Confirmation of Peanut Protein Using Peptide Markers in Dark Chocolate Using Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS)

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Detection of peptides from the peanut allergen Ara h 1 by liquid chromatography–mass spectrometry (LC-MS) was used to identify and estimate total peanut protein levels in dark chocolate. A comparison of enzymatic digestion subsequent to and following extraction of Ara h 1 from the food matrix revealed better limits of detection (LOD) for the pre-extraction digestion (20 ppm) than for the postextraction digestion (50 ppm). Evaluation of LC-MS instruments and scan modes showed the LOD could be further reduced to 10 ppm via a triple-quadrupole and multiple-reaction monitoring. Improvements in extraction techniques combined with an increase in the amount of chocolate extracted (1 g) improved the LOD to 2 ppm of peanut protein. This method provides an unambiguous means of confirming the presence of the peanut protein in foods using peptide markers from a major allergen, Ara h 1, and can easily be modified to detect other food allergens.

KEYWORDS: Peanut protein; Ara h 1; dark chocolate; allergen

INTRODUCTION

Peanut allergies are a recognized public health problem, with reports estimating that up to 1.1% of the U.S. population has an allergenic reaction to peanuts (*Arachis hypogaea*) (*1*). Current evidence suggests that the prevalence of this affliction has been rising for the past 20 years in both children and adults (*1–4*). The amount of peanut protein that has been reported to induce allergic reactions varies among individuals. Relatively small amounts, as low as 100 g of peanut protein, have been reported to induce an allergic reaction in sensitive individuals (*5, 6*). Reactions due to peanut allergen sensitivity can range from mild gastrointestinal reaction to severe anaphylaxis in sensitive individuals. Some studies have shown peanut or tree nut allergy to cause ≈80% of anaphylactic reactions and half of the deaths associated with food hypersensitivity (*7*).

Peanut allergies are caused by adverse immune responses to particular proteins in the peanut. There are several proteins in the peanut that can potentially cause an immunological response, known as Ara h 1–Ara h 8 (*8–15*). These proteins range in size between 10 and 70 kDa, but can form multimers and complexes with molecular masses of >70 kDa. One abundant protein that is associated with peanut allergy is Ara h 1, which accounts for roughly 12–16% of the total protein in a peanut (*16*). Ninety-five percent of all people with peanut allergy react to Ara h 1 (*17*). Ara h 1 is a vicilin protein that has three 62 kDa polypeptide chains that form a homotrimer (*18*).

Accidental ingestion of peanut-contaminated foods is one way that sensitive individuals come into contact with peanuts. In most cases, testing for contaminated foods is typically done using enzyme-linked immunosorbent assay (ELISA) based analytical methods. These methods, which are based on the response of an antibody to an antigen, are very quick, easy, and useful tests in an industrial environment. Poms et al. performed an interlaboratory validation study of five test kits, testing biscuits and dark chocolate (*19*). They demonstrated good reproducibility with the chocolate samples, but false negatives were observed in as many as 25% of the dark chocolate samples for some kits. Other studies have shown wide variability in measured protein level in ELISA due to food matrix, test kit used, and sampling size (*20, 21*). Recently, DNA-based detection methods have been introduced to detect peanut proteins in food. Real-time Polymerase Chain Reaction (PCR) methods are very specific and sensitive to the low parts per million range (*22*). Although this is a promising approach, real-time PCR detects DNA, not the offending allergen. Depending on protein expression conditions, there may be significant variations in the relationship between the quantity of DNA present and the amount of allergen present. For purposes of confirming the presence of an allergen, methods are needed that can directly measure a signal related to the allergen protein itself. Although some ELISA kits measure a specific allergen protein (*19*), many respond to total peanut protein, including those shown to be allergens. Thus, natural variations in the amount of allergens relative to total protein can also affect results from immunoassays.

Immunoassays and other methods are also subject to complications that result from food matrices. Dark chocolate has been a problematic matrix for peanut allergen detection with ELISA. This complex matrix can contribute to both false-positive and false-negative responses in immunoassay-based

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A)

MRGRVSPLMLLLGILVLASVSATHA**KSSPYQKK**TENPCAQRCLQSCQ**QEPDDLKQKA**CESRCTKL*EYDPRCV*YDPRGHTGTTNQR
SPPGERTRGRQP**GDYDDDRR**QP**RREEGGRW**GPAGPRER**EREEDWRQ**PREDW**RRPSHQQPRKIRPEG**REGEQEWGTPGSHVR
EETSRNNPFYFPSRRFSTRYGNQNGRIRVLQRFDQRSRQFQNLQNHRIVQIEAKPNTLVLPKHADADNILVIQQGQATVTVANGNNR
KSFNLDEGHALRIPSGFISYILNRHDNQNLRVAKISMPVNT**PGQFEDFF**PASSRDQSS*YLQGFSRN*TLEAA**FNAEFNEIRR**<u>VLLEENA</u>
<u>GGE**QEER**</u>GQRRWSTRSSENNEGVIVKVSKEHVEELTKHAKSVSKKGSEEEG**DITNPINLRE**GEPDLS**NNFGKLFEVK**PDKKNPQL
QDLDMMLTCVEIKEGALMLPHFNSKAMVIVVVNKGT**GNLELV**AVRKEQQQRGRREEEEDEDEEEEGSNREV**RRYTARLKEG**DVFI
MPAAHPVAINASS**ELHLLGFGIN**AENN**HRIFLAGDKDN**VI**DQIEKQAK**<u>DLAFPGSGE</u>QVEKLIKNQ**KESHFVSARP**QSQSQSPSSP**E**
KESPEKEDQEEENQGGKGPLLSILKAFN

B)

VLLEENAGGEQEER

MS

C)

DLAFPGSGEQVEK

MS

Product ion markers in Red

MS/MS
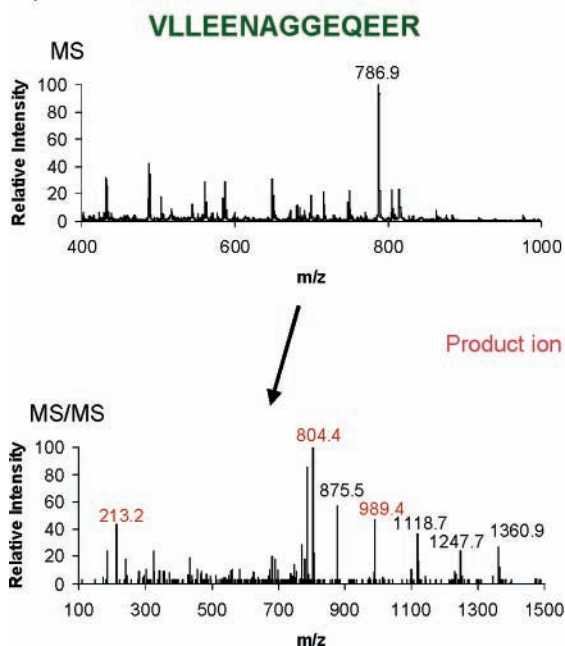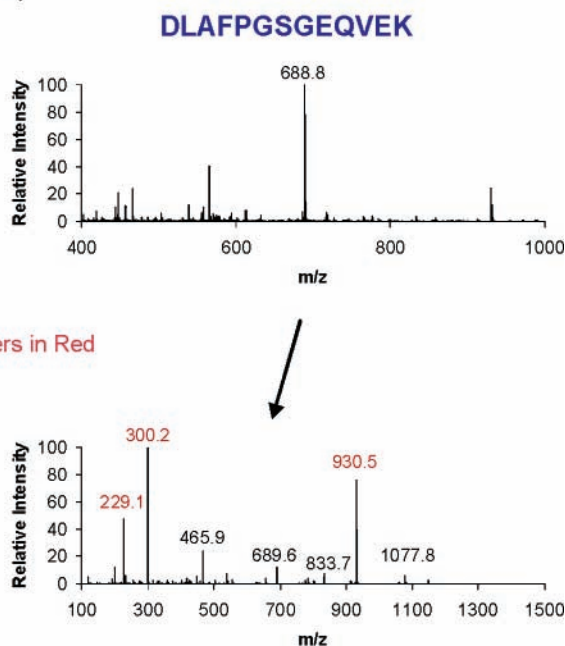
Figure 1. (**A**) Ara h 1 P17 precursor sequence. Known epitopes are in boldface type. The markers for doubly charged ions at *m/z* 786.9 and 688.9 are shown in green and blue type, respectively. (**B**) Mass spectrum and tandem mass spectrum of peptide marker VLLEENAGGEQEER. (**C**) Mass spectrum and tandem mass spectrum of peptide marker DLAFPGSGEQVEK.

techniques (*19*, *23*, *24*). Proteins in chocolate can interact with tannins, which complicates the extraction procedure and can reduce or eliminate the detection of allergenic proteins. Covalent, ionic, hydrophobic, or hydrogen bonding interactions are responsible for tannin−protein binding. Hydrogen bonding is the most prevalent and is possibly one of the main reasons proteins are difficult to extract from tannins. Frazier et al. (*25*). demonstrated that tannins can interact both specifically or nonspecifically, depending on the protein. Therefore, the success of any peanut protein extraction method will largely be based on the ability to interrupt these interactions. Consequently, the extraction procedure is a very important element of the peanut protein detection.

Some of the difficulties associated with the correct identification of peanut proteins in food matrices can be relieved by the use of more specific, proteomics methods that employ mass spectrometry (MS). Because MS can directly measure a specific molecular property of the allergen, the possibility of false positives is greatly reduced. We have shown in earlier investigations that MS-based proteomic techniques can be used to detect peanut proteins in food (*26*). Many proteomics-based approaches employ enzymatic digestion of protein mixtures to produce characteristic peptides. The resulting peptides are often more efficiently extracted and are more easily separated by reverse-phase liquid chromatographic techniques. By combining high-resolution liquid chromatography (LC) separations with electrospray ionization and tandem mass spectrometry, it is possible to identify the mass of the peptide and also its amino acid
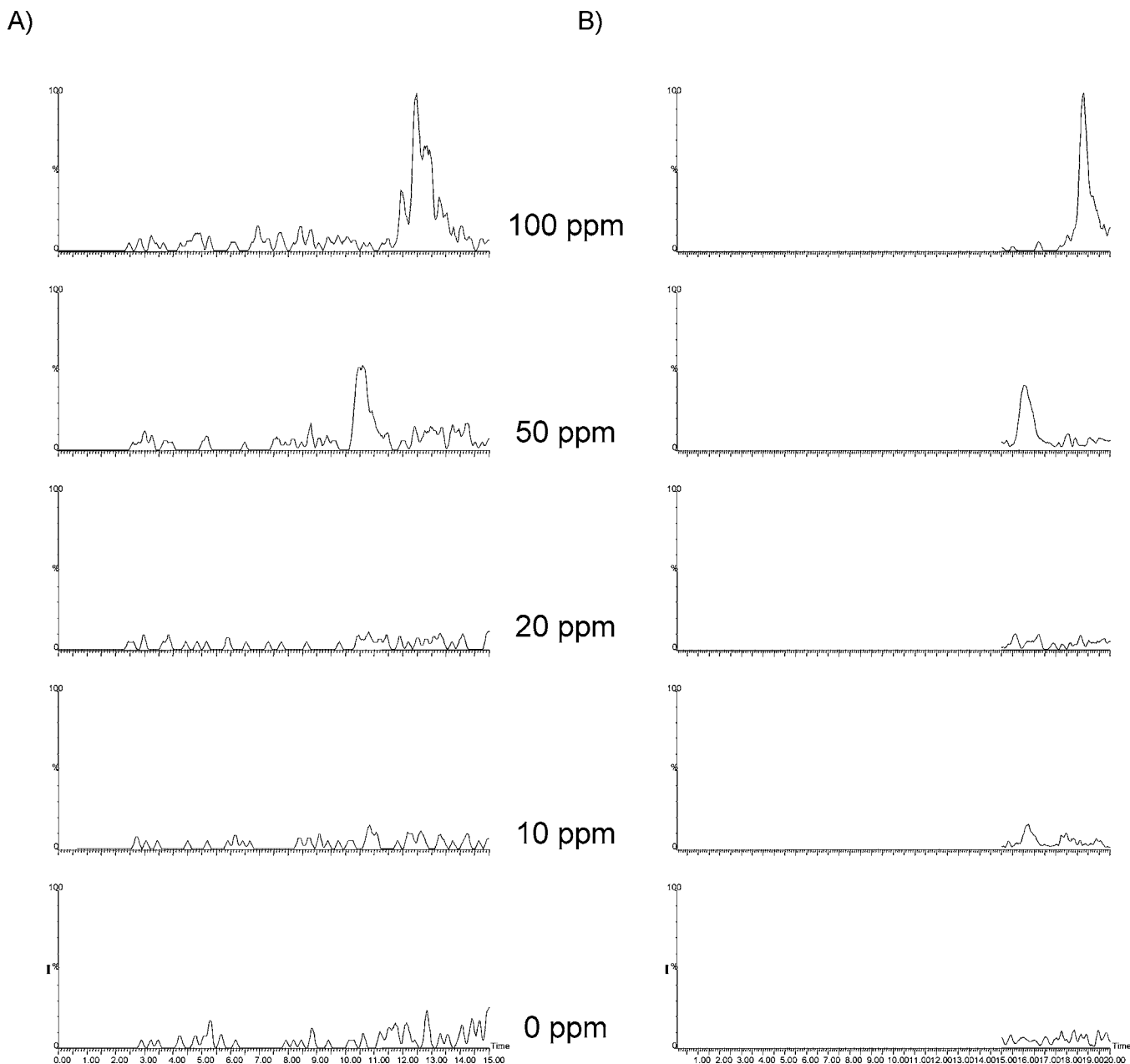
sequence. The latter provides a highly definitive signature for the peptide and, by extension, the protein from which it arises. One advantage of an enzymatic digestion is that the measurement regime is shifted from the realm of large molecules (proteins and their associated interactions) to that for smaller molecules (peptides). This greatly reduces the matrix interactions that render analyte proteins difficult to extract, and it also simplifies chromatographic procedures. In this study we demonstrate that MS-based proteomics methods provide an approach by which peanut allergens can be directly identified at the low parts per million level in chocolate matrices. This approach provides a powerful complement to immunoassay-based approaches.

MATERIALS AND METHODS

Chocolate Samples. Six dark chocolate samples (courtesy of Dr. Susan L. Hefle, University of Nebraska, Lincoln) containing known concentrations of peanut protein were used: A (10 ppm), B (100 ppm), C (50 ppm), D (20 ppm), E (0 ppm), and F (2 ppm).

Ara h 1 Digestion and Peptide Marker Identification. Markers were identified and chosen by digesting 100 μg of Ara h 1 (TNO, Zeist, The Netherlands) with 1 μg of trypsin (Pierce, Rockford, IL) in 50 mM ammonium bicarbonate (pH 8). The sample was incubated overnight at 37 °C and analyzed by LC-MS, as noted below. Peptides were identified using peptide sequence tag analysis and database searching with PeptideSearch (http://www.mann.emblheidelberg.de/GroupPages/PageLink/peptidesearchpage.html)(EMBL-Heidelberg).

Whole Protein Extraction and Digestion of Ara h 1 from Dark Chocolate. One hundred milligrams of each chocolate sample was

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A)

B)

Figure 2. (**A**) Reconstructed ion chromatogram (RIC) for *m/z* 786.9 from peptide VLLEENAGGEQEER using whole protein extraction and HPLC-QTOF analysis. (**B**) RIC for *m/z* 688.9 from peptide DLAFPGSGEQVEK using whole protein extraction and HPLC-QTOF analysis.

incubated overnight in 2.5 mL of 50 mM ammonium bicarbonate (pH 8) at 60 °C with vigorous shaking. The resulting mixture was centrifuged at 8000 rpm for 30 min. The supernatant was filtered through a 0.45 μm centrifugal filter (Millipore, Billerica, MA) and washed twice with 10 mL of 50 mM ammonium bicarbonate (pH 8). The supernatant and wash were filtered through a 5 kDa molecular weight cutoff filter (MWCO) centrifugal filter and washed five times with 5 mL of 50 mM ammonium bicarbonate (pH 8). The retained concentrate was removed and the filter washed with 50 mM ammonium bicarbonate (pH 8); the wash retentate was added to the retained concentrate. One microgram of trypsin was added to the retained concentrate and allowed to incubate overnight at 37 °C.
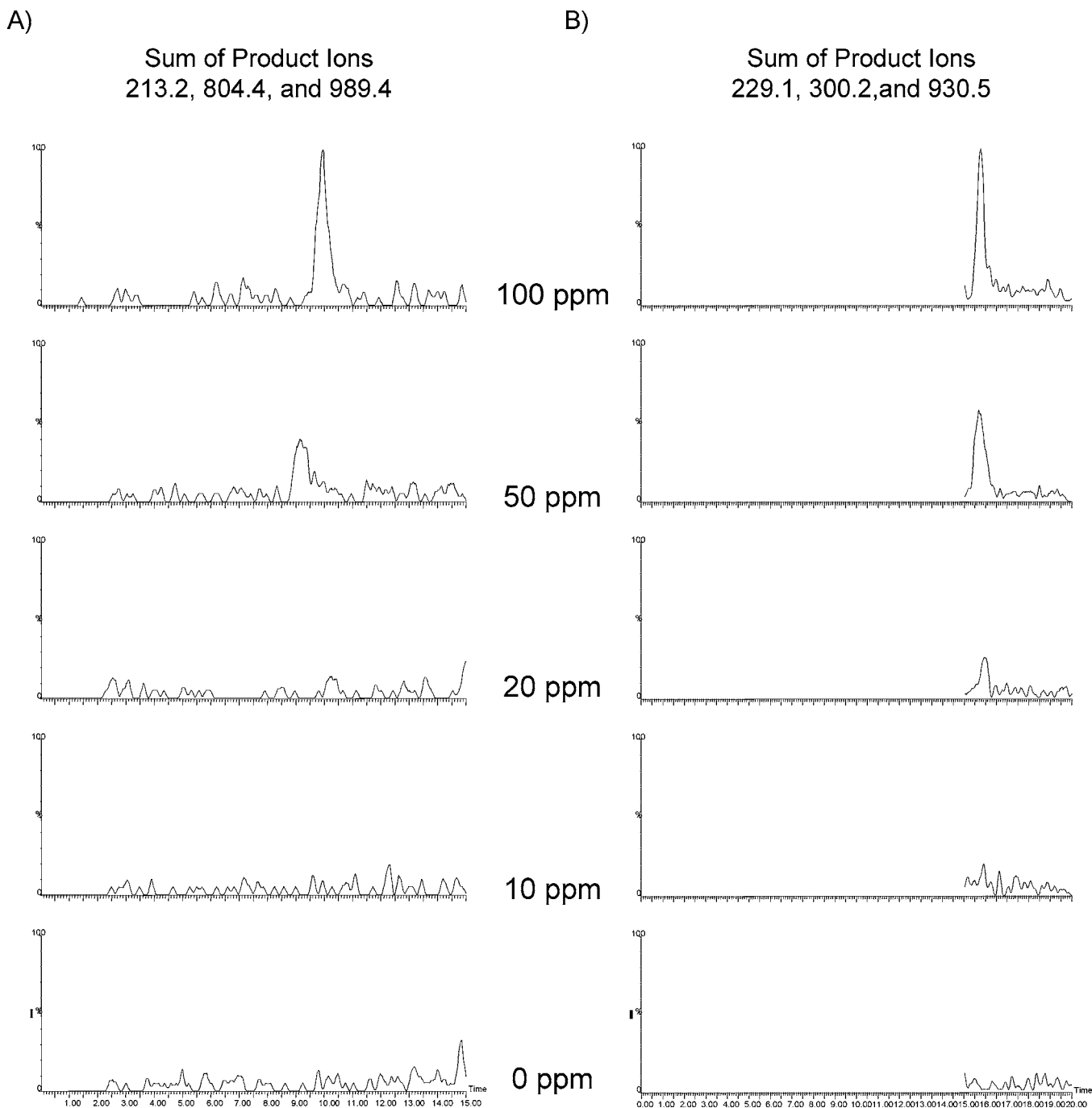
Simultaneous Protein Digestion and Extraction of Marker Peptides from Dark Chocolate. One hundred milligrams of each chocolate sample was incubated for 2 days in 2.5 mL of 8 μg/mL trypsin in 50 mM ammonium bicarbonate (pH 8) at 37 °C with vigorous shaking. The resulting mixture was centrifuged at 8000 rpm for 30 min. The supernatant was filtered through a 100 kDa MWCO centrifugal filter (Millipore) and washed twice with 5 mL of 50 mM ammonium bicarbonate (pH 8). To further improve the detection limits of our

analysis, we increased the amount of sample to 1 g and extraction buffer volume to 25 mL. The samples were washed 10 times with the same amount of ammonium bicarbonate.

Peptide Cleanup. The extract was further purified with Strata-X 33 μm polymeric sorbent multimode solid-phase extraction media (Phenomenex, Torrance, CA). The peptides bound to the SPE media were washed twice with 50 mM ammonium bicarbonate (pH 8) and eluted with 500 μL of 70% acetonitrile−0.1% formic acid. The samples were dried and reconstituted in 50 μL of 0.5% formic acid.

LC-MS Analysis of Peptides from Ara h 1 and Peanut Digests. LC (Agilent 1100 series, Agilent Technologies, Palo Alto, CA) was performed by injecting 5 μL of the digest mixture onto a 0.32 × 150 mm Symmetry300 C_{18} 5 μm particle size column (Waters, Bedford, MA) at a flow rate of 20 μL/min. A binary gradient consisting of water/ 0.5%formic acid and acetonitrile/0.5% formic acid was used for the separation; the organic component was increased from 0 to 50% over 40 min.

Method development and initial peptide characterization were performed on a Micromass Q-TOF Micro (Waters) mass spectrometer. Electrospray ionization was used. Tandem mass spectrometry

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A)

Sum of Product Ions
213.2, 804.4, and 989.4

B)

Sum of Product Ions
229.1, 300.2, and 930.5

Figure 3. (**A**) RIC for *m/z* 786.9 from peptide VLLEENAGGEQEER using peptide extraction and HPLC-QTOF analysis. (**B**) RIC for *m/z* 688.9 from peptide DLAFPGSGEQVEK using whole protein extraction and HPLC-QTOF analysis.
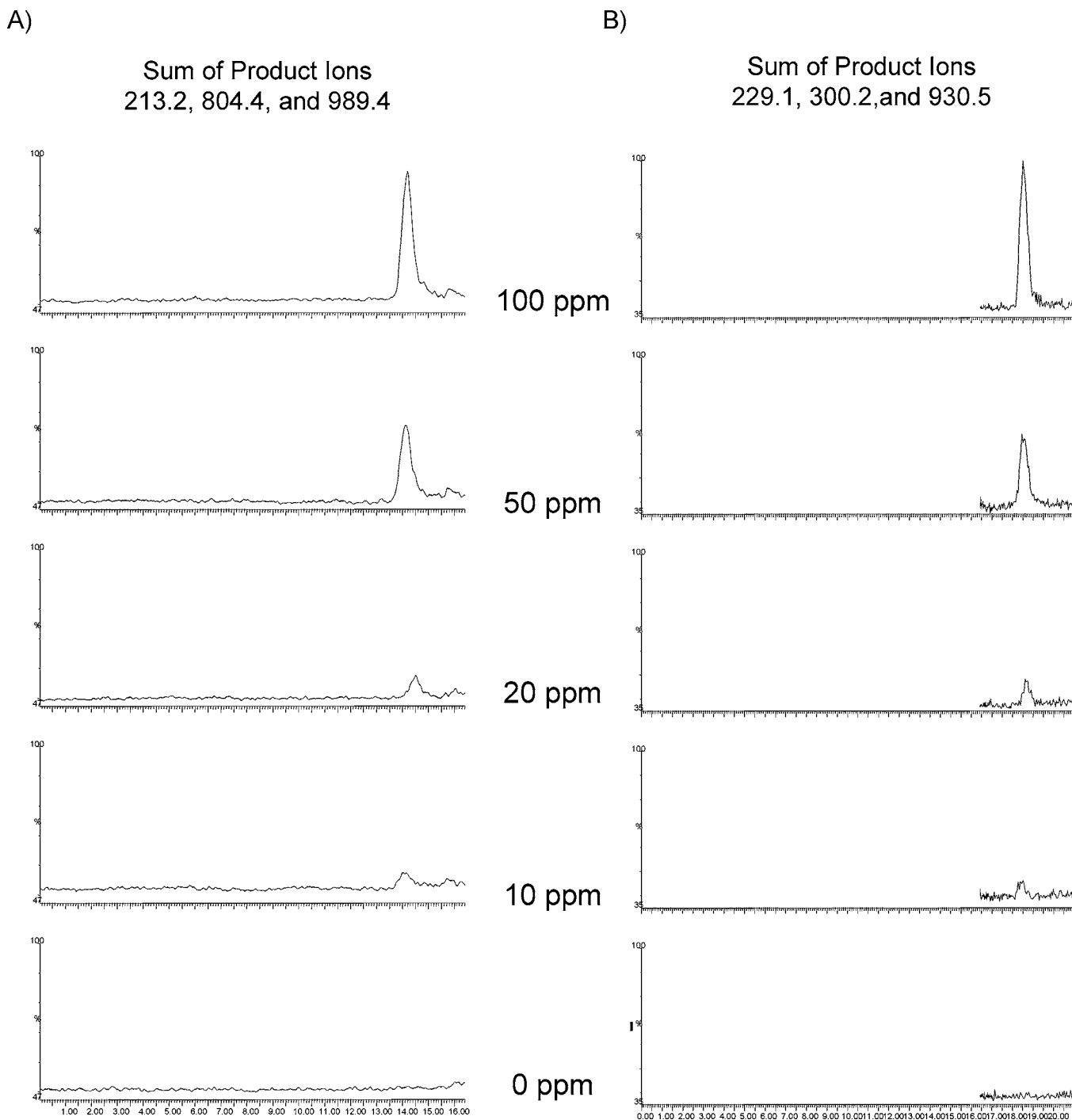
(MS/MS) of peptides eluted from the chromatographic separation was performed in a data-dependent scanning mode, using argon as the collision gas at collision energies determined by the charge state of the ion (typically 20−30 eV for doubly charged ions).

Identification of Ara h 1 was carried out using a Q-TOF Micro or a Micromass Quattro Premier (Waters) triple-quadrupole mass spectrometer. Identification with the Q-TOF instrument was performed using MS/MS of the selected target peptides at collision energies optimized for the mass and charge state of the peptide. Multiple-reaction monitoring (MRM) was performed on the triple-quadrupole instrument. Three product ions were monitored for each selected parent mass.

RESULTS AND DISCUSSION

Marker Peptide Identification. Previously we showed that we could use biomarkers for the identification of peanut proteins

in a food matrix (*26*). We chose two tryptic peptides from Ara h 1 for use as markers for peanut in chocolate. The doubly charged ions of the tryptic digest peptides VLLEENAGGEQEER (*m/z* 786.9) and DLAFPGSGEQVEK (*m/z* 688.9) were chosen on the basis of signal intensity, retention time position, and deficiency of missed cleavages. **Figure 1A**) shows these peptides in the Ara h 1 sequence. Marker VLLEENAGGEQEER overlaps with a known immunologically active epitope, EQEERGQRRW (*27*). DLAFPGSGEQVEK overlaps with two different immunologically active epitopes; it overlaps only slightly with IDQIEKQAKD (*27*), whereas almost all of KDLAFPGSGE (*27*) is included within the tryptic digest marker. The parent ion mass spectrum and tandem mass spectrum of peptide VLLEENAGGEQEER is shown in **Figure 1B**. Among

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A)

B)

Figure 4. (**A**) RIC for *m/z* 786.9 from peptide VLLEENAGGEQEER using peptide extraction and MRM analysis. (**B**) RIC for *m/z* 688.9 from peptide DLAFPGSGEQVEK using whole protein extraction and MRM analysis.

the product ions produced from the doubly charged parent ion at *m/z* 786.9, three specific product ions, shown in red, were chosen as markers: *m/z* 213.2, 804.4, and 989.4. Similarly, the markers produced from the doubly charged parent ion of DLAFPGSGEQVEK (*m/z* 688.9) are shown in **Figure 1C**; they are *m/z* 229.1, 300.2, and 930.5. The product ion at 930.5 is especially abundant and useful as a marker because it includes a proline at the N terminus, which provides stability and enhances the abundance of the resultant product. Using these markers we tried two methods of sample extraction and detection: whole protein extraction/digestion−reverse phase LC-Q-TOF and protein digestion−peptide extraction−reverse phase LC-Q-TOF. Once the approach was developed, the protein digestion−peptide extraction−reverse phase LC method was

translated to a triple-quadrupole mass spectrometer for MRM-based analysis.

Detection of Ara h 1 through Whole Protein Extraction and Digestion with LC-Q-TOF Mass Spectrometry. In previous experiments (*26*) we used a hybrid Q-TOF mass spectrometer for method development. For purposes of comparison, the same type of instrumentation was used in this work. Panels **A** and **B** of **Figure 2** show the reconstructed ion chromatograms of the product ion markers of VLLEENAGGEQEER and DLAFPGSGEQVEK, respectively, when the protein is first extracted from the chocolate and then digested. The results show that there are slight shifts in retention time from sample to sample. However, the product ions for the peptides definitively identify the chromatographic peak and peptides

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from Ara h 1. The method is limited in sensitivity; peptide markers can be detected only at concentrations of ≥50 ppm. Tannin−protein interactions and poor protein recovery are the most likely reasons for the relatively high detection limits for this method (*25*).

Detection of Ara h 1 through Peptide Digestion Extraction with LC-Q-TOF Mass Spectrometry. Efficient extraction of the Ara h 1 from chocolate prior to detection is difficult and limits the sensitivity of the approach. We attempted to improve the overall detection limits by reducing the role of protein−tannin interactions by enzymatically digesting the protein. In addition, this also reduces the mass of the analytical target as peptides are formed from the protein, shifting the target analyte mass away from the mass range of many interfering matrix components. By using a method that involves digestion of the protein during extraction (the simultaneous protein digestion/extraction method described above), the data in **Figure 3** were generated. **Figure 3A** shows the reconstructed ion chromatogram of the product ions of marker VLLEENAGGEQEER. The lowest concentration that could be detected was 50 ppm, which constitutes no improvement over the previous approach. However, a difference was observed with the marker DLAFPGSGEQVEK. The reconstructed ion chromatogram of the sum of three product ions shows improved detection levels, down to 20 ppm. There are several possible explanations for the difference in detection levels for the two peptides, but the most likely is that there are differences in their respective ionization efficiencies. The sample matrix and its contribution to ionization suppression also have an influence on the observed ionization efficiency, and this can vary with retention time as different impurities and concentrations coelute with the target peptides. Finally, the fragmentation spectrum itself plays a significant role in the observed difference. One of the amino acid residues in the marker is proline, which tends to direct fragmentation into a y-ion with proline at its N terminus. Consequently, much of the fragmentation signal is concentrated into one channel, improving the sensitivity in the product ion spectrum.

Detection of Ara h 1 through Peptide Digestion−Extraction with LC−Triple-Quadrupole Mass Spectrometry and MRM. Improving the detection limits of our analysis is the primary goal of this work. Detection limits can often be improved for MS/MS-based methods by using MRM on a triple-quadrupole instrument. In MRM, the portion of the duty cycle used to measure the product ions of interest is significantly higher, thereby enhancing signal-to-noise ratio, because only the product ions of interest are monitored. Because the peptide digestion−extraction approach gave somewhat lower detection limits, we combined this approach with MRM to produce a more sensitive overall approach. Panels **A** and **B** of **Figure 4** show the results, which demonstrate that both peptide markers can be detected for concentrations as low as 10 ppm of peanut protein in chocolate. The method was slightly modified to see whether the sensitivity of our analysis could be improved if we increased the size of our extraction. The sample size was increased 10-fold while the extraction volume was increased 4-fold. **Figure 5** illustrates the MRM chromatograms of DLAFPGSGEQVEK using 1 g of starting material and 25 mL of extraction buffer. The detection limit for the method was improved, and concentrations as low as 2 ppm of total peanut protein were detected. This confirms that the method is robust enough to utilize larger sample sizes.

Concluding Remarks. Using unique peptide markers for the peanut allergen Ara h 1, we developed a method to identify and measure peanut protein in chocolate at concentrations as
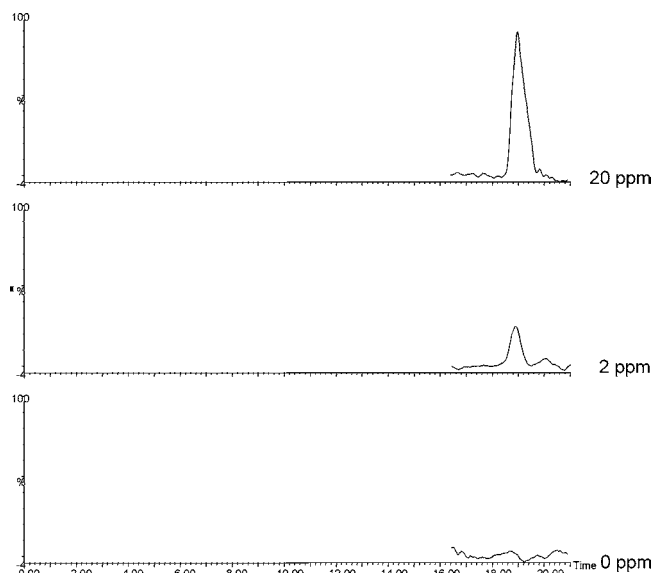
Figure 5. RIC for *m/z* 688.9 from peptide DLAFPGSGEQVEK using whole protein extraction and MRM analysis of 1 g of chocolate.

low as 2 ppm. The LC-MS analysis of the selected markers provides two tiers of identification and confirmation for peanut protein in chocolate. First, the retention time and mass of selected Ara h 1 peptide markers identify the presence of the peanut allergen. Second, mass selection and fragmentation of the peptide generates structurally indicative ions, thereby confirming the identity of the peptide and the protein from which it arises. Furthermore, fragmentation of the peptide can improve quantification because isobaric, coeluting interferences will not give rise to the same fragments as those selected as analyte targets. A semiquantitative method can be performed by using standard addition analysis with MRM.

This work also demonstrates that lower detection levels can be obtained by digesting the peptide during the process of extracting it from the chocolate, rather than extracting the protein first and then digesting it. When combined with MRM, the detection limit of the method was decreased by a factor of 5. Further improvements in detection limit, up to a factor of 10, were obtained by increasing the amount of chocolate digested. The time required to prepare samples for this method probably dictates that, at present, it can be used only as a confirmatory method for the detection of allergens in food. However, although not developed as a quantitative method, the results clearly demonstrate that estimates can be made relative to the amount of allergenic protein present in the chocolate. With the development of appropriate internal standards, it can be converted into a quantitative method. Finally, a key feature of this method is that it may easily be adapted to other food matrices and allergenic proteins, thus providing a platform for the development of new analytical methods for the detection of proteins in food matrices.

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