The Detection of Allergens in Bread and Pasta by Liquid Chromatography Tandem Mass Spectrometry

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Overview

A rapid, robust, sensitive and specific LC-MS/MS assay has been developed for the simultaneous detection of four major food allergens peanut, milk, wheat and egg. Peptides of allergens were detected at low parts-per-million (ppm) levels after simple homogenization, digestion with trypsin and SPE cleanup.

Introduction

The prevalence of food allergies in the United States is estimated at around 6% for children and 3.7% for adults[1], and reports suggest that the number of food allergies is rising.[2] Allergens themselves come from a variety of sources and are a complex mix of different chemicals but include proteins from buckwheat, egg, peanut, cereals containing gluten, tree nuts, crustaceans, fish, soybean, sesame, mustard and celery but can also be chemicals such as sulphites.[3] Allergic reactions can range from mild to severe and during the period 1999-2006, 48 fatal allergic reactions were recorded in the United Kingdom.[4] Currently, the only therapy available for food allergy is avoidance, and self-treatment with epinephrine[5] so there is a need amongst food producers and regulators for specific and sensitive methods to detect allergens at trace levels.

The Codex Alimentarius, the food standards commission for the United Nations Food and Agriculture Organization and the World Health Organization, recommends that eight potential allergens should always be declared on pre-packaged foods: peanuts, tree nuts, eggs, milk, cereals containing gluten, shellfish, fish, and sulphites.

Screening for allergens in food is traditionally performed using enzyme-linked immunosorbent assay (ELISA), which employ antibodies raised against proteins specific for the allergenic food.[3, 6] Qualitative and quantitative analyses regularly generate variable results, together with false positives and false negatives, constituting a severe limitation of this technique; additionally, each target allergen requires a separate ELISA test kit. Another approach is the use of real-time polymerase chain reaction (PCR). This has the drawback of being an indirect method where the presence of the allergen is not monitored only the presence of material from the organism, which can produce false negatives and positives. Therefore, a method that could unambiguously confirm the identification of multiple allergenic proteins simultaneously would be invaluable for allergen screening in food.[7-8]

Our original research into using liquid chromatography with tandem mass spectrometry (LC-MS/MS)[9] used an extraction method described by Careri et al.[10] This method was time consuming and when applied to the extraction of real samples lead to a coefficient of variation (CV) of >20% at low allergen levels. Here we present some new data using a modified and shorter sample preparation method incorporating solid phase extraction (SPE) to simplify the procedure which has been developed using information provided by a food testing laboratory.[11-12]
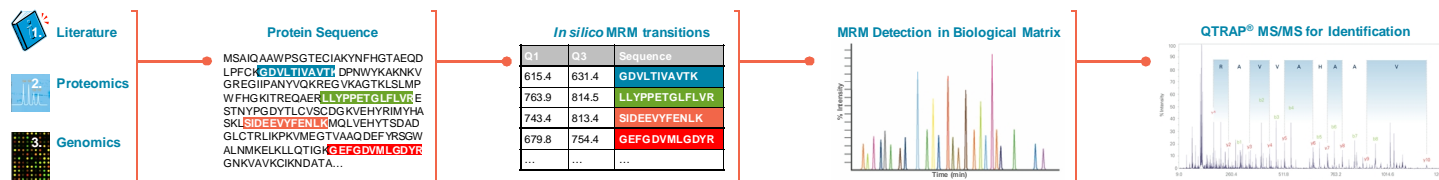
Figure 1. The MIDAS™ workflow (MRM-initiated detection and sequencing)

Experimental

Standards

For the initial development work some of the target allergens were commercially available and therefore purchased. Where allergens were not available the unprocessed food, e.g. peanuts, were purchased and the allergens extracted[9], these extracts were then used for method development.

Sample Preparation

The test sample, bread or pasta, was homogenized using a food processor and then the required amount of allergen protein was added to the sample to produce a spiked sample. Powdered spiked sample (5g) was mixed with the extraction buffer containing ammonium bicarbonate, urea and dithiothreitol. The mixture was broken up by shaking and agitated further using a roller mixer.

This mixture was centrifuged and 1 mL of the top liquid layer was mixed with iodoacetamide, incubated in the dark for 20 min, and digested by addition of a digestion buffer containing ammonium bicarbonate, acetonitrile and trypsin. After overnight incubation at 37°C the sample was acidified and filtered.

The filtrate was purified using a conventional conditioned polymeric SPE cartridge from Phenomenex. The peptides were extracted from the cartridge using acetonitrile and the extract was evaporated to dryness and reconstituted in acidified aqueous acetonitrile.

LC

Initial method development was carried out using an Eksigent Technologies Tempo™ LC system with 75mm x 150 mm C18 reversed phase HPLC column (LC Packings) at 300 nL/min using a gradient of water and acetonitrile where both solvents contained formic acid. This HPLC system was used to determine what MRM transitions were suitable for allergen detection.

Final extracted samples were separated over a 12 minute gradient from water to acetonitrile, by reversed-phase HPLC on a polar end capped column running at a flow of 300 µL/min, using a Shimadzu UFLC System. Both the water and acetonitrile mobile phases contained formic acid and trifluoroacetic acid.

MS/MS

All analyses were performed on an AB SCIEX 4000 QTRAP® LC/MS/MS system using electrospray ionization (ESI).

Initial method development was carried out using a NanoSpray® source at a flow rate of 300 nL/min. MRM Pilot™ software was used with the MIDAS™ workflow (MRM-initiated detection and sequencing).

Using the MIDAS™ workflow, a set of MRM transitions were predicted from the known protein sequence and then used as a survey scan to trigger the acquisition of full scan hybrid triple quadrupole linear ion trap (QTRAP®) MS/MS spectra (Figure 1). This data was then submitted to a database search engine for confirmation of peptide identification and confirmation of the feasibility of the MRM transition for allergen detection. With this workflow MRM transitions were designed without the need for synthetic peptides which was essential where commercial available allergen proteins were not available.

The final LC-MS/MS method to detect allergens in food samples was performed on an AB SCIEX 4000 QTRAP® system equipped with Turbo V™ source and ESI probe at a flow rate of 300 µL/min.

Results and Discussion

In the method development care was taken to make sure that peptides chosen were unique to the allergen. The list was further consolidated by removing peptides that could be susceptible to modification during food processing, e.g. undergo post translational modification or the Maillard reaction. This reduced the number of peptides used as triggers for detection and generation of peptide finger prints. For each allergen multiple triggers were used.

Figure 2 shows the total ion chromatogram for the MRM transitions used for the detection of peanut, milk, egg and wheat proteins. Here a total of 55 MRM transitions corresponding to 19 unique peptides for the allergens are shown.
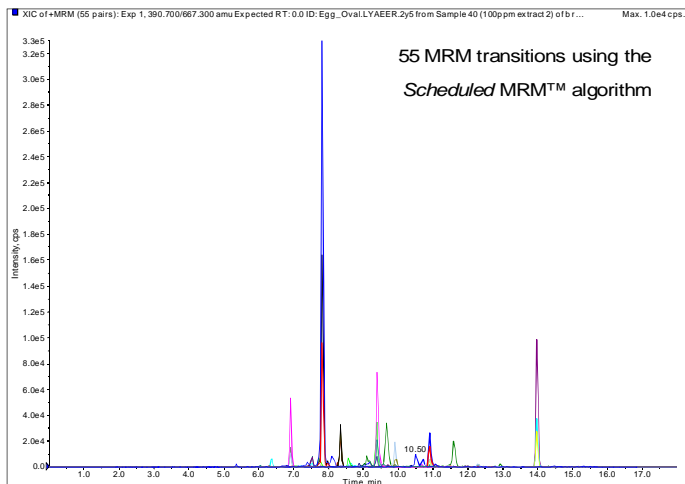

Figure 2. *Scheduled* MRM™ screen for peanut, milk, egg and wheat allergens in a bread sample spiked with 100 ppm milk and egg proteins

The *Scheduled* MRM™ algorithm was used in this method. Using this approach each MRM is monitored only across its expected retention time, decreasing the number of concurrent MRM transitions at any one time and maintaining both the cycle time and the dwell time.[6] This approach maximizes sensitivity but will also enable the easy addition of additional allergen markers as the method expands in the future.

This final list of MRM transitions was used as a survey scan to trigger the acquisition of QTRAP® MS/MS spectra. These spectra can be submitted to database search engines, providing confirmation of peptide identification.

Examples of this are shown in Figure 3a and 3b, here a pasta and a bread sample were spiked at 100 ppm with allergens of milk and egg, extracted and analyzed.

The extraction of both spiked pasta and bread yielded identical MS/MS spectra for the same peptides from egg and milk. This additional MS/MS information together with MRM ratio data gave multiple points of identification of allergen contamination in food and, as these peptides are unique, false positive allergen detection was dramatically reduced.
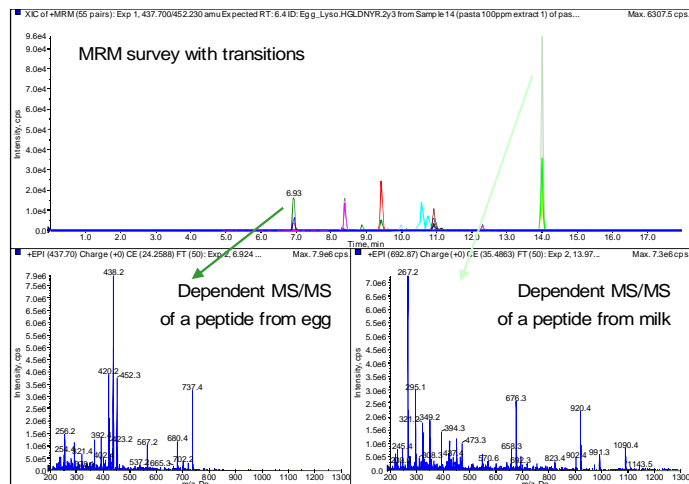

Figure 3a. MIDAS™ workflow for the detection of allergens in pasta. Analysis of an extract from pasta spiked at 100 ppm with egg and milk allergens. The top pane shows the total ion chromatogram for all MRM transitions; the bottom left pain shows the QTRAP® MS/MS spectrum which has been automatically generated by an egg peptide, and the bottom right pain is the spectrum generated by a milk peptide
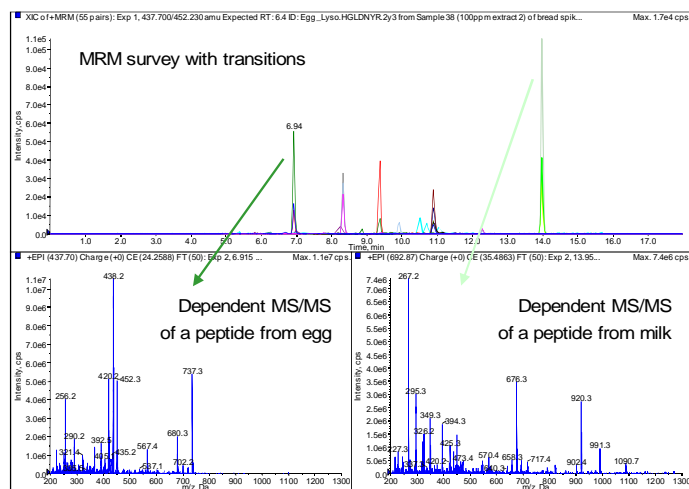

Figure 3b. MIDAS™ workflow for the detection of allergens in bread. Analysis of an extract from bread spiked at 100 ppm with egg and milk allergens. The top pane shows the total ion chromatogram for all MRM transitions; the bottom left pain shows the QTRAP® MS/MS spectrum which has been automatically generated by an egg peptide, and the bottom right pain is the spectrum generated by a milk peptide

Figure 4 shows a comparison of the tryptic peptide maps of 3 of the 4 investigated allergens.
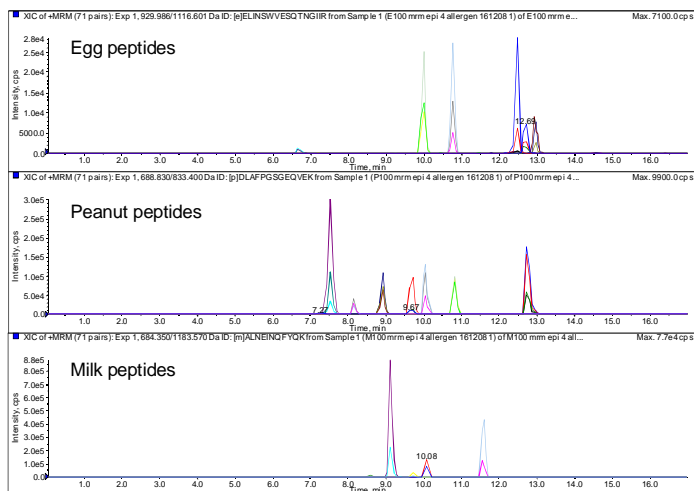

Figure 4. MRM transitions of a 100 ppm standard for egg (top), peanut (middle) and milk (bottom) allergens

This shows that each allergen protein produces a different peptide map with different intensities. The fact that some allergen peptides are of lower intensity will mean that detection limits will vary between different allergens. In Figure 4 egg peptides produce lower intensity signals compared to peanut and milk will therefore have a higher limit of detection.

To fully evaluate this approach bread samples were spiked at different concentrations with milk and egg proteins (highest and lowest sensitivity of the 4 allergens). Samples were spiked in duplicate and analyzed in triplicate to assess both linearity and robustness of the method. In this instance internal standards were not available so all results are without the positive effect of internal standardization. Results therefore show the reproducibility of the LC-MS/MS method as well as the extraction protocol.

Figures 5a and 5b show both egg and milk peptides give a linear response. In these tests milk peptides were detected at less than 2 ppm whereas egg peptides had a limit of detection between 5 and 10 ppm.
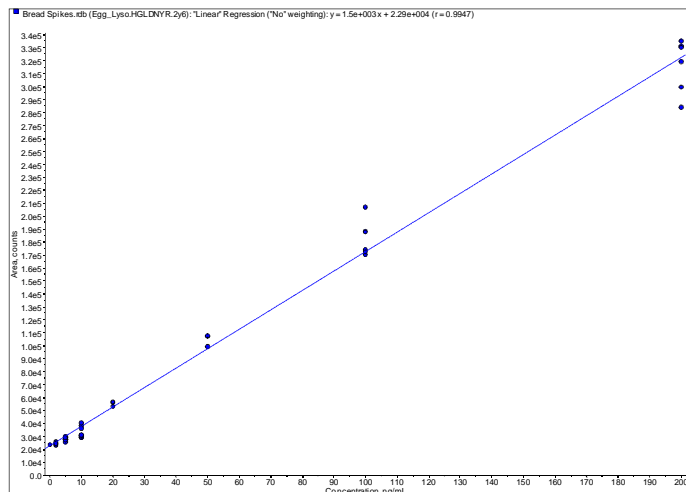

Figure 5a. Example of a calibration line obtained for an egg peptide
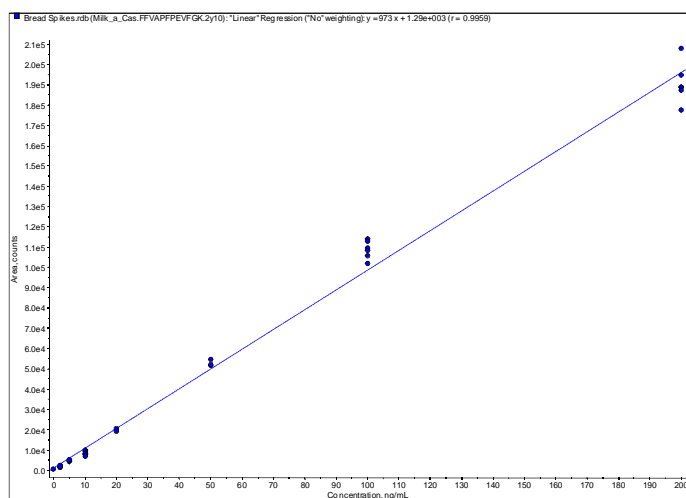

Figure 5b. Example of a calibration line obtained for a milk peptide

Milk peptide CVs were less than 5% at 100 ppm and less than 10% at 10ppm showing that the full procedure was reproducible (Table 1).

Table 1. Examples of reproducibility from the duplicate extraction and triplicate injection of a 10 and 100 ppm spike of milk proteins into bread

Extract	Injection	Calculated concentration (ppm)	
		Milk spiked at 10 ppm	Milk Spiked at 100 ppm
1	1	7.76	102.7
1	2	9.67	114.9
1	3	8.89	113.7
2	1	7.42	106.5
2	2	7.71	110.3
2	3	6.64	109.2
	Mean	8.02	109.5
	Std Deviation	1.09	4.58
	CV	9.3%	3.9%

Summary

A rapid, robust, sensitive and specific LC-MS/MS assay has been developed for the simultaneous detection of four major food allergens peanut, milk, wheat and egg. The initial sample preparation has been significantly simplified. The detection of allergens in processed foods was possible at low part per million levels.

Sensitivities achieved were equivalent to sensitivities of some currently available methods based on ELISA and real-time PCR, but the CV without any internal standards were better than have been previously reported by users[9] and were significantly better than those that can be obtained at low levels by ELISA. The LC-MS/MS approach has the additional advantage of being a multi allergen screen unlike ELISA where individual allergens are detected by separate kits. By using the MIDAS™ workflow full scan QTRAP® MS/MS spectra were obtained at the same time as quantitative information, confirming peptide identification and reducing the occurrence of false positives associated with other techniques.

ACKNOWLEDGEMENTS

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