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Microfluidic separation coupled to mass spectrometry for quantification of peanut allergens in a complex food matrix

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ABSTRACT Peanut is an important food allergen but cannot currently be reliably detected and quantified in processed foods at low levels. Three mg protein/Kg is increasingly being used as a reference dose above which precautionary allergen labeling (PAL) is applied to food products. Two exemplar matrices (chocolate dessert and chocolate bar) were prepared and incurred at 0, 3, 10 or 50 mg/Kg peanut protein using a commercially available lightly roasted peanut flour ingredient. After simple buffer extraction employing an acid labile detergent, multiple reaction monitoring (MRM) experiments were used to assess matrix effects on detection of a set of seven peptide targets derived from peanut allergens using either conventional or microfluidic chromatographic separation prior to mass spectrometry. Microfluidic separation provided greater sensitivity and increased ionisation efficiency at low levels. Individual monitored transitions were detected in consistent ratios across the dilution series performed, independent of matrix. The peanut protein content of each sample was then determined using ELISA and the optimised MRM method. Whilst other peptide targets were detected with three transitions at the 50 mg/Kg peanut protein level in both matrices, only Arah2(Q6PSU2)¹⁴⁷⁻¹⁵⁵ could quantify reliably, and only in the chocolate dessert at 10 mg/Kg peanut protein. Recoveries were consistent with ELISA analysis returning around 30-50% of the incurred dose. MS coupled with microfluidic separation shows great promise as a complementary analytical tool for allergen detection and quantification in complex foods using simple extraction methodology.

INTRODUCTION

Food allergens have posed the food industry with a new paradigm in chemical food safety, since small amounts of an allergenic food, innocuous to the vast majority of consumers, can pose a significant risk of eliciting a reaction in allergic consumers. There is currently no accepted cure, so individuals with food allergies have to practice food avoidance. To help them, regulations have been enacted across the world which require that priority allergenic foods are listed on ingredient labels, at whatever their level of inclusion in a recipe¹. However, the unintended presence of allergens can occur through the contamination of raw ingredients and the use of shared production lines and processing equipment. With as little as 3 mg of protein capable of triggering IgE-mediated reactions in 90% of peanut-allergic population²⁻⁵, manufacturers warn consumers of the potential hazard posed by the unintended presence of allergens by voluntarily applying precautionary allergen labels (PAL)⁶. Surveys of

foods with and without PAL have shown that levels of unintended allergens can vary and often bear no relation to the presence of PAL^{1,7}. One allergen management approach that has been developed to ensure PAL is only applied when required is the Voluntary Incidental Trace Allergen Labelling system (VITAL[®]). The VITAL approach uses reference doses of allergens for priority allergenic foods based on clinical threshold doses collected in food allergic subjects and serving sizes to identify when PAL should be applied⁸. When using risk assessment approaches, such as VITAL, it is important to know how much unintended allergen is present in a food. Currently immunological tests are the methods of choice for allergen detection and quantification in foods since they determine the protein hazard in foods. However, antibody recognition is often modified because of the effect of thermal processing or interactions with other components in the food⁹⁻¹². Mass spectrometric (MS) methods offer an alternative, complementary tool for detecting and quantifying allergens.

Several proteomic approaches have been published regarding the quantification of allergens in foods, such as peanut, using both labeled and un-labeled quantification¹³⁻¹⁵. Targeted methods using multiple reaction monitoring (MRM) experiments offer a means of absolute quantification using equivalent synthetic heavy-labeled peptides (AQUA) of target sequences. MRM methods have previously been reported with linearity over 4-5 orders of magnitude and assay sensitivities in the amole range¹⁶. They also have the advantage of being compatible with harsh extraction conditions, such as sonication and employing reducing agent in extraction buffers^{10,11,17,18}. However, few MS studies have validated methods for allergen detection using incurred matrices, and lack the sensitivity required at the 3-5 mg total allergen protein/Kg food, required to determine levels around those indicated by allergen management approaches, such as VITAL, that might trigger the use of PAL. To that end AOAC now require that MS methods for allergen determination must have an LOD $\geq$ 3 mg/Kg and a LOQ $\geq$ 10 mg/Kg of peanut¹⁹. One approach to improving sensitivity of MS methods is to employ nano-flow separation; the narrow diameter of the analytical channel from which the sample enters the ion source produces a smaller electrospray plume, improving sampling efficiency. The small size of microflow droplets also allows ions to desolvate more readily before reaching the MS inlet, improving ionisation efficiency and reducing any ion suppression effects associated with analysis of complex mixtures, thereby increasing analyte signal. Novel ceramic based (high-pressure monolithic substrate) micro-flow devices offer enhanced sensitivity compared to capillary columns. This report describes the application of such technology to the detection and quantification of peanut incurred into two different food matrices using a simple extraction method and MRM experiments with a range of peanut allergen peptide targets. It demonstrates promise of being able to detect peanut at levels of 10 mg/Kg peanut protein with equivalent sensitivity but greater technical reproducibility compared to an immunoassay test method.

MATERIALS AND METHODS

Reagents: All reagents and chemicals were of analytical grade unless otherwise stated. 2D Quant-Kit™ was obtained from GE Healthcare (Buckinghamshire, UK). HPLC grade acetonitrile and water, and tris(hydroxymethyl)aminomethane, were purchased from Fisher Scientific UK Ltd (Loughborough, UK). Iodoacetamide (IAA), dithiothreitol (DTT), and formic acid were purchased from Sigma (Poole, Dorset, UK). Mass spectrometry grade Trypsin Gold from Promega, WI, USA. RapiGest™ (Sodium 3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)methoxy]-1-propanesulfonate) was the gift of Dr Lee Gethings (Waters Corporation, Wilmslow, UK). Isotopologues of the target peptide sequences were synthesised with either ¹³C₍₆₎ ¹⁵N₍₄₎ C-term R or ¹³C ¹⁵N₍₂₎ C-term K by JPT Peptide Technologies GmbH (Berlin, Germany) and reconstituted in 10 μ L of 0.1% (v/v) formic acid, 2% (v/v) acetonitrile in HPLC-grade water. Peptides were produced with trifluoroacetic acid as a counter ion, target mass confirmed by LC-MS and purity confirmed as >95% by HPLC¹⁶. Raw peanuts were obtained from the Golden Peanut Company (GPC), LLC, Alpharetta, GA 30022, USA.

Preparation of complex food matrices: Lightly roasted mechanically defatted peanut flour manufactured by the

Golden Peanut Company (GPC), LLC, Alpharetta, GA 30022, USA was obtained from Byrd Mill (Byrd Mill Co., Ashland, VA 23005, USA). Peanut flour was incurred into chocolate dessert and chocolate bars to provide 100 mg/Kg peanut protein, and using a blank (0 mg/Kg peanut protein), three levels of allergen-incurred matrix (3, 10 and 50 mg/Kg peanut protein) were prepared. Details of ingredients and preparation of matrices are supplied in the supplementary material (Table S1-S3).

Sample extraction: Defatted raw peanut (prepared as described by Sayers *et al.*,¹⁶) and the lightly-roasted peanut flour ingredient used in preparation of incurred matrices (20 mg per sample) were extracted using 50 mM Tris-HCl containing 50 mM DTT and 0.04% (w/v) RapiGest™ at a 1:50 (v/v) sample:buffer ratio. Incurred matrices (50 mg per sample) were extracted in triplicate using the same buffer, and at a 1:20 (w/v) sample:buffer ratio. Extraction was performed at 60°C in a sonicating water bath for 15 min; samples were vortexed every 5 min to ensure the particulate remained suspended in the buffer. Extracts were clarified by centrifugation at 14,000 x g for 20 min and supernatant removed into fresh tubes.

Sample preparation for MS: The protein content of the extracts was quantified using a 2D Quant-Kit™ (GE Healthcare Life Sciences, Buckinghamshire, UK). Samples were diluted and prepared for MS as described previously¹⁶. Briefly, samples were reduced by addition of 50 mM DTT to a final concentration of 5 mM and alkylated by addition of 150 mM iodoacetamide to a final concentration of 15 mM. Trypsin digestion was performed by addition of 0.1 mg/mL trypsin at a ratio of 1:10 (w/w) protease:protein and incubating for 3 h at 37°C in a heat block. The trypsin addition was repeated and followed by an overnight incubation at 37°C. Formic acid was then added to a final concentration of ~0.1 % (v/v) to hydrolyse the acid-labile detergent and inactivate any remaining trypsin. Samples were heated to 37°C for 30 min, clarified by centrifugation at 14,000 x g for 10 min and the supernatant stored at -20°C.

SDS-PAGE analysis: Raw and roasted peanut extracts were analysed by 1D-PAGE using the Nu-PAGE gel system (Invitrogen, Paisley, UK). LDS sample buffer was added (1:4 sample to buffer ratio (v/v)) and samples heated for 5 min at 70°C. Samples (5 μ g protein/well) and molecular weight markers (Mark-12, Invitrogen, Paisley, UK), were loaded onto 4-12% Bis-Tris precast gradient gels and proteins separated at 200 V for 35 min. Gels were stained using Simply Blue Safe Stain (Invitrogen, Paisley, UK) and visualized using a Typhoon TRIO variable mode imager (GE Healthcare, Buckinghamshire, UK). Densitometry was performed using ImageQuant Software (GE Healthcare, Buckinghamshire, UK), and allergenic protein content determined after normalisation to 5 μ g protein of raw peanut extract loaded in another lane.

Data acquisition: A Skyline method was derived using the target peptide sequences and a background proteome comprising a curated database containing all available peanut sequences in UniProt (<http://www.uniprot.org/>) (protein sequences, n = 1131)²⁰. Fixed modifications were set as carbamidomethylation of cysteine and for custom synthesized AQUA peptides, isotopically labeled C-terminal lysine or arginine. For each target peptide three MRM transitions were selected corresponding to precursor ions with a 2⁺ charge

1 paired with the resulting y fragment ions and peptide
2 transitions exported for use with Xevo® TQ-S mass
3 spectrometer (Waters, Milford, USA) (**Table S4-S5**). A
4 peptide mix stock solution, containing 1 mM of each heavy-
5 labelled AQUA peptide, was used to prepare a dilution series
6 from 100 and 33.3 nM of each peptide in either buffer alone
7 (0.1% (v/v) formic acid in HPLC-grade water) or blank matrix
8 digest diluted in 0.1% (v/v) formic acid in HPLC-grade water.
9 Each sample was analysed using three replicate injections.
10 Peanut-incurred matrix samples were diluted in 0.1% (v/v)
11 formic acid in HPLC-grade water at a ratio matched to the
12 selected SID and spiked with peptide mix stock solution to
13 give a final concentration of 2.5 nM. A digest of the peanut
14 flour ingredient was spiked with 2.5 fmol/ μ L heavy peptide
15 mix solution.

16 Peptides were analysed using the Xevo® TQ-S (Waters,
17 Milford, USA) operated in positive ion MRM mode coupled
18 with either (i) an ACQUITY M-Class UPLC equipped with a
19 BEH C18, 130 Å, 1.7 μ m, 2.1 mm x 150 mm column or (ii) an
20 ACQUITY M-Class equipped with the iKey source and iKey
21 peptide BEH C18, 130 Å, 1.7 μ m, 150 μ m x 50 mm. The
22 column and iKey were heated to 35°C whilst samples were
23 maintained at 8°C prior to analysis by triplicate injection of
24 either 5 μ L (conventional chromatography) or 2 μ L (iKey).
25 Peptides were separated over a 15 min gradient at a flow rate
26 of either 100 μ L/min (conventional chromatography) or 1.2
27 μ L/min (iKey). Equilibration was with 98% HPLC-grade
28 water containing 1% (v/v) acetonitrile and 0.1% (v/v) formic
29 acid (buffer A) and 2% (v/v) of 98% acetonitrile containing
30 1% (v/v) HPLC-grade water and 0.1% (v/v) formic acid
31 (buffer B). The gradient was then ramped from 2 - 45% buffer
32 B over 10 min and then to 80% (v/v) over a further 2 min to
33 rinse column before re-equilibrating at 2% buffer B.

34 **Data analysis - Stable isotopic dilutions (SID):** After
35 importing into Skyline, raw data files were examined to assess
36 data quality and if required peak selection was adjusted based
37 on expected chromatographic retention time. Retention time-
38 matched peak areas corresponding to the total ion intensity
39 were monitored for three transitions (unless otherwise stated).
40 Data were exported and analysed as previously described¹⁶.
41 Briefly, log-transformed data was used to construct calibration
42 plots and a linear regression using least-square fitting
43 performed (GraphPad Prism). Assay sensitivities were
44 calculated for each peptide in each matrix using the calibration
45 plot method²¹.

46 **Data analysis:** Target peptide peaks were confirmed by
47 retention time and heavy peptide fragment ions. Peak area
48 ratios (PARs) of the heavy-labeled peptide standard to the
49 light peptide in the sample were calculated based on the total
50 ion intensity (three MRM transitions) for each target peptide
51 in samples (peanut flour or incurred matrices). Using the
52 PARs, and the femtomolar amount of the heavy peptide on
53 column, and taking account of sample dilutions during
54 preparation, the number of moles of peptide present in each
55 extract was calculated per mg of sample. Assuming molar
56 equivalence between peptides and parent protein, these values
57 were converted to amount of protein per mg of sample using
58 the molecular weight of the dominant isoform for each
59 protein²². Mature protein sequences were calculated after
60 removal of the signal peptide and any residues prior to the
known N-terminal sequence²³. Each of the peanut samples (n
= 6; 20 mg) were extracted at a ratio of 1:50 (w/v) in buffer.

Using a Kjeldahl conversion factor of 54.9%, and taking into
consideration the expected extraction efficiency¹⁶ and
subsequent dilution factors during sample preparation, the data
were used to calculate conversion factors to convert from
nmoles peptide target to total peanut protein.

Statistical Analyses: Dilution series were analysed in
triplicate technical replicates. The peanut standard was
analysed with six extracts, each with three technical replicates.
Sample extracts were prepared in triplicate and each analysed
in duplicate; resulting mean and standard deviations (SD) were
calculated and used for graphical representations. All
statistical analyses, including 2-way ANOVA and pairwise t-
tests, were performed using GraphPad Prism version 6.05 for
Windows, GraphPad Software, San Diego California USA,
www.graphpad.com.

**Detection and quantification of peanut by enzyme-linked
immunosorbent assay (ELISA):** Each of the prepared blank
and peanut-incurred matrices were extracted and analysed in
triplicate by AgraQuant® Peanut ELISA (COKAL0148,
Romer, UK). The kit manufacturer instructions were followed
and calculation of peanut converted to peanut protein using
typical % of protein in raw (25%).

RESULT AND DISCUSSION

Chromatographic separation; UPLC compared with iKey:
A set of eight previously described peptide targets for the
major peanut allergens was applied to the detection of peanut
incurred into two food matrices, a chocolate dessert and
chocolate bar¹⁶. A simple, single-step extraction procedure
employing reducing agent and an acid labile detergent was
used which had previously been shown to be effective in
extracting protein from raw and thermally-processed peanuts¹⁶.
Validation experiments were undertaken using serial isotopic
dilution series prepared in either buffer alone, or reduced,
alkylated and digested extracts of blank matrices diluted 1:10
(v/v). Initial experiments were undertaken using a
conventional chromatographic separation step prior to MS
(**Figures S1, S2; Tables S6, S7**) and subsequently using a
microfluidic separation step (**Figures 1,2; Tables S8, S9**).
Monitoring three MRM transitions for each peptide, the
contribution of each to the total ion intensity across the
dilution series was assessed and calibration plots constructed
from the summed peak areas of all three transitions. Assay
sensitivities were determined when a minimum of four data
points were included in the linear regression analysis, using
the calibration plot²¹. Values above the highest concentration
in the isotopic dilution series have not been reported.

Ara h 1: Using conventional chromatography (**Figure S1-S2**)
the ratio between monitored transitions for peptide
Arah1(P43237)³²⁹⁻³⁴² remained consistent when applying ≥ 5
fmols on column. Below this level peptide behavior was
more varied and all three transitions were not reproducibly
detected. The resulting SIDs were only linear over a single
order of magnitude, corresponding to the highest
concentrations of the dilution series, so assay sensitivities
were not determined. Transitions associated with the second
Ara h 1 peptide, Arah1(P43237)⁵⁵⁵⁻⁵⁷⁷ were unevenly
distributed as a result of fragmentation being directed towards
formation of N-terminal proline, y9 ion¹⁵. Although reliant on
the y9 ion, transitions were stable at 5 fmols on column in
extracts of both matrices. Although Arah1(P43237)³²⁹⁻³⁴² gave

SIDs which were less sensitive than Arah1(P43237)⁵⁵⁵⁻⁵⁷⁷, Arah1(P43237)⁵⁵⁵⁻⁵⁷⁷ displayed greater sensitivity in the chocolate bar compared to chocolate dessert matrix (**Table S7**). Microfluidic separation extended the dynamic range of the assays in both matrices, giving LODs of 0.08-0.70 and LOQ values of 0.23-2.10 fmoles on column depending on the matrix, with the same pattern of matrix effects as observed using conventional chromatography (**Tables S8 - S9**). Transitions related to Arah1(P43237)³²⁹⁻³⁴² spiked into chocolate dessert extract were stable at 0.2 fmoles on column, compared to 5 fmoles when separated by UPLC (**Figure 1**). Arah1(P43237)⁵⁵⁵⁻⁵⁷⁷ transitions behaved similarly and were stable at between 0.02-0.2 fmoles on column in matrix extracts, and fragmentation was again directed towards formation of the y9 ion.

Ara h 3: Arah3(Q647H4)²⁵⁻⁴¹ performed poorly, with stable transitions only being observed at either 5 fmoles (buffer) or 17 fmoles on column (matrix extracts) (**Figure S1-S2**); below these levels a transition was often lost. It was possible to perform linear regression analysis for the peptide in buffer, but values were outside the range of the assay. The other Ara h 3 peptide, Arah3(Q647H4)³⁷¹⁻³⁸³ was detected at 0.5 fmoles on column, but due to the varying contribution of the y10 transition throughout the analysis, consistent transition ratios were not observed. Due to the instability of Arah3(Q647H4)³⁷¹⁻³⁸³ using both conventional and microfluidic chromatography, data for this peptide target was not analysed further.

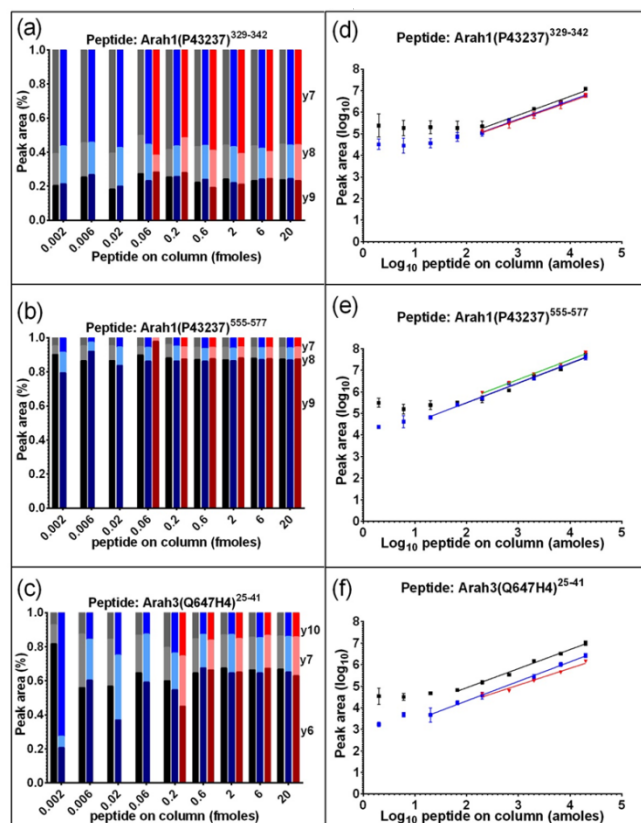


Figure 1. Effect of microfluidic separation prior to MS analysis on cupin peptide transitions and SIDs. Graphical representation of the contribution of each monitored transition to the total ion intensity as a function of peptide load on column when using microfluidic separation prior to MS analysis (a-c) and

corresponding matrix-matched calibration curves using isotopically labeled cupin peptide targets (d-f). Peptide targets were as follows: (a,d) - Arah1(P43237)³²⁹⁻³⁴²; (b,e) Arah1(P43237)⁵⁵⁵⁻⁵⁷⁷; (c, f) Arah3(Q647H4)²⁵⁻⁴¹. Peptides were prepared in either buffer (black) or extracts of chocolate dessert (blue) or chocolate bar (red). Each of the monitored transitions is shaded and labelled. Calibrations were constructed from log-transformed data (**Table S8**). Each SID is shown prepared in either buffer (black) or extracts of chocolate dessert (blue) or chocolate bar (red). LOQ ranges are given in **Table S9**.

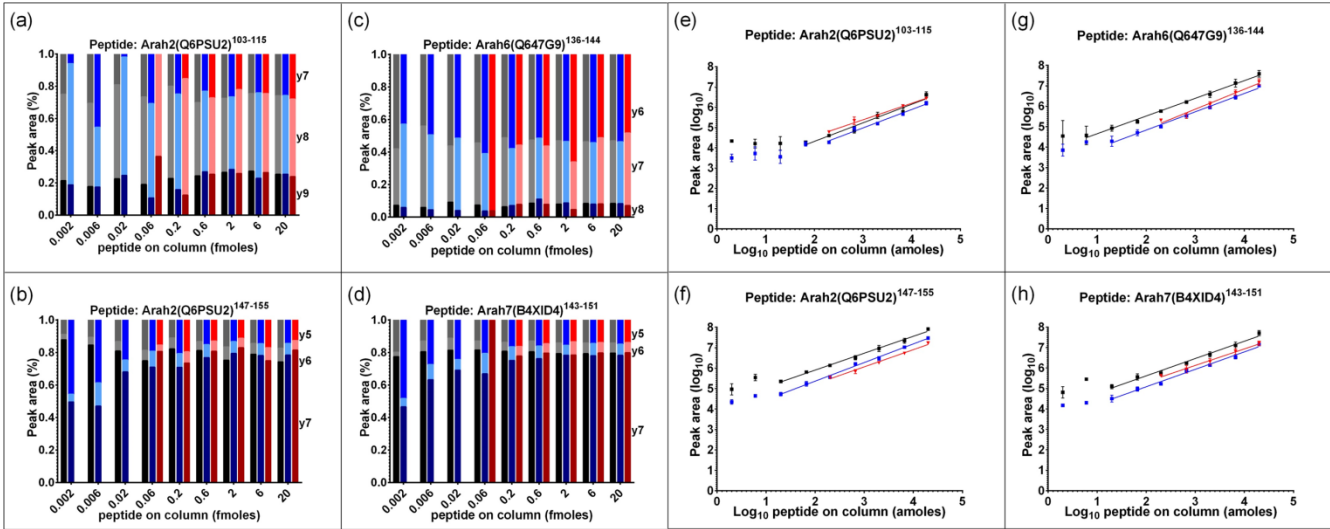
Arah3(Q647H4)²⁵⁻⁴¹ showed a significantly improved performance when analysed using microfluidic separation (**Figure 1**). Stable transition ratios were observed for the peptide in chocolate dessert matrices down to 0.2 fmoles on column and LODs of 0.06 and LOQs of 0.18 fmoles on column were reported (**Table S9**). Significant matrix interference was observed in the chocolate bar extract, with transitions being stable only above 0.6 fmoles on column. This was reflected in the LOD and LOQ values which were >5 fmoles on column.

Ara h 2: When analysed using conventional chromatography detection of Arah2(Q6PSU2)¹⁰³⁻¹¹⁵ was very unstable and the ratio between transitions was not evenly distributed even at the highest spike level of 50 fmoles on column (**Figures S1-S2**). Consequently, linear regression analyses could not be performed and no LOD or LOQ value calculated for this peptide. Arah2(Q6PSU2)¹⁰³⁻¹¹⁵ performed more consistently when using microfluidic compared to UPLC separation and was stable above 0.6 fmoles on column (**Figure 2**). Using this platform, the peptide calibration was sufficiently linear to perform regression analysis, giving calculated LOD and LOQ values of 0.75 and 2.26 fmoles on column for the chocolate dessert, and 1.94 and 5.82 fmoles on column for the chocolate bar (**Table S9**). In contrast Arah2(Q6PSU2)¹⁴⁷⁻¹⁵⁵ displayed stable transitions in both matrices when spiked at greater than 1.7 fmoles on column using the conventional chromatographic separation prior to MS analysis (**Figure S1**). Although fragmentation appeared to be directed towards formation of the y7 ion, the ratios between transitions were consistent. Calibration plots were linear over at least two orders of magnitude. Assay sensitivities were calculated for both matrices giving LODs of 0.33 and 0.68, and LOQs of 0.98 and 2.04 fmoles on column in the chocolate dessert matrix and chocolate bar, respectively (**Table S7**). This peptide also performed well in all matrices using the microfluidic device. Transitions were stable at 0.02 fmoles in chocolate dessert and 0.06 fmoles in chocolate bar. Assay sensitivities (LOD/LOQ) were determined as 0.08 and 0.23 fmoles in chocolate dessert. Sensitivity was significantly reduced when spiked into the chocolate bar digest with LOD of 2.82 and LOQ 8.46 fmoles on column (**Table S9**).

Ara h 6/7: The ratio of transitions monitored for Arah6(Q647G9)¹³⁶⁻¹⁴⁴ was stable at 5 fmoles on column in buffer using the conventional chromatography (**Figures S1-S2**). In both chocolate containing matrices, ratios remained consistent at 1.7 fmoles on column. Linear regression analysis was performed on all SIDs and gave LODs of 0.61 and 0.68 and LOQs of 1.83 and 2.05 fmoles on column in the chocolate dessert and chocolate bar, respectively (**Table S7**). The final target, Arah7(B4X1D4)¹⁴³⁻¹⁵¹, has a similar amino acid composition to Ara h2(Q6PSU2)¹⁴⁷⁻¹⁵⁵ and performed almost equivalently. The y7 ion again dominated the response while

1 stability was observed at 5 fmoles on column. Good linearity
2 was again observed in all conditions giving calculated LODs
3 of 0.32 and 0.83 and LOQs of 0.95 and 2.48 fmoles on column
4 in the chocolate dessert and chocolate bar (Table S7). When
5 analysed using the microfluidic separation Arah6(Q647G9)¹³⁶⁻¹⁴⁴
6 yielded stable y6 and y7 ions but the contribution of the y8
7 ion was reduced in all matrices below 0.2 fmoles. This target
8 gave good sensitivity in all matrices with LODs and LOQs
9 ranging from 0.11-0.82 and 0.34-2.45 fmoles on column
10 (Table S9). Arah7(B4X1D4)¹⁴³⁻¹⁵¹ was stable in chocolate
11 dessert and bar at 0.2 fmoles on column. The lowest assay

sensitivities were determined for peptide spiked into chocolate
dessert which gave a LOD of 0.21 and a LOQ of 0.63 fmoles
on column (Table S9).
Initial application of the methods to incurred matrices showed
that when diluted 1:10 (v/v) matrix:buffer, the endogenous
light peptide was no longer detectable. The experiments were
therefore repeated using microfluidic separation with a
reduced dilution factor of 10:1 (v/v) matrix:buffer (Figure S3-
S4, Table S10-S11). Whilst this allowed an increased loading
of target peptide and matrix components on to the column,
sensitivity was lost due to components in the



29 **Figure 2.** Effect of microfluidic separation prior to MS analysis on 2S albumin peptide transitions and SIDs. Graphical representation of
30 the contribution of each monitored transition to the total ion intensity as a function of peptide load on column when using microfluidic
31 separation prior to MS analysis (a-d) and corresponding matrix-matched calibration curves using isotopically labeled 2S albumin peptide
32 targets (e-h). Peptide targets were as follows: (a, e) Arah2(Q6PSU2)¹⁰³⁻¹¹⁵; (b, f) Arah2(Q6PSU2)¹⁴⁷⁻¹⁵⁵; (c, g) Arah6(Q647G9)¹³⁶⁻¹⁴⁴; (d, f)
33 Arah7(B4X1D4)¹⁴³⁻¹⁵¹. Calibrations were constructed from log-transformed data (Table S8). Peptides were prepared in buffer (black) or
34 extracts of chocolate dessert (blue) or chocolate bar (red). Each of the monitored transitions is shaded and labeled.

36 matrix co-eluting and competing for ionisation with target
37 peptides (Figures S3, S4; Table S10). In an attempt to
38 maintain sensitivity whilst not limiting light peptide detection,
39 a compromised dilution factor of 1:5 (v/v) matrix:buffer
40 was chosen for analysis of the incurred samples. Further method
41 optimization included increasing the number of serial isotopic
42 dilution points within the expected range of the samples.
43 Transitions were analysed and calibration curves constructed
44 for the serial isotopic dilutions prepared in 1:5 (v/v) ratio with
45 buffer (Figures S5, S6; Tables S12, S13). Assay sensitivities
46 were determined for each peptide target using all three sample
47 dilutions and the microfluidic separation summarized in
48 Figure S7 and were as follows for each peptide target in
49 fmoles on column; Arah1(P43237)³²⁹⁻³⁴² [LOQ – 0.97-8.99];
50 Arah1(P43237)⁵⁵⁵⁻⁵⁷⁷ [LOQ- 0.23-10.07]; Arah3(Q647H4)²⁵⁻⁴¹
51 [LOQ - 0.18-550]; Ara h2(Q6PSU2)¹⁰³⁻¹¹⁵ [LOQ – 0.90-6.27];
52 Ara h2(Q6PSU2)¹⁴⁷⁻¹⁵⁵ [LOQ – 0.11-1766];
53 Arah6(Q647G9)¹³⁶⁻¹⁴⁴ [LOQ - 0.06-137]; Arah7(B4X1D4)¹⁴³⁻¹⁵¹ [LOQ -0.08-10.68].

54 **Analysis of peanut flour and incurred food matrices:**
55 Initially an extract of the lightly roasted peanut flour
56 ingredient used to prepare the incurred matrices was analysed
57 to quantify the major allergens (Figure 3). The performance of
58 light peptide transitions was assessed using the peak area
59 percentages and comparison of the ratio of light:heavy

peptides (peak area ratio, PAR). Overall, the contribution of
individual transitions to the total ion intensity was consistent
with ratios observed for heavy targets apart from
Arah1(P43237)⁵⁵⁵⁻⁵⁷⁷ (Figure S8). The ratio between heavy to
light y7 ions was lower than expected suggesting that this ion
was not generated equivalently from the heavy peptide and
therefore not a suitable reporter ion for quantification.
Fragmentation of the y8 and y9 ions from heavy and light
peptides was equivalent. As expected, Ara h 3 was the most
abundant allergen. Interestingly the molar abundance of Ara h
6 was greater than that of Ara h 2, and collectively the 2S
albumins were present at equivalent amounts to Ara h 3 at a
molar level (Figure 3a).

Assuming that the peptides are present in molar equivalents to
the parent protein from which they are derived, the amount of
peptide can be converted to the amount of allergen protein
using the mature subunit molecular weight (Figure 3c).
Conversion of molar concentrations to protein amount brought
the 2S albumin allergens in line with their expected
abundances based on mass because of their significantly lower
molecular weight than the cupin allergens. This also
emphasized that the Arah3(Q647H4)²⁵⁻⁴¹ peptide was over-
estimating the amount of protein since it returned a value
greater than the total protein content of the sample of peanut
flour extracted. The relative proportion of each allergen

quantified in the same extracts by MS and densitometry proportions of allergens identified by the best performing targets in MS analysis are consistent with those determined using densitometry.

Based on the profiling of the peanut flour, conversion factors were calculated for each individual peptide reporter to convert nmoles of peptide target determined in the extract into mg of peanut protein (**Figure S9 and Table S14**). This approach assumes the extraction and digestion efficiencies of the peanut

analysis of SDS-PAGE (**Figure 3e**) showed that the flour are mirrored in the food matrices. The factors were then applied to detecting and quantifying the peanut protein incurred into the different matrices using an approach based on that of Parker *et al* 2015. Although several targets could be detected in the 50 mg/Kg peanut protein samples with three transitions, the only target which was reliably quantified below 50 mg/Kg of peanut was Ara h2(Q6PSU2)¹⁴⁷⁻¹⁵⁵. It reported 43% of the

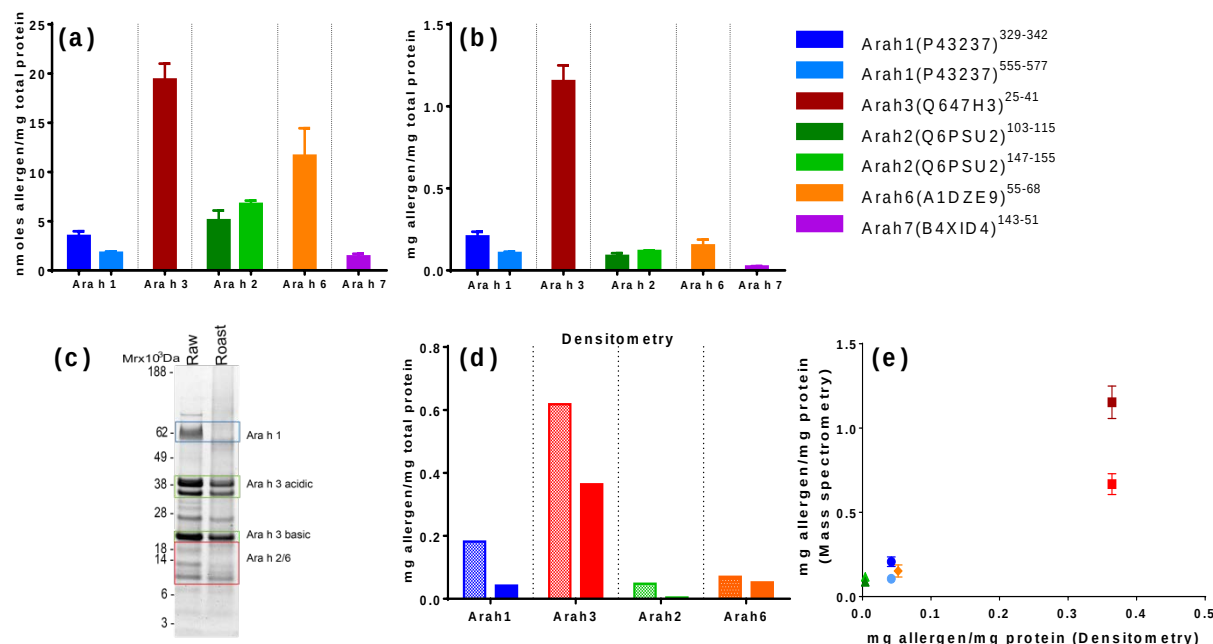


Figure 3. Quantification of allergen peptide targets in roasted peanut flour using the MRM-MS method. MS data are expressed as the mean of six replicate extracts analysed in triplicate, (+/-) SD in (a) nmoles of allergen per mg of peanut protein or (b) mg allergen per mg peanut protein. (c) and (d) Densitometry performed on 1D-PAGE analysis of raw (pattern fill) and roasted (solid fill) peanut flours extracted using 50 mM Tris-HCl pH 8.8 containing 50 mM DTT and 0.04% (w/v) RapiGest and (e) comparison of MS and densitometry data from (b) and (d) respectively.

peanut at the 10 mg/Kg level and 28% at the 50 mg/Kg level (**Figure 4**). Peak areas and transition ratios of the heavy peptide spike mix used in all samples remained consistent across the different matrices. Analysis of the incurred matrices by ELISA showed no detectable peanut in the 0 mg/Kg sample and peanut could only be determined at the 3 mg/Kg level in the chocolate dessert matrix (**Figure 4; Table S15**). Data from MS analyses provided technical replicate CVs of ~3% for Ara h2(Q6PSU2)¹⁴⁷⁻¹⁵⁵ in the dessert, and CVs ranging from 21-32% between replicate extracts (**Table S15**). For the equivalent ELISA, CVs ranged from 0.7-20% in the same matrix (**Table S15**).

CONCLUSIONS

Food is complex and its analysis is often difficult due to the presence of compounds, such as lipids, sugars, tannin and phenolic compounds as well as other proteins. These components can interfere in a targeted MRM assay by co-eluting with the peptide target and suppressing its ionisation. Initially the effect of such interfering substances co-extracted from the chocolate dessert and chocolate bar was assessed

using the SIDs for eight previously identified peanut allergen peptide targets¹⁶. Using a single-step extraction with no sample clean up resulted in matrix effects when conventional liquid chromatographic separation was used prior to MS. This was especially severe for the chocolate containing matrices, probably due to the presence of cocoa phenolics, which may become associated and form large complexes²⁴. However, when microfluidic separation was employed matrix interference was much reduced and showed increased target peptide stability over a wider dynamic range and with sensitivity reported in the amole range.

The lower than expected recovery of the allergen Ara h 1 from the flour by both MS analysis and densitometry of SDS-PAGE analysis probably relates to the poor solubility of the protein following roasting and it is therefore not efficiently extracted by the buffer used in this study¹⁶, although the extraction efficiency of the buffer has previously been shown to be around 70%¹⁶. The Ara h1(P43237)⁵⁵⁵⁻⁵⁷⁷ peptide derived from this protein also contains residues that can be modified either post-translationally, or as a result of thermal processing.

Any modification of this type, which alters the amino acid sequence of the peptide target, will result in it not being detected in targeted MS experiments. Thus, hydroxylation of proline has been observed in the natural peptide in the peanut flour sample, which may result in the target not being detected and could result in underestimation of the protein compared to untargeted methods²². Reduced abundance of Ara h 1 indicated by Arah1(P43237)⁵⁵⁵⁻⁵⁷⁷ may also be linked to Maillard modification of the N-terminal lysine^{11,16,25} in the parent protein. Peptide targets flanked by lysine residues have already been shown to be more susceptible to Maillard modifications¹⁶ and studies suggest these can also crosslink, leading to linked b ion formation^{26,27}. The presence of proline residues in a target sequence can also enhance specific backbone cleavage of peptides, directing fragmentation towards a specific y ion. For these reasons the Ara h 1 peptides used in this study may not act as effective reporters for the accurate quantification of peanut due to under-reporting. In contrast, analysis using the Ara h 3 peptide target Arah3(Q647H4)²⁵⁻⁴¹ resulted in over-reporting of the level of Ara h 3. Arah3(Q647H4)²⁵⁻⁴¹ is located within the N-terminal sequencing region of the acidic subunit which in combination with ragged processing could explain its presence in the seed at levels higher than the parent protein²³. Over reporting may also be linked to peptide stability and the preparation of standards. In contrast to the cupin allergen peptide reporters, those for the 2S albumin allergens, Ara h 2, 6 and 7 gave abundances of the intact protein more consistent with what was expected and may reflect the fact these proteins are less affected by thermal processing¹⁶.

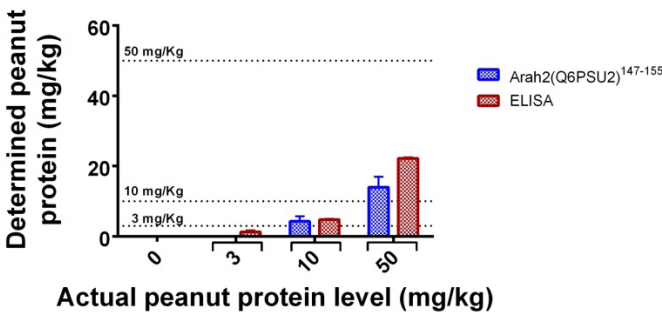


Figure 4. Quantification of peanut by mass spectrometry and ELISA. MS analysis (blue) used peptide target Ara h2(Q6PSU2)¹⁴⁷⁻¹⁵⁵ which functioned above LOD for chocolate dessert. ELISA analysis (red) for the quantification of peanut in chocolate dessert.

Subsequently, the peptide targets were applied to the analysis of peanut in the incurred matrices. Most peptides proved to be effective qualitative targets for confirming the presence of peanut; however only the 2S albumin reporter Arah2(Q6PSU2)¹⁴⁷⁻¹⁵⁵ was able to quantify at the low levels required for allergen analysis. Recoveries were of the order of 28-43%, similar to those achieved by the ELISA, although quantification was only possible down to the 10 mg/Kg level using the MS methods, whilst the ELISA was able to quantify at the 3 mg/Kg level. Whilst the chocolate matrix components were not interfering with the SIDs it is evident that they did affect the extraction and digestion steps that give rise to the peptides analysed. It may be that the cocoa adversely affects both the extraction and trypsin digestion steps in the sample

workflow for MS analysis. Proteomic workflows have been developed and optimized for human and animal proteins and may not be suitable for analysis of plant proteins in food matrices. For example, peanut target proteins also function as trypsin inhibitors and their activity is increased by roasting²⁸. Further studies will be required for a better way of standardizing proteomic workflow for food allergen analysis to resolve these issues and gain insight into such phenomena. It also suggests that all assays developed need to use labeled peptides for full validation of assays.

Previously detection limits have been reported using a similar MRM method for Arah2(Q6PSU2)¹⁴⁷⁻¹⁵⁵ analysing for peanut incurred at 100 ppm in muffin and 1000 ppm in cereal bar²⁹. Although there were several experimental differences between the two studies, peanut was also underestimated with recovery ~60% in cereal bar and ~70% in muffin. In another study using NIST peanut butter incurred into several matrices, including a cookie and chocolate matrix, with a Tris-urea extraction followed by solid-phase clean-up and conventional chromatographic separation and only two transitions, detection of peanut at the 2 mg/Kg peanut protein was reported using different peptide targets to the ones reported here³⁰. Molecular mass cut off filters are an alternative to solid-phase extraction and have been used previously to enrich target proteins in an extract and allow filter-aided digestion^{29,31}. This method has the advantage of being compatible with extracts containing detergents which support complete denaturation of proteins, and can therefore maximize both extraction and digestion efficiency. Removal of some of the interfering components present in the food matrix is also achieved by the filtration step. Such approaches allow a more robust detection of allergens irrespective of the nature of the incurred matrix and fully exploit the potential sensitivity of MS methods to both detect and quantify allergens, as indicated by the SIDs in this study³¹.

This work demonstrates the targeted MS analysis has the capacity to detect allergens in incurred, complex food matrices at levels of 10 – 50 mg/Kg peanut protein. It is evident that there is a need for multiple peptide targets for each allergen source when analysing complex food matrices as the components and processing effects can modulate the performance of reporter ions in different ways. It may be that different reporters are used for a given allergen, to give a flexible method of analysis required for deployment in the food arena where a diverse range of food matrices is encountered. The peanut flour which has been used in clinical studies² is stable over long periods of time and shows little batch to batch variation, properties which make it a potential standard²², the use of which would allow for conversion from allergen peptide to peanut protein.

ASSOCIATED CONTENT

SUPPORTING INFORMATION:

The following files are available free of charge at ACS website <http://pubs.acs.org>:

Supplementary Materials - Microfluidic separation coupled to mass spectrometry for quantification of peanut allergens in complex food matrices.

Table S1. Ingredient sources.

Table S2. Recipe used for production of blank and peanut-incurred dessert matrix.

Table S3. Recipe used for production of peanut-incurred chocolate bars.

Table S4. Waters Xevo® TQ-S conditions.

Table S5. MRM transitions and collision energies for heavy and light target peptides generated by Skyline.

Figure S1. Graphical representation of each monitored transition's contribution to the total ion intensity displayed as parts of a whole analyzed using conventional chromatographic separation prior to MS analysis.

Figure S2. Matrix-matched calibration curves for each isotopically labelled peptide target determined using conventional chromatographic separation prior to MS analysis.

Table S6. Linear regression parameters for SIDs derived using data obtained with conventional UPLC chromatographic separation prior to MS analysis.

Table S7. SID calibration parameters using data obtained with conventional UPLC chromatographic separation prior to MS analysis.

Table S8. Linear regression parameters for SIDs derived using data obtained with microfluidic separation prior to MS analysis using matrix extracts diluted 1:10 (v/v).

Table S9. SID calibration parameters using data obtained with microfluidic chromatographic separation prior to MS analysis using matrix extracts diluted 1:10 (v/v).

Figure S3. Graphical representation of each monitored transition's contribution to the total ion intensity displayed as parts of a whole analysed using microfluidic separation prior to MS analysis with extracts diluted 10:1 (v/v).

Figure S4. Matrix-matched calibration curves for each isotopically labelled peptide target determined using microfluidic separation prior to MS analysis using matrix extracts diluted 10:1 (v/v).

Table S10. Linear regression parameters for SIDs derived using data obtained with microfluidic chromatographic separation prior to MS analysis using matrix extracts diluted 10:1 (v/v).

Table S11. SID calibration parameters using data obtained with microfluidic chromatographic separation prior to MS analysis using matrix extracts diluted 10:1 (v/v).

Figure S5. Graphical representation of each monitored transition's contribution to the total ion intensity displayed as parts of a whole analyzed using microfluidic separation prior to MS analysis with extracts diluted 1:5 (v/v).

Figure S6. Matrix-matched calibration curves for each isotopically labelled peptide target determined using microfluidic separation prior to MS analysis with extracts diluted 1:5 (v/v).

Table S12. Linear regression parameters for SIDs derived using data obtained with conventional UPLC chromatographic separation prior to MS analysis using matrix extracts diluted 1:5 (v/v).

Table S13. SID calibration parameters using data obtained with microfluidic chromatographic separation prior to MS analysis using matrix extracts diluted 1:5 (v/v).

Figure S7. Comparison of assay limit of detection calculated from serial isotopic dilution series of peptide targets prepared in buffer and blank food matrix extracts.

Figure S8. Light transition performance expressed as peak area ratio (PAR) comparative to heavy observed in roasted peanut flour extracts for Ara h1(P43237)⁵⁵⁵⁻⁵⁷⁷ and Ara h2(Q6PSU2)¹⁴⁷⁻¹⁵⁵.

Figure S9. Conversion from amoles allergenic peptide to mg peanut protein for (a) calculation of conversion factor using peanut standard and (b) application of these factors in analysis of an unknown sample.

Table S14. Conversion factors calculated from MRM analysis of peanut allergens in peanut flour.

Table S15. Determination of peanut protein in incurred dessert matrix by (a) ELISA, and (b) MS using the peptide target Ara h2(Q6PSU2)¹⁴⁷⁻¹⁵⁵.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

Lee Gethings, Antonietta Wallace and James Langridge declare a financial relationship with Waters Corporation that could be perceived to influence or give the appearance of potentially influencing the work submitted as both as employed by the mass spectrometry vendor used in this paper. Adrian Rogers is employed by Romer Labs UK Ltd who sell immunoassay test kits for allergen analysis, including those used for the work described in this paper and could also be perceived to influence or give the appearance of potentially influencing the work submitted. Helen Brown is employed by Campden BRI (Chipping Campden) Ltd providing analytical services, including allergen analysis, to the food industry. The other authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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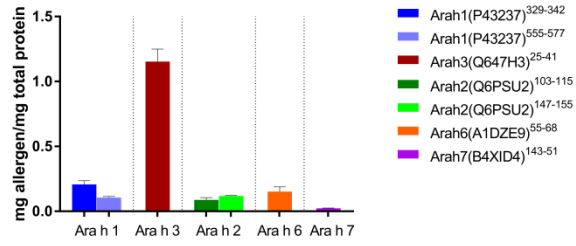
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Quantification of allergen peptide targets in roasted peanut flour



Quantification of peanut protein in chocolate dessert by ELISA and MS

