

Determination of peanut allergens in cereal-chocolate-based snacks: metal-tag inductively coupled plasma mass spectrometry immunoassay versus liquid chromatography/electrospray ionization tandem mass spectrometry

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A comparison of two methods for the identification and determination of peanut allergens based on europium (Eu)-tagged inductively coupled plasma mass spectrometry (ICP-MS) immunoassay and on liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) with a triple quadrupole mass analyzer was carried out on a complex food matrix like a chocolate rice crispy-based snack. The LC/MS/MS method was based on the determination of four different peptide biomarkers selective for the Ara h2 and Ara h3/4 peanut proteins. The performance of this method was compared with that of a non-competitive sandwich enzyme-linked immunosorbent assay (ELISA) method with ICP-MS detection of the metal used to tag the antibody for the quantitative peanut protein analysis in food. The limit of detection (LOD) and quantitation of the ICP-MS immunoassay were 2.2 and 5 μg peanuts g^{-1} matrix, respectively, the recovery ranged from $86 \pm 18\%$ to $110 \pm 4\%$ and linearity was proved in the 5–50 μg g^{-1} range. The LC/MS/MS method allowed us to obtain LODs of 1 and 5 μg protein g^{-1} matrix for Ara h3/4 and Ara h2, respectively, thus obtaining significantly higher values with respect to the ELISA ICP-MS method, taking into account the different expression for concentrations. Linearity was established in the 10–200 μg g^{-1} range of peanut proteins in the food matrix investigated and good precision (RSD <10%) was demonstrated. Both the two approaches, used for screening or confirmative purposes, showed the power of mass spectrometry when used as a very selective detector in difficult matrices even if some limitations still exist, i.e. matrix suppression in the LC/ESI-MS/MS procedure and the change of the Ag/Ab binding with matrix in the ICP-MS method. Copyright © 2008 John Wiley & Sons, Ltd.

Food allergies and related syndromes are one of the arising health issues in western countries:¹ recent estimations show that the affected population can be up to 1.5%.² Nuts, and namely peanuts,³ are the major players in the food allergy scenario, even resulting in fatalities both in infants and adults.⁴ Unfortunately, no effective clinical treatment is known, only relief of adverse symptoms. For this reason, prevention, i.e. a strict dietary control, is the only effective measure to be taken in the cases of severe allergy and therefore an accurate declaration of ingredients on food labels is essential, as stated in recent legislative changes

aimed at improving consumer protection such as the European Union (EU) legislation on food labeling that came into force in November 2005 (EU Directive 2003/89/EC amending Directive 2000/13/EC). Taking in account that accidental contamination with allergens can occur during food manufacturing or handling of the raw materials,⁵ fast and reliable analytical methods are required to assess allergen levels in the final products.

Although the fluorescence-based enzyme-linked immunosorbent assay (ELISA) is regarded as the 'gold standard' for general screening of food samples for allergen detection,^{6,7} and it is presently used as the official screening method, several problems such as selectivity, accuracy and cross-reactivity lead to severe limitations in the applicability of this screening technique. Several approaches have been proposed to overcome these issues,⁸ such as complementary detection techniques,⁹ real-time polymerase chain reaction or proteomics-based ones. Recently, inductively coupled

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plasma mass spectrometry (ICP-MS) via element-tagged immunoassay has been proposed as a selective and sensitive detection technique for immunoassays.^{10,11} ICP-MS detection have several advantages if compared with conventional fluorescence, such as lower matrix effects caused by the other food components, low detection limits, excellent response linearity, long-term sample storage, and the capability of a multi-analyte detection using different elemental tags. However, few applications of the metal-tagged ICP-MS immunoassay have been reported,^{10–16} and the potential of the technique has not been fully assessed up to now.

To overcome some limitations of immunoassay techniques in terms of false positive or false negative results, confirmatory methods are needed. In this case the selectivity of mass spectrometry could be of great benefit in identifying peanut proteins even in complex matrices and could be used for confirmation purposes. Very recently, the advantages of liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) methods for the detection of peanut allergens in various food matrices have been demonstrated.^{17–19} A proteomics-based approach based on the direct identification of Ara h1 was used as a new strategy to identify peanuts in food matrices.^{17,18} To improve the selectivity and thus the confirmation criteria in the allergen identification more than one target could be selected.¹⁹ For this reason our research group developed a LC/ESI-MS/MS method with a triple quadrupole analyzer for the identification and determination of two of the major peanut allergens in food: Ara h2 and Ara h3/4.²⁰

In this paper we discuss a comparison of the two methods recently developed by our group, based respectively on Eu-tagged ICP-MS immunoassay¹¹ and on LC/ESI-MS/MS identification, and determination of Ara h2 and Ara h3/4²⁰ applied to a complex food matrix like a chocolate rice crispy-based snack.

EXPERIMENTAL

Materials

Phosphate-buffered saline (1x PBS; 150 mM NaCl, 20 mM sodium phosphate, pH 7.4), 0.01% Tween 20 in 1x PBS, 7 M nitric acid (Carlo Erba, Milan), Eu-labeled anti-mouse affinity purified rabbit polyclonal Abs (DELFI[®], Perkin Elmer Life Sciences, MA, USA) were used. The holmium standard solution (1000 mg L⁻¹) used for ICP-MS was purchased from High-Purity Standards (Charleston, SC, USA). Ara h2 was purified from toasted peanuts following a procedure reported in the literature.²¹ Ara h1 and Ara h3/4 purified peanut proteins and anti-Ara h1 (Pn-t) and anti-Arah3/4 (Pn-b) monoclonal antibodies raised against proteins purified from toasted peanuts were kindly provided by the Leibniz Center for Medicine and Biosciences at the Research Center Borstel (Borstel, Germany). All antibody solutions were diluted with deionized water (Millipore, Bedford, MA, USA).

Acetonitrile (HPLC purity), trifluoroacetic acid and formic acid (analytical reagent grade) were purchased from Carlo Erba (Milan, Italy). Trypsin from bovine pancreas was from Sigma-Aldrich (St. Louis, Missouri, USA).

Toasted peanuts and food products (chocolate/rice crispy-based snacks) used in this work were obtained at a local food store.

Apparatus and procedures

ELISA ICP-MS method

The assay format was based on indirect ELISA using well-plates coated with polyclonal immunopurified antibodies targeting total soluble peanut proteins. The immunoassay and flow-injection analysis (FIA)-ICP-MS procedures are described in detail in a previous paper.¹¹

Briefly, the sample solutions were incubated for 15 min at room temperature in to well-plates coated with polyclonal immunopurified antibodies targeting total soluble peanut proteins. After washing, two incubation steps were performed: the first with a mixture of the mouse anti-Ara h3/4 and anti-Ara h1 monoclonal antibodies (Ab I) and the second with the Eu-labeled anti-mouse polyclonal antibodies (Ab II) for detection. After digestion with 7 M HNO₃ (100 μ L, 1 h, room temperature), 25- μ L samples were injected into the FIA-ICP-MS system.

Limits of detection and quantitation were calculated as three and ten times the standard deviation of blank solution on the slope of the calibration curve, respectively. Linearity was tested in the 2.5–500 μ g peanuts g⁻¹ matrix range. Accuracy was evaluated both in terms of matrix effect and recovery. In particular, a *t*-test was used to compare the slopes of calibration curves built up in the range 2.5–50 (n = 15) μ g peanuts g⁻¹ matrix and by using aqueous peanut protein solutions. The recovery experiments were carried out both on unspiked food matrix and on the same matrix fortified with peanuts at three different concentration levels (5, 20 and 50 μ g peanuts g⁻¹ matrix). Recovery was expressed as the ratio percentage of the determined total peanut concentration to the added peanut concentration. Precision expressed as percentage coefficient of variance (CV%) was calculated by carrying out the extraction procedure on three samples and performing three FIA-ICP-MS analyses for each sample.

Experimental measurements were made on an ICP-MS X Series^{II} (Thermo Electron Corporation, Waltham, MA, USA) equipped with the trigger card and operating under Xt interface standard conditions. The instrument optimization was performed daily in the high mass range to assure a response of at least 50 000 counts per second (cps)/ μ g L⁻¹ for indium and 80 000 cps/ μ g L⁻¹ for uranium in the high mass range. A GS50 chromatographic pump (Dionex Corporation, Sunnyvale, CA, USA) and an EV750-100 inert injection valve (Rheodyne, Rohnert Park, CA, USA) were used to perform fully automated FIA with a 2% (m/v) nitric acid aqueous solution as the mobile phase delivered at a flow rate of 0.5 mL min⁻¹. Both isotopes of ¹⁵¹Eu and ¹⁵³Eu together with the ¹⁶⁵Ho signal used as internal standard (10 μ g L⁻¹) were monitored in the experiment. For qualitative and quantitative purposes the signal of ¹⁵³Eu was considered.

The operating parameters of the ICP-MS instrument were as follows: RF power 1400 W; coolant gas flow 15.5 L min⁻¹; auxiliary gas flow 0.98 L min⁻¹; nebulizer gas flow 0.87 L min⁻¹; transient; nickel standard X_t cones; TRA data

acquisition mode: dwell time 100 ms; duration time 60 s, standard resolution.

Liquid chromatography/triple quadrupole tandem mass spectrometry

LC elution was carried out on a Poroshell C18 300 Å column (75 × 2.1 mm, 5 µm particles) (Agilent Technologies, Santa Clara, CA, USA) using a gradient solvent system [(A) aqueous formic acid 0.1% (v/v)/(B) 0.08% (v/v) formic acid in acetonitrile] as previously described.²⁰ The flow rate was 200 µL min⁻¹.

The mobile phase was delivered by a 2690 series Alliance chromatographic system (Waters, Milford, MA, USA) equipped with a 120-vial capacity sample tray. Injection volume was 30 µL.

A Quattro LC triple quadrupole instrument (Micromass, Manchester, UK) equipped with a pneumatically assisted ESI interface was used. The system was controlled by MassLynx software version 4.0.

The nebulizing gas (nitrogen, 99.999% purity) and the desolvation gas (nitrogen, 99.999% purity) were delivered at flow rates of 55 and 500 L h⁻¹ respectively.

Optimized conditions of the interface were as follows: ESI voltage 3.5 kV, cone voltage 35 V, RF lens 0.3 V, source temperature 130°C, desolvation temperature 250°C. MS/MS experiments were performed under selected reaction monitoring (SRM) conditions with a collision gas pressure of 2.3×10^{-3} mbar in the collision cell. The SRM transitions monitored were as follows: *m/z* 807/1050 collision energy (CE) 35 eV (CCNELNEFENNQR, Ara h2), *m/z* 950/1120 CE 35 eV (CMCEALQQIMENQSDR, Ara h2), *m/z* 649/1089 CE 30 eV (AHYQVVDSDNGDR, Ara h3/4), *m/z* 695/700 CE 35 eV (SPDIYNPQAGSLK, Ara h3/4). Leucine-enkephalin was used as internal standard (*m/z* 557/397; CE 20 eV).

Sample treatment

Peanut-containing food extracts were prepared by adding 25 mL of 1x PBS to 1 g of ground sample. Proteins were extracted by shaking for 15 min at 60°C, then centrifuged (14000 rpm, 5 min) and filtered on 0.2-µm nylon filter. The whole sample treatment procedure for the subsequent ELISA-ICP-MS method is described fully by Careri *et al.*¹¹

In the case of the LC/ESI-MS/MS method²⁰ peanut-containing food extracts were prepared by adding 10 mL of 0.2 M Tris HCl (pH 8.2) to 1 g of ground sample. Proteins were extracted by shaking for 2 h at 60°C; then the extract was centrifuged (14000 rpm, 5 min) and filtered on a 0.2-µm nylon filter. Sample concentration was performed on a 5 kDa molecular weight (MW) cut-off filter (Millipore, Billerica, MA, USA) by adding 5 mL protein extract and reducing the volume to 0.5 mL. The sample was then digested with trypsin (5 µL, 0.6 mM in Tris HCl, pH 8.2) for 24 h at 50°C before injection into the LC system.

RESULTS AND DISCUSSION

Performance of the ELISA-ICP-MS-based method

A non-competitive sandwich ELISA with ICP-MS detection was previously developed for screening purposes to detect

hidden peanut allergens in food.¹¹ The assay, which is based on the determination of the element used to tag the antibody, exhibited good performances in terms of detection limits, linearity and selectivity on cornflakes.

In addition, ICP-MS analysis conditions were carefully evaluated. Due to the small sample volume available and the nature of the samples, the FIA mode was chosen. The FIA mode offers several advantages in the sample analysis since it is easy to use, there is no sample loss and it is a high-throughput analysis. With this configuration, it was observed that the introduction of digest samples into the ICP source toward a LC mobile phase flow did not cause any significant clogging in the nebulization system and in the sample cone, usually seen in the normal analysis mode: this could more likely be due to the high nitric acid residual concentration. However, a significant memory effect on the europium signal coming from the six-port injection valve was observed, probably due to the sample sticking on the peek parts: it was necessary to inject 7 M nitric acid twice before introduction of the next sample to decrease the background noise to the level usually observed (700 cps). Such a configuration allows the analyses of a high number of samples to be performed making ICP-MS suitable for a rapid screening detection. The method performance was further investigated considering a more complex matrix based on chocolate, milk and rice.

The detection and the quantitation limits were 2.2 and 5 µg peanuts g⁻¹ matrix, respectively. In contrast to ELISA-UV reading, by injecting 25-µL aliquots into the ICP source, the signal reading for each sample is performed on three different aliquots of the each well-plate sample to account for the variability in the FIA-ICP-MS method. Assessment of the dynamic response range in the 5–500 µg peanuts g⁻¹ matrix range evidenced linearity between 5 and 40 µg g⁻¹, since a signal saturation starts to occur at the 50 µg g⁻¹ level (Fig. 1).

Trueness of the measurements was found to be affected by the presence of matrix effect on the antigen/antibody (Ag/Ab) binding and thus on the ICP-MS response of Eu-labeled Abs. By performing a *t*-test on the slope values of the matrix-matched calibration curve and the slope of the curve obtained by aqueous dilution of peanuts extracts, a significant difference at the 95% confidence level (*t*-calculated = 48, *t*-tabulated_{12, 0.05} = 1.78) was highlighted. Namely, regression equations evidenced that in the presence of matrix (*Y* = 3913 ± 140*X*) a ~20% signal suppression was observed with respect to that obtained analyzing aqueous peanut extracts (*Y* = 4874 ± 160*X*) to indicate a lower Ag/Ab binding in the presence of matrix interferences. The lowering in the Ab/Ag binding in such a complex extract compared to aqueous peanut solutions was also observed by analyzing the same matrix with a commercially available ELISA kit (Veratox quantitative peanut allergen test; Neogen Corporation, Lansing, MI, USA) based on colorimetric UV detection. This result strongly supports the critical role of the matrix on immunoassay performance and the lack of accuracy of quantitative analysis. For this reason the recovery values were calculated by using the standard addition method. Excellent recovery values were obtained on the three concentration levels tested (5 µg peanuts g⁻¹ matrix: 86 ± 18%; 20 µg peanuts g⁻¹ matrix: 110 ± 4%; 50 µg

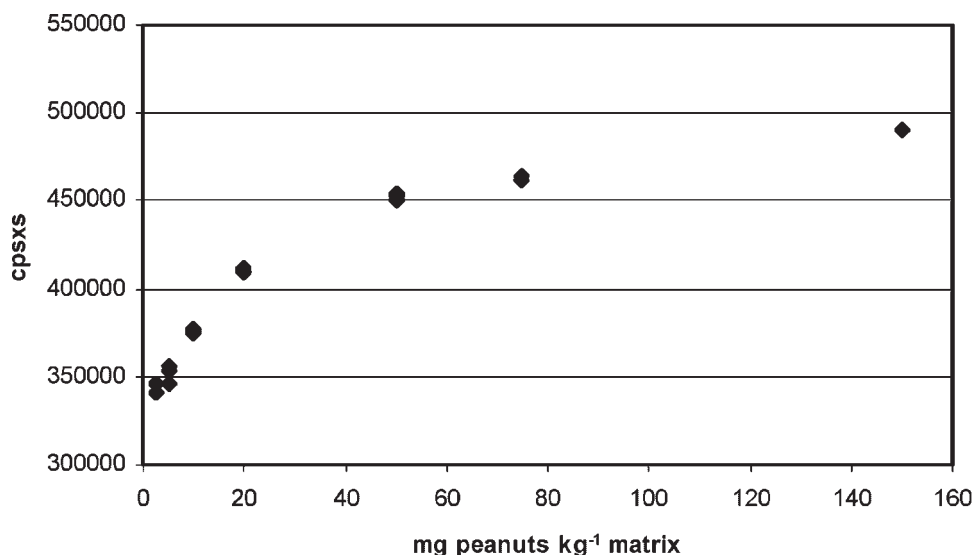


Figure 1. ICP-MS ELISA calibration curve obtained by using chocolate/rice crispy-based matrix fortified with different amounts of peanuts (2.5–160 μg peanuts g^{-1} matrix range).

peanuts g^{-1} matrix: $89 \pm 2\%$). From these results it should be noticed that there is a significant increase in recovery precision as the peanut concentration increases, suggesting a better repeatability of data at higher fortification levels.

As far as the cross-reactivity is concerned, the analysis of several food components (hazelnuts, Brazil nuts, almonds, walnuts, pecan nuts, macadamia, milk, soybeans, cacao powder) showed no significant effects and demonstrating that the monoclonal Abs used are very specific toward peanut proteins.

Performance of the LC/ESI-MS/MS method

The availability of triple quadrupole mass spectrometers has significantly increased in the last decade and the selectivity of operating acquisition modes can allow identification of peptides in complex matrices even without high-resolution data. The capability of the LC/MS/MS method as a confirmatory tool is strongly related to its sensitivity. As for the immunoassay, the sensitivity depends on the Ag/Ab binding affinity and thus on the quality of reagents used; in the case of the LC/ESI-MS/MS method this is strongly related to the ionization efficiency of selected peptides and to the presence of matrix suppression effects in the ESI source. In this work, a sudden degradation of performance in terms of MS/MS analyte response was observed after a few LC runs of the food sample extracts dependent on the source rapidly becoming dirty. In order to make detection and quantification limits less dependent on the effects of the matrix complexity, it clearly appears that a more selective sample enrichment treatment is required. However, by operating under SRM conditions, LOD and LOQ values for the peptide of Ara h3/4 at m/z 695 [SPDIYNPQAGSLK] were 1 and 3.7 μg protein g^{-1} matrix, respectively. Figure 2 shows the SRM chromatograms of peptide m/z 695/700 [SPDIYNPQAGSLK, Ara h3/4] in a chocolate/rice crispy-based matrix fortified with different amounts of peanuts. Detection and quantitation of the peptide biomarker of Ara h2 at m/z 807 [CCNELNEFENNQR] led to poor sensitivity; LOD and LOQ values being 5 and 14 μg protein g^{-1} , respectively. Accord-

ingly, better sensitivity was observed for the peptides selected for Ara h3/4 ($y = 10.48 \pm 0.23x$ and $y = 3.78 \pm 0.12x$; m/z 695 [SPDIYNPQAGSLK] and m/z 649 [AHYQV-VDSNGDR], respectively) with respect to those selected for Ara h2 ($y = 4.23 \pm 0.22x$ and $y = 2.68 \pm 0.18x$; m/z 807 and m/z 950 [CMCEALQQIMENQSDR], respectively). Excellent linearity was demonstrated for all the peptides selected by spiking the matrix extract with 1–20% peanut extract.

Good method precision for all the peptide biomarkers investigated was obtained, the relative standard deviation

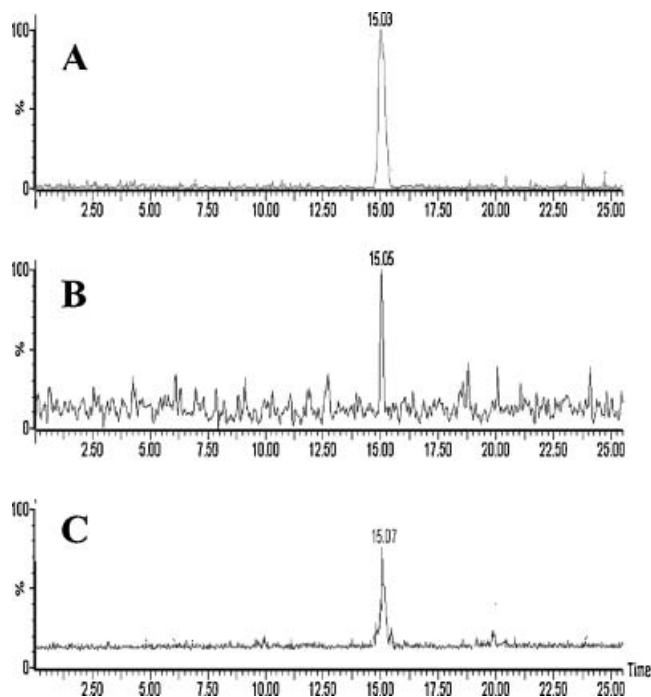


Figure 2. LC/ESI-QqQ-MS/MS SRM chromatograms of the peptide m/z 695/700 [SPDIYNPQAGSLK, Ara h3/4] in a chocolate/rice crispy-based matrix fortified with different amounts of peanuts: (A) 20 μg g^{-1} ; (B) 4 μg g^{-1} ; and (C) 1 μg g^{-1} .

(RSD) being lower than 10% ($n=10$) when measurements were performed in the model food extract fortified with 1.25 and 5% w/w peanuts/matrix. The signals for the Ara h2 peptides were found to be strongly affected by ion suppression (from 30–50%), whereas this drawback was more negligible in the case of the detection of Ara h3/4 peptides (lower than 10%). The use of an internal standard allowed a reduction in the matrix effect of between 5% and 30% for the measurement of Ara h3/4 and Ara h2 peptides, respectively.

The method was proved highly specific in the matrix investigated as a consequence of the specificity of the peptide biomarkers selected. Performing a search in protein databases, the sequences of the selected peptides were found to be unique for peanuts.

Comparison of the ELISA-ICP-MS and LC/ESI-MS/MS methods and conclusions

In this work mass spectrometry is presented as a detection technique for either screening and confirmation purposes. Findings highlight that this technique has versatility in different approaches for the detection and determination of hidden peanut allergens.

Even though the methods used have two different purposes, they share some major limitations. In fact, their application to a complex matrix pointed out that in both cases trueness of the quantitative analysis is strongly influenced by the food under investigation and the complexity of the matrix still remains the main determining factor to improve detectability. In particular, as discussed before in the case of the LC/MS/MS method, the whole food matrix extract is injected into the LC system causing a significant signal suppression effect on the analyte signals at the ESI interface with respect to the signal obtained by analyzing standard solutions. In the case of the ELISA the matrix is able to influence the Ag/Ab binding by reducing the amount of bound protein. It should be remarked that the reasons for the lack of accuracy demonstrated for the two methods have different origin. In the case of the LC/MS/MS method the main limitation is related to the working principle of the ESI source; by contrast, in the ICP-MS-based method, no significant instrumental drawbacks were observed apart from the memory effect on the injection valve, and the bottle-necks were found to be related to the immunoassay. As for the LOD values, very good detection was obtained with ICP-MS detection even by using an Ab not optimized for this kind of detection, whereas, in the case of the LC/MS/MS method, poorer sensitivity was observed. These findings suggest that

actually the immunoassay still represents the most powerful technique for the evaluation at trace level of hidden allergens in foods. On the other hand the development of a sample treatment able to enrich Ara h2 and Ara h3/4 extract content could allow a significant improvement in the LOD values of the LC method and fully take advantage of the highly selective MRM analysis.

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