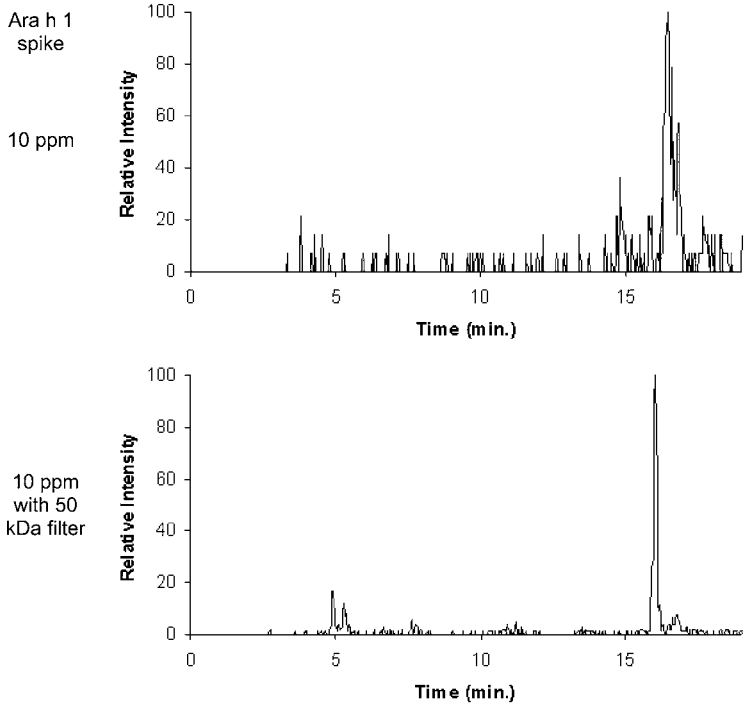

Figure 5. Comparison of filtered and unfiltered 10 ppm Ara h 1 spiked samples. Selected ion chromatograms of fragment *m/z* 797.2 from an MS/MS scan of peptide *m/z* 629.8.

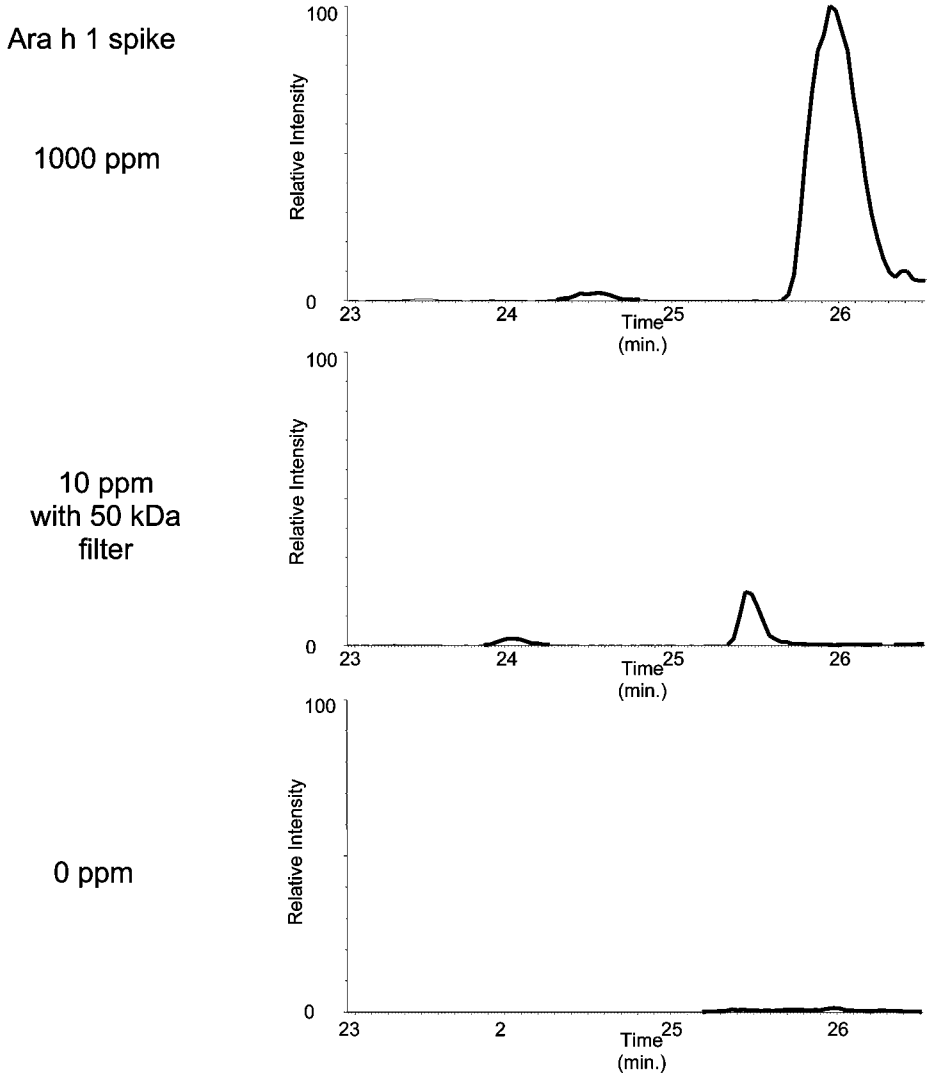

Figure 6. Detection of Ara h 1 peptides in ice cream. Selected ion chromatograms of product ion *m*/*z* 779.3 from an MS/MS scan of peptide *m*/*z* 606.6 at different concentrations of Ara h 1.

maximize the sensitivity, MS/MS experiments were conducted for a single biomarker peptide over its retention time. **Figure 4** illustrates the selectivity of the MS/MS method and demonstrates excellent signal-to-noise ratios for the peptide biomarkers of interest, despite the overwhelming presence of unrelated peptides from the ice cream. This MS/MS scanning technique not only allows the selection of the biomarker peptide of interest but further distinguishes the peptide as a biomarker for Ara h 1 based on its fragmentation pattern and corresponding sequence information.

Enrichment of Peptides of Ara h 1. Identification of Ara h1 biomarker peptides in ice cream samples spiked at 1000 ppm was accomplished with good signal-to-noise being observed for all peptides. However, ice cream samples spiked with 10 ppm Ara h 1 were very difficult to confirm. This led us to evaluate different molecular mass cutoff filters, 30 and 50 kDa, as means of enriching the Ara h 1 in the total protein extracts. The selected ion chromatograms for the Ara h 1 peptide *m*/*z* 629.8 (**Figure 5**) illustrate the improvement in signal observed when using molecular mass cutoff filters as an additional sample cleanup step. The signal-to-noise ratio in the 10 ppm sample without the filter is approximately ~10-fold lower than in the signal-to-noise ratio of the 10 ppm sample concentrated with the 50 kDa cutoff filter. The cutoff filter acts in two ways to improve the signal-to-noise in the 10 ppm sample. One, it simply concentrates the Ara h 1 protein in the protein extract. Second, it removes lower molecular mass ice cream proteins and other small molecules, which are present in high abundance that can interfere with the ionization efficiency of the Ara h 1 peptides. For example, the milk protein lactalbumin with a molecular mass of 14 kDa comprises approximately 3.7% of the total milk proteins and can be greatly reduced by using the molecular mass cutoff filters. The Ara h 1 peptides were enriched with both the 30 and the 50 kDa cutoff filters. A comparison of the two showed a slight increase in intensity of the peptides with the 50 kDa cutoff filter over the 30 kDa cutoff filter (figure not shown).

Identification of Ara h 1 in Ice Cream. The use of a molecular mass cutoff filter allows us to more easily identify the Ara h 1 in the 10 ppm Ara h 1 sample. **Figure 6** demonstrates this in selected ion chromatograms of the product ion *m*/*z* 779.3 obtained from the doubly charged biomarker peptide at *m*/*z* 606.6. While a small signal is observed in the blank, careful examination of the full scan spectrum shows no fragment ions common to the MS/MS spectrum of this biomarker peptide. The fact that a small signal is observed highlights the importance of acquiring as much spectral information as possible to avoid reporting false positives. **Figure 7** illustrates the important differences in the full scan spectra from samples that contain Ara h 1 vs the blank. The product

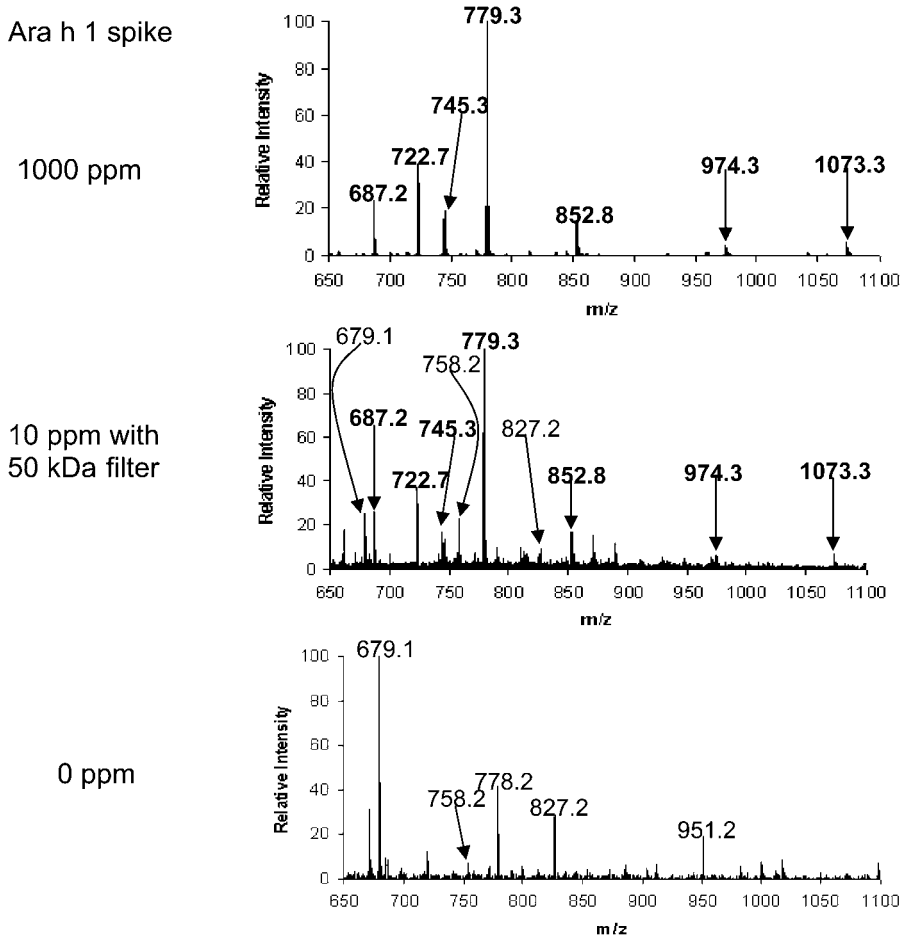

Figure 7. Detection of Ara h 1 in ice cream. MS/MS of peptide *m*/*z* 606.6 at different concentrations of Ara h 1. *m*/*z* ratios in bold represent peptides included in the Ara h 1 peptide, and other *m*/*z* ratios are other matrix peptides.

Table 1. Detection of Four Ara h 1 Biomarkers in Ice Cream[a]

Ara h 1 peptide biomarker	concentration of Ara h 1 in vanilla ice cream sample		
	1000 ppm	10 ppm	0 ppm
m/*z* 629.8	6	6	0
m/*z* 571.3	5	4	0
m/*z* 606.6	7	7	0
m/*z* 869.9	6	6	0

[a] The table represents the number of fragment ions from each biomarker seen at different concentrations of Ara h 1.

ions of the peptide *m*/*z* 606.6 are apparent in the 1000 ppm Ara h 1 sample and are completely identical to those observed in the digest of the Ara h 1 standard. The 10 ppm Ara h 1 sample also includes all of the product ions from the peptide *m*/*z* 606.6. However, there are many background ions that are not in the peptide fingerprint. The blank spectrum only includes background ions, none of which are observed in the *m*/*z* 606.6 fingerprint. Seven out of seven product ions are seen in the 10 ppm Ara h 1 sample spectrum, which gives us a definitive confirmation that the allergen is contained in the sample. **Table 1** shows the results for all of the biomarkers tested. Of these, only one product ion from one of the biomarkers tested was unaccounted for in the 10 ppm Ara h 1 sample. This particular product ion was in very low abundance even in the 1000 ppm sample.

In conclusion, unique peptide biomarkers were identified for the peanut allergen, Ara h 1. These peptides were used to identify Ara h 1, which was spiked into vanilla ice cream. All of the peptides were detected with a high degree of certainty at the 10 ppm concentration. A key feature of this method was the use of a 50 kDa molecular mass cutoff filter, which enriched the Ara h 1 for straightforward characterization. This method has broad applicability as a confirmatory test for ELISA. It can be adapted for use with any well-characterized protein in virtually any matrix including clinical and industrial samples. Additionally, the method has the flexibility of not needing specific antibodies, so it can easily be used for the analysis of other food allergens such as in eggs and milk. Any number of peptides from the respective protein can be employed as biomarkers. Also, this method can be used for allergens in different matrices. Finally, MS has the ability to detect any differences in the sequences of the peptides for potential changes in the immunological response of the food allergen.

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