

FOOD COMPOSITION AND ADDITIVES

Towards Absolute Quantification of Allergenic Proteins in Food—Lysozyme in Wine as a Model System for Metrologically Traceable Mass Spectrometric Methods and Certified Reference Materials

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Current routine food allergen quantification methods, which are based on immunochemistry, offer high sensitivity but can suffer from issues of specificity and significant variability of results. MS approaches have been developed, but currently lack metrological traceability. A feasibility study on the application of metrologically traceable MS-based reference procedures was undertaken. A proof of concept involving proteolytic digestion and isotope dilution MS for quantification of protein allergens in a food matrix was undertaken using lysozyme in wine as a model system. A concentration of lysozyme in wine of 0.95 ± 0.03 µg/g was calculated based on the concentrations of two peptides, confirming that this type of analysis is viable at allergenically meaningful concentrations. The challenges associated with this promising method were explored; these included peptide stability, chemical modification, enzymatic digestion, and sample cleanup. The method is suitable for the production of allergen in food certified reference materials, which together with the achieved understanding of the effects of sample preparation and of the matrix on the final results, will assist in addressing the bias of the techniques routinely used and improve measurement confidence. Confirmation of the feasibility of MS methods for absolute quantification of an allergenic protein in a food matrix with results traceable to the International System of Units is a step towards meaningful comparison of results for allergen proteins among laboratories. This approach will also underpin risk assessment and risk management of allergens in the food industry, and regulatory compliance of the use of thresholds or action levels when adopted.

Food allergy is an increasingly prevalent global health problem, particularly in the industrialized world (1, 2), with well-documented detriment to the quality of life for allergic consumers and their families (3, 4). In susceptible individuals an immunological response (5) develops to specific food proteins, and thereafter, ingestion elicits inflammatory reactions in sensitized individuals, ranging from mild lip tingling to life-threatening anaphylaxis (6). Current cures for food allergies remain experimental (7–9); for most people with food allergies, lifelong avoidance of the eliciting food(s) is required (10, 11). In response, legislation has been introduced (12). In Europe, the inclusion in prepacked food of any of 14 major allergens, defined by Annex IIIa to Directive 2000/13/EC (as amended), triggers specific labeling requirements (13). Such labeling requirements will be extended in 2014 to nonprepacked food including catering establishments, by the European Union Food Information Regulation (14). Cross-contamination with allergens may trigger general principles of European and UK food law that make it an offense to sell food that is unsafe for, or not of the nature, substance, or quality demanded by, allergic consumers, particularly if specifically intended for their consumption.

There are significant unmet clinical needs and much that remains unknown about the causes of allergy generally (15). Food allergen risk assessment and management are suboptimal for consumers with widespread use of “may contain” statements seen as unhelpful (16). For food businesses, there are potentially serious financial impacts and reputational risks of increased food recalls (17, 18). Many consider that the introduction of thresholds or action levels by means of a probabilistic approach to a minimum eliciting dose for food allergen to be risk management that will deliver improvements for all stakeholders (19). However, there remains (20) a need for fit-for-purpose analytical methods to underpin risk assessments and risk management, including cross-contamination and clean down efficiencies, and to secure compliance and regulate the use of thresholds or action levels.

Food Allergen Analysis

Current analytical methods for allergens have been described elsewhere (21–24). Only ELISA and PCR assays have the flexibility, ease of use, and economy to be routinely applied

by food businesses and regulators to assist in allergen risk management. However, for both approaches, quantification can be problematic, and currently there are few published validated methods. ELISA exhibits variable and manufacturer specific sensitivities (25, 26) and cross-reactivity (27). Structural changes induced in the target molecules as a result of food processing or sample extraction may prevent detection. PCR assays are probative of the source species rather than the allergen protein. Hence, there is a requirement for methods that confirm molecular identity and are capable of validated quantification. MS-based methods, e.g., multiple reaction monitoring (MRM) of peptides arising from enzymatic digestion of proteins, offer such advantages, along with the possibility of multiplexed high-throughput analysis, and an increasing number of papers have been published (28–33). However, the complexity of most food matrixes represents a significant challenge in the development of MS methods for quantification of food allergens where matrix homogeneity, protein content, protein stability and solubility, and process-induced modifications of the proteins are generally not fully understood. To meet the requirements of all stakeholders, it is important that progress is made to further the viability of such techniques. Guidelines have been published dealing with criteria for the selection of target protein analytes, and peptides, MS transitions; optimization of protease digestion, quantification (e.g., through standard addition or isotopically labeled peptide standards), effective validation of methods, and harmonization of results through the use of naturally incurred reference materials spanning several types of food matrix (34). However, metrological traceability has so far been neglected.

Metrological Traceability

Metrological traceability of allergen proteins is currently possible only by MS-based absolute quantification, and is the property of an analytical result that allows measurements made under different conditions (e.g., at different times, by different people, in different locations, and/or using different procedures) to be compared in a meaningful way. It is achieved through a series of unbroken calibration chains for each parameter in the measurement process that influences the final result, and its associated measurement uncertainty, within an international infrastructure, the International System of Units (the SI; 35, 36). To our knowledge, SI traceable assignment of the concentrations of allergenic proteins in food matrixes has yet to be achieved, and would greatly facilitate the standardization of current analytical techniques for food allergens.

Isotope Dilution MS (IDMS)

Isotope dilution MS (IDMS) is a primary ratio method that relates directly to the SI with small measurement uncertainty, and is commonly used for the production of certified reference materials (CRMs) in the small molecule area (37). The principles of exact matching (EM)-IDMS have been recently applied to absolute quantification of proteins and peptides to obtain results traceable to the SI and to decrease the uncertainty of the measurements by precluding instrumental bias (38–44). IDMS is not easily applied to the analysis of intact proteins due to the lack of well-characterized protein reference materials and the significant amount of labeled analog that would be required. Furthermore, the structure of the labeled analog

protein and its impact on the quantification results must be fully understood (45). The approach commonly applied is based on proteolytic (commonly tryptic) digestion of the protein, and the use of isotopically labeled peptides as internal standards (ISs) and of synthetic unlabeled peptides as primary standards. Isotopically labeled and unlabeled peptides are more readily available, less expensive than isotopically labeled proteins, and better characterized. However, it must be demonstrated that they can be used as ISs in complex matrixes. Their stability and full recovery at the different steps of the process have to be verified. Furthermore, the enzymatic digestion has to be optimized to obtain complete release of the peptides of interest from the protein to be quantified. Confirmation of complete digestion can only be achieved by monitoring multiple peptides, necessitating the use of a minimum of two or preferably three isotopically labeled standards. Finally, the peptides chosen for quantification must be unique to the protein of interest. Ensuring that these criteria have been met is an important part of method development and validation of quantification of protein allergens in food matrixes and results in increased levels of complexity with respect to routine MS (29).

Egg Allergy

Egg allergy is one of the most common food allergies of childhood, affecting 0.5 to 2.5% of children. Although most patients with egg allergy are likely to develop tolerance by adolescence, a significant minority do not. The prevalence in the adult population has been estimated at 0.1%, and, of 31 reported cases of fatal food-induced anaphylaxis between 1990 and 2005, egg accounted for 7%. Five major allergens, Gal d 1–5, have been characterized in hen's egg. Egg white contains the immunodominant protein ovomucoid (Gal d 1: 11%), ovalbumin or conalbumin (Gal d 2: 55%), ovotransferrin (Gal d 3: 12%), lysozyme (Gal d 4: 3%), and ovomucin (4%; 46–48). Egg white powder is often used as a fining agent, particularly in red wines with high tannin content. Furthermore, lysozyme from hen's egg is used as an antimicrobial stabilizer in some wines and cheeses. Risk assessments have concluded that, while bentonite-treated wines contain negligible residual lysozyme, some 34–73% remains in nonbentonite-treated white wines and some 10% in nonbentonite-treated red wines (49). Thus, adverse allergic reactions to wine treated with lysozyme cannot be ruled out in susceptible individuals (50). This was confirmed by the European Food Safety Authority following a Commission request for an evaluation of an application for exemption of lysozyme from 2000/13/EC Annex IIIa labeling (51). The application for labeling exemption was unsuccessful (52).

Lysozyme

Lysozyme, a nonglycosylated protein of 129 amino acids, EC No. 3.2.1.17, has been determined in wine by ELISA (53–55), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting, LC-UV (56–58), and LC-fluorescence (59). The Office International de la Vigne et du Vin considers ELISA as generally suitable for quantification of potentially allergenic fining agent residues in wine, but rather than recommend specific approaches, prefers to adopt method performance criteria (60), including an LOD of 0.25 µg/g and LOQ of 0.5 µg/g (61). Despite efforts towards comprehensive

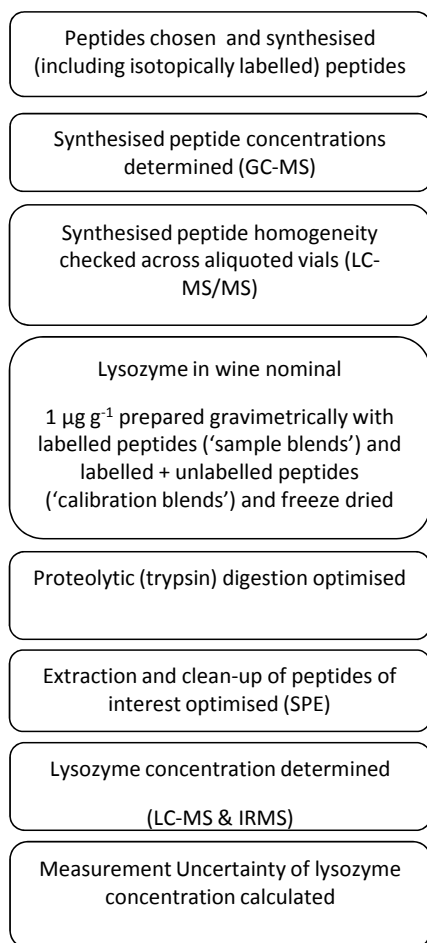


Figure 1. Flow diagram of experimental work.

validation of egg ELISA (62), disadvantages described above led to investigation of MS detection of fining residues in wine (63). Lysozyme has been determined in cheese by immunocapture MS (64) and by surface enhanced laser desorption-time of flight-MS (SELDI-TOF-MS; 65). However, to the best of our knowledge only one paper describes the detection of egg proteins including lysozyme in wine by MS (66), and no paper reports quantification of egg allergen proteins in wine by MS leading to results traceable to the SI.

Absolute protein quantification of lysozyme in wine is a challenging analysis. Wine composition is not fully characterized and differs from wine to wine. Herein is described a method for quantification of lysozyme spiked at 1 µg/g in white wine (Sauvignon Blanc) via exact matching IDMS by using isotopically labeled peptides as ISs. The conditions for the complete release of the peptides of interest from lysozyme in wine were optimized, the stability of the peptides during the digestion and sample cleanup were investigated, and the suitability of using labeled peptides as ISs for quantification of lysozyme in a food matrix were demonstrated. Quantification of 1 µg/g of lysozyme spiked in white wine was achieved with results traceable to the SI with an expanded uncertainty of 3% ($k = 2$, confidence level 95%). Figure 1 illustrates the experimental workflow developed for quantification of lysozyme in wine. The method developed can be applied for

quantification of lysozyme in wine at lower concentrations with the advantages associated to SI traceable quantification. However, optimization of the sample cleanup procedure is required for red and rose wine.

Experimental

Materials

Lysozyme from chicken egg white (powder, white, approximately 70 000 U/mg) and bovine serum albumin (BSA) were purchased from Fluka-Sigma-Aldrich (St. Louis, MO). The peptides used for IDMS quantification T5 (sequence HGLDNYR), T5* (sequence HG-[U-¹³C₆, ¹⁵N-Leu]-DNYR), T7 (sequence FESNFNTQATNR) and T7* (sequence FESN-[U-¹³C₉, ¹⁵N-Phe]-NT-QATNR), and T16 (sequence GTDVQAWIR) and T16* GTD-[U-¹³C₅, ¹⁵N]-QAWIR were custom synthesized (Biopolymers, ThermoFisher Scientific, Loughborough, UK). Peptides were delivered in 96 1 nmol aliquot solutions 5 ± 0.25 nmol/mL.

The National Institute of Standards and Technology SRM 2389a containing 17 stable amino acids at nearly equimolar concentrations was used as primary standard for amino acid quantification by IDMS. Labeled amino acids L-alanine lab (U-¹³C₃, ¹⁵N), L-glycine lab (U-¹³C₂, ¹⁵N), L-leucine lab (U-¹³C₆, ¹⁵N), L-phenylalanine lab (U-¹³C₉, ¹⁵N), and L-arginine: HCl lab (U-¹³C₆) were purchased from Cambridge Isotope Laboratories (Yewksbury, MA).

Additional materials were acetonitrile from LGC Standards (Teddington, UK), ultrapure water (18.0 MΩ cm⁻¹), formic acid from Fisher Scientific (Loughborough, UK), and sequencing grade trypsin from Roche (Burgess Hill, UK). Hydrochloric acid (37 wt. % in water). Tris buffer, tris-(2-carboxyethyl)phosphine (TCEP), S-methylmethanethiosulfate (MMTS), urea, dithiothreitol (DTT), iodoacetamide (IAA), triethylammonium bicarbonate (TEAB), and ammonium acetate were all purchased from Sigma-Aldrich. SDS 20% was purchased from MP Biomedicals (Cambridge, UK).

Hydrophilic interaction LC (HILIC) SPE columns were purchased from Hichrom (Reading, UK) SeQuant and the STRATA X polymeric SPE sorbent from Phenomenex (Macclesfield, UK). Low binding Durapore PVDF membrane 0.22 µm filters from EMD Millipore (Billerica, MA) were used for filtration of the eluates after sample cleanup. An Atlantis C18 150 × 2.1 mm id column from Waters Corp. (Milford, MA) equipped with an equivalent guard column was used for the separation of the peptides.

The wine used, a commercially available Sauvignon Blanc, was purchased from a local supermarket.

LC/MS

The analysis of the standard peptides and optimization of the tryptic digestion and sample cleanup were performed by injecting the appropriate samples on an Agilent Technologies (Santa Clara, CA) QToF 6530 coupled to the Agilent nano-HPLC Chip technology. ProtID-Chip-150 (II) from Agilent Technologies was used for these analyses. The separation column was 150 mm × 75 µm, and the enrichment column 40 nL. Both columns were packed with Zorbax material 300SB-C18 5 µm. Mobile phases were 0.1% formic acid in water (A) and 0.1%

formic acid in acetonitrile (B). The gradient used for separation of the peptides was 0–1 min 0% B, 1–5 min 0–5% B, 5–10 min 5–20% B, 10–15 min 20–40% B, 15–20 min 40–70% B, 20–21 min 70–90% B, and 21–23 min 90% B. The flow rate applied on the capillary pump was 4 $\mu\text{L}/\text{min}$, and that applied on the nano pump was 600 nL/min. A 1 μL amount of sample was injected. MS experiments were performed in the positive ion mode. Data were acquired between 250 and 2000 m/z units. The operating conditions were gas temperature 325°C, drying gas 4 L/min, fragmentor 210 V, skimmer 62 V, and capillary 2100 V.

EM-IDMS experiments were performed using a Waters Corp. Ultima triple-quadrupole instrument coupled with a Waters Corp. Alliance 2965 HPLC instrument. Separation of the peptides was carried out on an Atlantis dC18 5 μm column, 150 $\times$ 2.1 mm, with an Atlantis dC18 5 μm 10 $\times$ 2.1 mm guard column at 30°C. Both columns were from Waters Corp. Mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). A curved gradient (slope 8) was applied for the separation of the peptides: 0–2 min 0–10% B, 4–10 min 10–12% B, 10–12 min 13–15% B, 12–14 min 15–20% B, 14–16 min 20–30% B, and 16–17 min 30–40% B. The column was then washed at 98% B for 6 min. A flow rate of 200 $\mu\text{L}/\text{min}$ was applied. The $[\text{M}+2\text{H}]^{2+}$ ions for unlabeled and labeled peptides were monitored using selected-reaction monitoring (SRM): T5 437.9 > 737.3, T5* 441.4 > 744.1, T7 715.0 > 277.0, T7* 719.9 > 277.0, T16 523.5 > 256.1, and T16* 526.5 > 256.1. Collision energies of 20 V (T5), 24 V (T7), and 25 V (T16) were used, with a dwell time of 0.1 s for all transitions. The resolution of the first quadrupole was reduced to low setting 10 to assure selective transmission of all the isotopes of the multiply charged precursor peptides. The resolution of the third quadrupole was set as unit resolution.

Samples were injected five times each, bracketed with the respective calibration blend (CB), and the results were determined using the following EM-IDMS equation (37–45):

$$Cx = Cz \frac{m_z}{m_{yc}} \frac{m_y}{m_x} \frac{R'_B}{R'_{BC}}$$

where Cx is the concentration (nmol/g) of the protein to be quantified; Cz is the concentration of the pure standard material in the standard solution; m_z and m_{yc} are, respectively, the mass of the natural standard and the labeled standard used to prepare the calibration solution; m_y and m_x are the mass of labeled standard and sample in the sample blend (SB), respectively, and R'_B and R'_{BC} are the measured ratios of the natural-labeled peptide signals in the sample and in the calibration blend, respectively.

Ion suppression effects were monitored by injecting a sample of digested blank wine after sample cleanup. A solution containing approximately 690 pmol/g of the unlabeled peptides was then infused postcolumn at a flow rate of 7 $\mu\text{L}/\text{min}$. LC/MS/MS traces were recorded by applying the above method.

GC/MS

GC/MS was used for the analysis of the hydrolyzed peptides. The instrument used was a Finnigan Trace DSQ mass spectrometer (ThermoFisher Scientific, Waltham, MA) equipped with a Dean switching valve using electron ionization coupled to a GC Pal autosampler (CTC, Basingstoke, UK) and a Broad Trace Ultra GC system (ThermoFisher Scientific). A 1 μL splitless injection was performed.

Separation was carried out using helium carrier gas (continuous flow, 1 mL/min) and a splitless injector with injector temperature 280°C and splitless time 0.75 min. Amino acids were separated using a Zebron ZB-5HT INFERNO GC column (30 m $\times$ 0.25 mm, 0.25 μm film thickness) with a Z-guard column (length 2 m, 0.53 mm id, both from Phenomenex). The GC temperature program used throughout was 90°C for 2 min, 25°C/min to 330°C, and hold 3 min. The MS conditions were transfer line temperature 300°C and source temperature 250°C. Data were recorded in the selected ion monitoring mode, and the m/z values monitored for the unlabeled and labeled analogs were 116 and 119 for alanine, 102 and 104 for glycine, 158 and 164 for leucine, 218 and 221 for phenylalanine, and 256 and 261 for arginine.

Evaluation of the Standard Peptides

A solution of 5 nmol/g was delivered in 96 vials, each containing 1 nmol peptide. The peptides were first analyzed by using the Agilent QToF 6530 and the method previously described to check for peptidic impurities that may compromise the amino acid quantification via hydrolysis.

The concentration peptides was measured by acid hydrolysis and EM-IDMS. Seven vials of each standard unlabeled peptide were combined. An equimolar amount of labeled amino acid was added gravimetrically, and the samples were split into three aliquots to give three SBs. A CB solution containing the same amount of labeled amino acids and equimolar amount of NIST standard amino acid was prepared gravimetrically and split into two aliquots resulting in two calibration blends. All the samples were dried and hydrolyzed. One vial containing only the unlabeled amino acids, one vial containing only the labeled amino acids, and one vial containing a blank solution were also dried and hydrolyzed with the samples. Hydrolysis was performed in an Ethos EZ microwave digestion system (Milestone, Bergamo, Italy) equipped with a protein kit. A 3 μL amount of hydrochloric acid was added to each vial, and a temperature of 190°C was maintained in the vessel for 30 min. The temperature ramp was set to 10 min. After hydrolysis, samples were dried and solubilized in *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide containing 1% trimethyl chlorosilane derivatization reagent. Derivatization was performed at 75°C for 2 h. Samples were injected five times each, bracketed with the respective CB, and the results were calculated using the EM-IDMS equation (37–45).

Finally, the homogeneity of the peptides aliquoted in 96 vials was checked by comparing the MS measured ratio of unlabeled to labeled peptide of 10 aliquots of the unlabeled peptides. Two vials of labeled peptides were combined, and 20 μL of the solution was mixed with 20 μL unlabeled peptide from 10 aliquots (five/box). The preparation of those vials was performed gravimetrically. Samples were diluted to obtain a solution around 400 pmol/g. Each preparation was further diluted 1:10 with water and analyzed by LC/MS/MS. The LC/MS/MS analysis was performed by using the Quattro Ultima triple quadrupole instrument and the SRM method previously described. Samples were injected three times, randomized across the sequence. The ratios of the areas of unlabeled to labeled peptides were calculated for each peptide and corrected for the actual weights taken during the gravimetric preparation.

Sample Preparation

All samples were prepared gravimetrically with weighings carried out in triplicate. The wine spiked at 1 µg/g was freshly prepared before each quantification experiment.

(a) *Preparation of lysozyme standard stock solutions.*—All standards were prepared gravimetrically. A stock solution of lysozyme was prepared in water from the solid material at 1000 µg/g and stored in a freezer at −20°C. Serial dilutions were prepared in wine at 100 and 10 µg/g by first adding the blank wine to ensure no binding of lysozyme to the vessel walls occurred, and subsequently adding the standard containing lysozyme. Standards of 100 and 10 µg/g were stored in 500 µL aliquots at −20°C; 1 µg/g were prepared from those aliquots by diluting the 10 µg/g standard 1:10 in the manner described above. The prior addition of the wine proved to be very important to assure consistency of the results, since absorption of the lysozyme on the walls of the vials was observed in the absence of wine.

(b) *Preparation of SBs and CBs.*—SBs were prepared by adding equimolar amounts of the three labeled peptides to 1 mL of 1 µg/g lysozyme wine standard. CBs were prepared by spiking 1 mL of blank wine with unlabeled and labeled peptides. Mass fractions of labeled peptides were equivalent in both blends and added such that equal instrument response for the unlabeled and labeled signals was observed. Before tryptic digestion, CBs, and SBs were held at −80°C and freeze-dried using a vacuum centrifuge (AVC 2-22, Christ, Osterode am Harz, Germany). A 3 h freeze drying time was found to be optimal.

Tryptic Digestion

A 300 µL amount of Tris buffer (1 M, pH 8.5) was added to the samples to be digested. The pH had to be between 8 and 9 for consistent tryptic digestion. A 4 µL amount of 2% SDS and 2 µL 50 mM TCEP were added. Samples were left for 30 min at room temperature for denaturation and reduction. Following addition of 1 µL 200 mM MMTS, samples were left at room temperature for 15 min. Trypsin was solubilized in Tris buffer to obtain a concentration of 1 µg/µL, and three additions of trypsin at time point 0, after 30 min, and after 1 h were performed. Samples were incubated overnight at 37°C. The tryptic digested samples were freeze dried and solubilized in 100 µL water before analysis.

Sample Cleanup

An RP sample cleanup method (STRATA X RP column, 3 µm, 100 mg/6 mL from Phenomenex) at high pH was optimized to cleanup the peptides of interest from the wine matrix after tryptic digestion. Before SPE, the samples were diluted with 1 mL 50 mM ammonium acetate, pH 12, and left at room temperature to equilibrate. After 20 min the pH of the samples was checked to assure it was higher than 10.5. The pH of samples in wine was generally above 11. SPE columns were conditioned with 5 mL methanol and 5 mL 10 mM ammonium acetate, pH 10.5. Samples were loaded and a wash step was performed with 5 mL ammonium acetate buffer 10 mM–acetonitrile (95 + 5, v/v). The peptides of interest were eluted with 5.4 mL ammonium acetate 10 mM–acetonitrile (95 + 5, v/v). The eluate was centrifuged at 3000 rpm for 3 min with an MSE centrifuge, stored at −80°C

for 2 h, and freeze-dried overnight. Recovery of the standard peptides spiked in wine was also evaluated by using silica ZIC-HILIC cartridges (3 µm, 100 mg/6 mL) from Hichrom.

Calculation of Uncertainties

The standard and combined uncertainties were calculated in accordance with the EURACHEM/CITAC Guide CG 4 (67) and with the ISO Guide to the Expression of Uncertainty in Measurement (68).

Calculation of the Uncertainty of Lysozyme in Wine

The uncertainty (*u*) associated with the EM-IDMS values corresponding to the concentration of protein (*c_{x,p}*) in each sample from each standard peptide was calculated as previously reported (38, 69). The combined uncertainty of the concentration of lysozyme in wine was then calculated using the following equation:

$$uC_{lyso} = \sqrt{\sum_{i=1,n} \left(\frac{\sigma_{C_{lyso}}}{\sigma_{C_{x_i,p}}} u(C_{x_i,p}) \right)^2}$$

where *uC_{lyso}* is the SD of the average concentration of lysozyme calculated from the two peptides T5 and T7 in each SB (*n* = 6, three SBs obtained from two set of experiments, *C_{x_i}*).

Calculation of the Uncertainty of the Standard Peptides

The uncertainty (*u*) associated with the EM-IDMS values corresponding to the concentration of peptide (*C_{x_i}*) in each sample from the individual standard amino acids was calculated as previously reported by Mussell et al. (69) and Burkitt et al. (38).

The combined uncertainty was then calculated using the following equation:

$$uC_{pep} = \sqrt{\sum_{i=1,n} \left(\frac{\sigma_{C_{pep}}}{\sigma_{C_{x_i}}} u(C_{x_i}) \right)^2} + uh^2$$

where *uC_{pep}* is the average concentration of the peptide calculated from the individual amino acids in each sample blend (*n* = 3), and *uh* the uncertainty associated to the homogeneity of the aliquots of peptide and calculated as the SD of the mean of the amount of unlabeled peptide in 10 aliquots of the sample from a combined solution of labeled peptide (*n* = 10).

Results and Discussion

Selection of Peptides for Quantification

The selection of peptides to be used as ISs in protein quantification via IDMS is an important aspect of analysis. For accurate quantification to be achieved, peptides must meet the following criteria: the peptide must be efficiently released from the protein chain; it must be unique to the protein of interest; an isotopically labeled analog of the peptide must be readily available; and the peptide must be resistant to chemical modification throughout the method. If a peptide does not fulfil these criteria, the results obtained will be inaccurate.

The selection of the peptides for the quantification of

Table 1. Summary of hen's egg lysozyme peptides considered for quantification by IDMS

Position	Amino acid sequence (6–15 residues)	Observed LC/MS/MS	BLAST Search of Swissprot (Organism variants)	Quantification by amino acid analysis	Cysteine and methionine free	Missed cleaved likely
T3	CELAAMK	Yes	Unique ^a	L, A, K	No	Yes
T5	HGLDNYR	Yes	Unique ^a	G, L, R	Yes	Yes
T6	GYSLGNWCAAK	Yes	Unique ^a	G, L, V, A, K	No	No
T9	WWCNDGR	Yes	Unique ^a	G, R	No	No
T10	TPGSR	Yes	Not unique	G, R	Yes	No
T7	FESNFNTQATNR	Yes	Unique ^a	F, A, R	Yes	No
T16	GTDVQAWIR	Yes	Unique ^a	G, V, A, I, R	Yes	Yes

^a Unique avian lysozyme peptide.

lysozyme is detailed below. To ensure effective synthesis of unlabeled and isotopically labeled analogs, the peptide length had to be between 6 and 15 residues. If IDMS amino acid analysis is to be performed, the peptide should ideally contain at least three residues that are suitable for this type of analysis (44). This corresponds to the amino acids alanine, glycine, valine, leucine, isoleucine, proline, phenylalanine, lysine, and arginine. The lysozyme tryptic peptides that conformed to these criteria were then assessed for the uniqueness of their sequence against all other known protein sequences on the Swissprot database using a Basic Local Alignment Search Tool (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). All but one peptide was found to be unique to lysozyme amino acid sequences. Peptides with a sequence common to the fewest lysozyme variants were preferentially chosen. Lysozyme is found in a wide variety of organisms, including birds, mammals, and plants. Avian lysozyme sequences are homologous and only vary by between four and 20 amino acids. The selection criteria allow for more confident quantification of hen egg lysozyme, with possible organism variant contribution minimized.

Chemical modification to peptides during the digestion protocol can also be problematic. Modifications that occur preferentially to the free peptide, as opposed to the peptide when protected within the protein, can result in biased results and complicate maintaining method traceability (43). Methionine residues were avoided due to their propensity to oxidize. Peptides containing cysteine residues were not selected due to the high reactivity of cysteine residues. Many of the popular digestion protocols in use employ a cysteine blocking step, whereby the cysteine residues of a denatured protein are alkylated to prevent the random refolding of the higher order structure. Differences in the extent of derivitization between the peptide spike and denatured protein could cause inaccurate results.

An initial test digestion of lysozyme was undertaken to assess which peptides are released with good efficiency and have good MS response. The results of this initial screen of peptide properties are shown in Table 1. The three peptides chosen were T5, T7, and T16. However, the T16 peptide in the 5 nmol/g solution was found to be unstable after 1 month and was, therefore, not used for quantification, but only to check consistency of the enzymatic digestion

Evaluation of the Standard Peptides

The quality of the peptides employed as ISs is extremely important for the quantification of the proteins via IDMS. A number of QC checks were, therefore, performed on the peptides obtained from ThermoScientific.

The unlabeled and labeled peptides were analyzed by LC/MS using a QToF mass spectrometer coupled with a nano LC system to check for potential peptidic impurities that may interfere with the amino acid analysis. No major impurity was detected (less than 10%, corresponding to approximately 50 fmol/g).

The homogeneity of the peptides in the 96 aliquots was also evaluated. For the T5 peptide, an average MS measured area ratio of unlabeled to labeled of $0.905 \pm 2.6\%$ was obtained; for the T7 peptide, a ratio of $0.950 \pm 1.9\%$ was observed. The ratio precision obtained by injecting the same sample three times was below 1 and 1.5% for the T5 and T7 peptide, respectively. This suggests a low level of inhomogeneity across the vials, which was accounted for in the 5% uncertainty stated by the manufacturer. An average ratio of $0.973 \pm 19.3\%$ was calculated for the T16 peptide, with a calculated ratio precision below 2%. The high RSD obtained by injecting 10 aliquots of the T16 peptide was discussed with the manufacturer, and was due to instability of the peptide in the 5 nmol/g solution.

The unlabeled standard peptides were then quantified by amino acid analysis to confirm the values provided by the manufacturer. A modification of the method reported by Pritchard et al. (44) was applied. The calculated concentration of the T5 peptide was 5.04 ± 0.12 nmol/g ($k = 2$, 95% confidence) and of the T7 was 5.25 ± 0.20 nmol/g ($k = 2$, 95% confidence). The amino acids used for the quantification of the T5 peptide were glycine, leucine, and arginine, whereas those used for the quantification of the T7 peptide were phenylalanine (present twice in the peptide) and arginine.

LC/MS Optimization

A C18 Waters Corp. Atlantis 150 $\times$ 2.1 mm column with a guard column was chosen for LC separation of peptides prior to MS analysis, as this provided suitable separation and resolution of tryptic peptides for quantification. Optimization of the gradient was fundamental to improve sensitivity and reduce ion suppression effects. Ion suppression is one form of matrix effect from which LC/MS techniques suffer, regardless of the sensitivity or selectivity of the mass analyzer used.

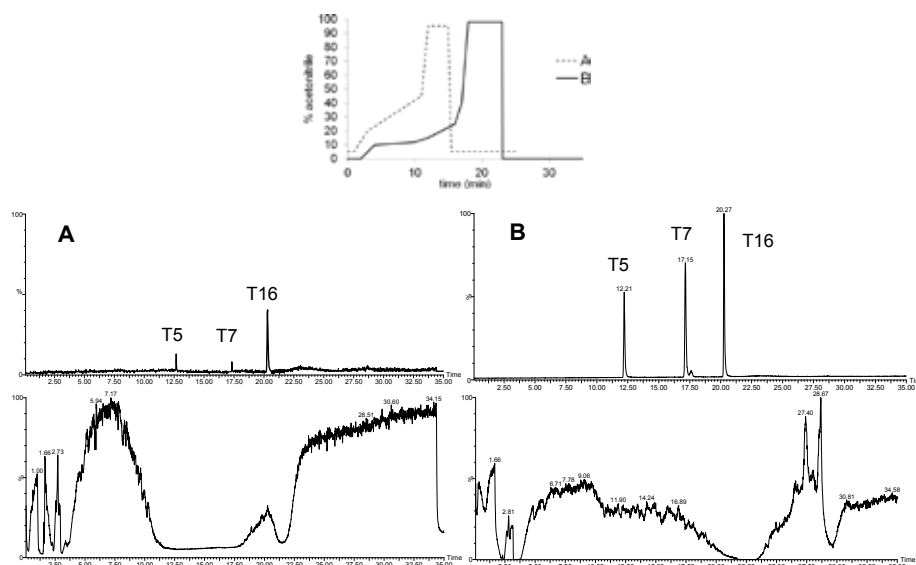


Figure 2. Ion suppression profile obtained by injecting a blank wine sample and infusing after column a solution containing the T5, T7, and T16 peptides. In A, a linear gradient was applied and in B, a curved gradient was applied. Details of the method are reported in the *Experimental* section. The three peptides eluted between 12 and 22 min.

Ion suppression negatively affects sensitivity, precision, and accuracy, and can generally be reduced by changing components of the method developed, such as the chromatographic separation or sample cleanup.

Figure 2 shows the ion suppression profiles obtained by running a linear gradient (A) and a curve gradient (B). The drop in the baseline indicates suppression in ionization of the analyte due to the presence of interfering material. It is possible to observe that the application of a curve gradient minimizes the matrix suppression effects between 12 and 22 min, where the peptides of interest elute.

SRM was used. The transitions monitored were based upon the observed product ion spectra of each peptide and the most intense fragment ions. To improve ratio reproducibility, the most abundant combined isotopologs were selected. The resolution of the first quadrupole was reduced to assure selective transmission of all the isotopes of the multiply charged precursor. Deamidation of the T7 peptide was detected; however, the same rate of deamidation was observed for the labeled and unlabeled peptide (data not shown). In order to reduce the impact of deamidation on quantification, the b2 product ion corresponding to the sequence FE of the T7 peptide was selected for SRM final quantification experiments.

Collision energies were optimized for each peptide. Product ions were carefully selected to minimize any cross-contributions between unlabeled and labeled peptides, and to maximize sensitivity.

Sample Cleanup Optimization

While water (70–90%) and alcohol (10–20%) are the major components of wine, natural ingredients (e.g., acids, sugars, pigments, phenolics, vitamins, terpenes, and proteins) and additives (e.g., sulfite) that vary from wine to wine are present at low concentrations, and may interfere with the quantification of the lysozyme in wine. This makes it difficult to predict potential interferences during the LC/MS/MS analysis or nonspecific

interactions that may occur between the wine components and the lysozyme or the standard peptides.

Three sample cleanup strategies were investigated to isolate the peptides to be used for quantification from wine: RP at high pH and HILIC at low and neutral pH. The recovery of the unlabeled standard peptides was initially evaluated by spiking an equimolar amount of isotopically labeled peptides in the solution obtained from the sample cleanup. The recovery was then calculated as a percentage of the ratios of the areas of unlabeled to labeled peptides obtained by LC/MS/MS analysis. A stock solution of unlabeled peptides in wine was prepared by volumetrically spiking the appropriate amount of peptides into wine to obtain a final concentration of approximately 69 pmol/g, corresponding to approximately 1 µg/g of lysozyme in wine.

Nine 1 mL aliquots were freeze dried overnight and resolubilized in 100 µL digestion buffer to mimic the post-digestion conditions. Samples were then diluted with 1000 µL of loading buffer [10 mM ammonium acetate, pH 9.7, for the RP column; 10 mM ammonium acetate, pH 3.0–acetonitrile (30 + 70, v/v) for the HILIC low pH; and 10 mM ammonium acetate, pH 7.0–acetonitrile (30 + 70, v/v) for the HILIC high pH]. The aliquots were loaded on nine sample cleanup cartridges, three for each sample cleanup method. In order to identify the optimal washing and elution conditions, samples were loaded and elution steps were performed by increasing the organic or aqueous content. Aliquots were collected during the loading step and after each elution step. The elution steps for RP-SPE were performed by increasing the content of acetonitrile (10, 20, 40, 60, 80, and 100%). The elution steps for the HILIC were carried out by increasing the content of ammonium acetate buffer, pH 3.0 or 7.0. Samples were then analyzed. It was noticed that a relatively large amount of T5 and T7 peptides (higher than 10%) was lost during the loading step on the HILIC cartridges. The total recoveries of the peptides were calculated as a sum of the recovery of the peptides for each elution step. Recovery of the T5 peptide was 40% from HILIC low pH cleanup and 31% from HILIC neutral

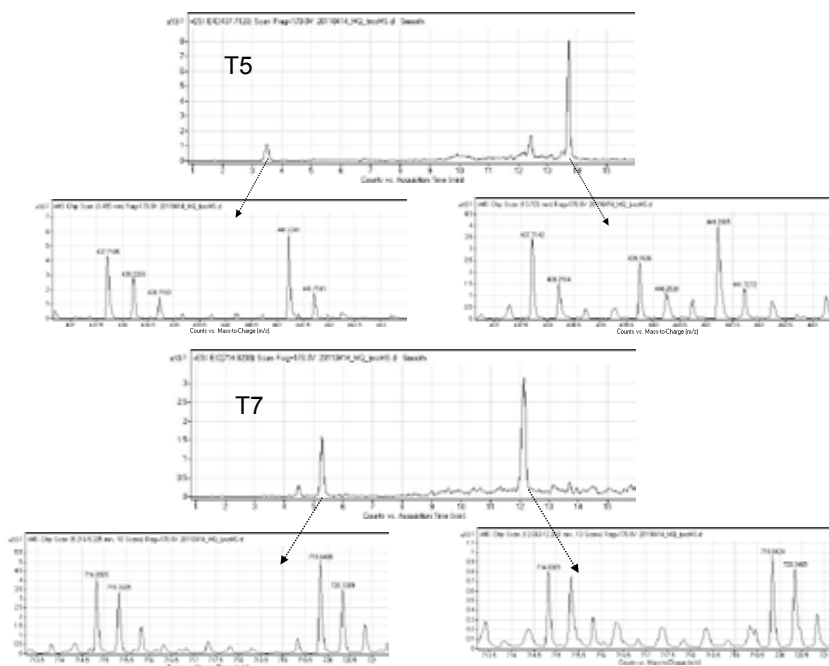


Figure 3. Extracted ion chromatogram for the T5 and T7 peptides and MS spectrum detected at the respective retention times. The doubly charged ion 437.7 ± 0.1 m/z for the T5 peptide and the doubly charged ion 714.8 ± 0.1 m/z was extracted for the T7 peptide.

pH cleanup. Recovery of the T7 peptide was 21% at low pH and 15% at neutral pH, and finally, recovery of the T16 peptide was 24% at low pH and 5.4% at neutral pH. Better results were obtained by performing RP-SPE at high pH; 85% recovery was obtained for T5 peptide, 78% for T7, and 55% for T16. The method finally chosen for sample cleanup of the peptides spiked in wine was RP-SPE at high pH.

In order to verify the peptide content of the samples eluted during RP-SPE, samples were analyzed by ToF-MS (250–2000 m/z range) by using an Agilent QToF 6530 coupled with a nano-HPLC chip interface. The method optimized to check the quality of the standard peptides was applied. The extracted ion chromatograms for T7 and T5 are shown in Figure 3. It is possible to observe that two peaks were detected corresponding to the T7 peptide, and two peaks for the T5 peptide. The complexity of the spectra of the second eluting peak also reveals the presence of polymeric material coeluting with the peptide. This suggests that stable complexes are formed between the peptides and additives or natural ingredients present in wine. In order to understand the nature of the complexes, 10% dimethyl sulfoxide and 5% formic acid were added to the samples. Samples were reinjected into the QToF instrument, and no differences were observed.

Experiments were repeated by adding 1% of casein before the RP-SPE sample cleanup step, a protein known to form stable complexes with wine. It was observed that the second peak of the T5 and T7 peptides disappeared, leading to the assumption that tannins may be responsible for the formation of complexes with the peptides of interest. Tannins are polyphenolic compounds found principally in the bark, leaves, and immature fruit of a wide variety of plants. They are important components in wine that are principally responsible for its astringent properties. Tannins have an acid dissociation constant (pK_a) around 10,

whereas the lysozyme has a pK_a of 9.7, and the three standard peptides have pK_a between 5.5 and 6.7. At acidic pH, e.g., when lysozyme and standard peptides are spiked in wine, tannins, lysozyme, and standard peptides have the same charge. At high pH, e.g., during the loading step of the SPE cleanup, the tannins are neutral and the peptides negatively charged. During the tryptic digestion at pH 8.0, the tannins and the lysozyme are positively charged, but the peptides are negatively charged. This means that nonspecific ionic interactions between the peptides and the tannins may occur, and complexes may form between the peptides and the tannins at this stage that are not destroyed during sample cleanup. The pH of the buffer used to dilute the sample before the loading step of the SPE was, therefore, increased to 12 to assure the pH of the sample to be loaded was above pH 11, at which both tannins and peptides are negatively charged. Experiments were repeated and samples were analyzed by full scan nano-LC/QToF-MS. The second peak for the T5 and T7 peptides disappeared, confirming the initial theory regarding the formation of complexes between tannins and standard peptides during the digestion step.

Optimization of the Tryptic Digestion

Three digestion protocols were initially assessed using lysozyme spiked into water. Significant proteolytic release of the T7 peptide was observed for two of the digestion protocols. The first successful protocol was based upon protein denaturation through the use of digestion in 80% acetonitrile (20, 21), and the second was based on the use of 0.5 M TEAB buffer, SDS as the denaturing agent at 60°C, TCEP (50 mM) as the reducing agent, and MMTS (200 mM) as the alkylating agent (40, 41). The third unsuccessful protocol employed urea (6 M) as the denaturing

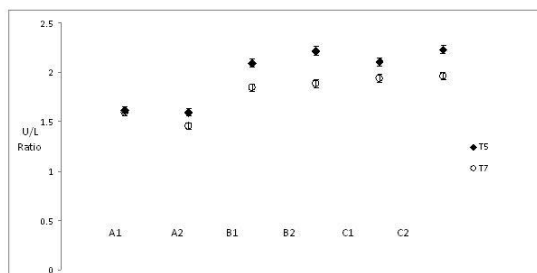


Figure 4. MS area ratios unlabeled (U)/labeled (L) of the peptides T5 and T7 normalized for the gravimetric preparation. A solution of wine spiked with labeled peptides was prepared and spiked with an equimolar amount of unlabeled peptides before the freeze drying/denaturation/alkylation step (A1 and A2), before addition of trypsin (B1 and B2), and after overnight digestion (C1 and C2). Error bars are SD of multiple injections ($n = 3$).

reagent, dithiothreitol (10 mM) as the reducing reagent, and iodoacetamide (30 mM) as the cysteine blocking reagent.

The two successful protocols were then assessed using lysozyme spiked into a model matrix at a concentration of 10 mg/kg. The model matrix consisted of 12% ethanol and 2 g/L tartaric acid at a pH of 3.2, with reference to work undertaken on quantifying lysozyme in wine by HPLC and postcolumn fluorescent derivatization (50). When used with the model matrix, the high percentage acetonitrile protocol resulted in precipitates forming in the solutions. Consequently, minimal T7 release was observed. The digestion protocol using TEAB buffer was selected from the preliminary results for continued optimization in white wine. Due to the acidic pH of the wine, the molarity of the buffer had to be increased, and the TEAB buffer had to be substituted with Tris buffer 1 M to assure the pH of the solution to be digested was above 7.5.

Time course experiments were performed to monitor the kinetics of release of the peptides. Several aliquots of lysozyme spiked with an equimolar amount of labeled peptide were prepared and analyzed before the trypsin addition at t_0 , after 30 min (t_1), after 1 h (t_2), after 2 h (t_3), after 3 h (t_4), and after overnight (15 h) digestion (t_5). The MS measured ratio of unlabeled to labeled areas was calculated, and release of the

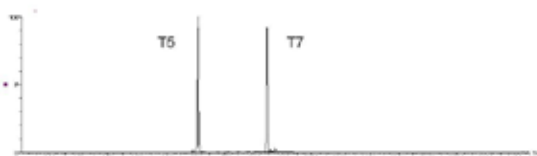
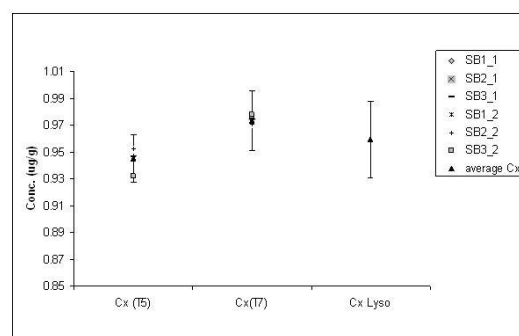


Figure 5. (a) Results obtained by EM-IDMS of lysozyme spiked into wine at 1 µg/g. SB1_1, SB2_1, SB3_1 and SB1_2, SB2_2 and SB3_2 are the results obtained from the release of the T5 and T7 peptides in two separate digestions (1 and 2). Average Cx is the average of the results obtained for the T5, T7 peptide and by combining the T5 and T7 results. Error bars are the uncertainty, calculated as reported in the *Experimental* section; (b) MRM peaks for the T5 and T7 peptide obtained after digestion of lysozyme in wine at 1 µg/g.

peptides was considered complete when the measured ratio of unlabeled to labeled peptide was constant. This appeared to be after 2 h digestion at 37°C. Samples were, however, left overnight to assure complete release was achieved. Consistent ratios assured stability of the peptides overnight.

Because labeled peptides were spiked in wine as ISs before tryptic digestion and have chemical and physical characteristics different from the intact lysozyme, their stability throughout the denaturation/alkylation and tryptic digestion steps had to be studied to assure no degradation occurred leading to quantification errors. Six samples were prepared in which the labeled peptides were spiked into wine at concentrations equivalent to 1 µg/g lysozyme. Two of the samples were gravimetrically spiked with an equimolar amount of unlabeled peptide. All of the samples were freeze dried by using a vacuum centrifuge and resolubilized in the digestion buffer, and the denaturation/alkylation step was performed. Two samples were then spiked with unlabeled peptides to check for stability of the peptides throughout the alkylation/denaturation step. Tryptic digestion was then performed on all of the samples, and, after digestion, an equimolar amount of unlabeled peptide was added to the two remaining samples containing only labeled peptides. A high pH RP sample cleanup step was finally performed, and samples were analyzed by LC/MS/MS. If no degradation occurred, constant MS measured area ratio of the unlabeled to labeled peptide was expected. The results are reported in Figure 4. It is possible to observe that the ratio of unlabeled to labeled peptide increased when unlabeled peptides were spiked after the denaturation/alkylation step. This suggests that degradation of the peptides may occur before or during the denaturation/alkylation step. Experiments were repeated

Table 2. Concentration of T5 and T7 calculated in the six SBs from two sets of hydrolysis (a and b)

	T5	T7
SB1_1	0.953	0.975
SB2_1	0.952	0.973
SB3_1	0.946	0.974
SB1_2	0.951	0.971
SB2_2	0.952	0.970
SB3_2	0.932	0.978
Average	0.948	0.974
SD	0.008	0.003
RSD, %	0.854	0.277

^a Results obtained from the release of the T5 and T7 in two separate digestions (1 and 2).

by performing denaturation at room temperature. However, no improvement was observed. It was, therefore, thought that loss of the peptides may occur during the freeze drying/resolubilization step. Experiments were repeated by freeze drying the samples for 3 h to reduce the digestion volume without reaching dryness of the sample to avoid resolubilization issues. Constant ratios were finally observed when unlabeled peptides were added before freeze drying, after freeze drying, and after trypsin digestion.

Quantification

A solution of 1.0273 ± 0.0016 $\mu\text{g/g}$ of lysozyme (approximately 1 $\mu\text{g/g}$) in wine was gravimetrically prepared as previously described and spiked with an equimolar amount of labeled peptides. The solution was then divided into three fractions to obtain three SBs. A solution containing the same amount of labeled peptides and equimolar amount of unlabeled peptides was also gravimetrically prepared in wine and divided into three aliquots to give three CBs. In order to check for losses of lysozyme due to potential absorption of the protein on the walls of the vials, a solution of 0.994 ± 0.0037 $\mu\text{g/g}$ of lysozyme in 1% BSA was also prepared and analyzed in duplicate. BSA is, in fact, well-known in molecular biology as an efficient carrier protein. Two CBs in 1% BSA were also prepared. All the samples were tryptic digested, extracted by RP-SPE at high pH, and analyzed by LC/MS/MS. A blank wine solution was also tryptic digested and analyzed by LC/MS/MS to check for interferences in the matrix. Equimolar release of the T5 and T7 peptides was observed. Consistent release of the T16 peptide was also detected; however, the values obtained from its release were not considered for quantification due to the inhomogeneity of the peptide in the stock solution. By considering the lysozyme MW equal to 14304.9 ± 0.3 g/mol, a final value of 0.727 ± 0.039 $\mu\text{g/g}$ was calculated for the lysozyme spiked in wine and 0.792 ± 0.042 $\mu\text{g/g}$ for the lysozyme spiked in 0.1% BSA. No significant loss of the lysozyme was observed, and the discrepancy between the gravimetric value and the calculated value was probably due to the purity of the standard lysophylized protein.

The aim of the project was to quantify lysozyme in wine at 1 $\mu\text{g/g}$, a concentration considered to be critical for the presence of lysozyme as an allergen in wine after consultation performed at the beginning of the project. Two individual 1 $\mu\text{g/g}$ preparations were gravimetrically prepared considering the purity of the lysozyme calculated from previous experiments. The two solutions were spiked with an equimolar amount of labeled peptides, and CBs were prepared. Samples were digested and, after sample cleanup, analyzed by LC/MS/MS. The results are reported Table 2 and Figure 5. Figure 5b shows the MRM peaks obtained by injecting the tryptic digested lysozyme in wine at the defined concentrations. A final concentration of 0.95 ± 0.03 $\mu\text{g/g}$ was calculated based on the calculated concentrations for T5 and T7.

Conclusions

A feasibility study of the application of MS-based reference measurement procedures for quantification of protein allergens in food matrixes was undertaken that was also suitable for the production of CRMs. The challenges associated with this promising method were demonstrated,

including peptide stability, chemical modification, enzymatic digestion, and sample cleanup required for accuracy and precision measurements. A proof-of-principle demonstration confirmed that this type of analysis is viable; however, the method development is expensive and time-consuming, and the requirement for method validation remains paramount. It is obvious that routine laboratories and public analysts lack of time or resources to develop those methods in-house. Furthermore, legislation does not require the level of accuracy and low uncertainty achieved here for routine measurements, but only for the production of CRMs. However, understanding the effects of sample preparation and of the matrix on the final results may help reduce the bias of the techniques routinely used and improve measurement confidence.

The requirement for reference materials to harmonize immunoassay results and enable cross-validation with MS-based reference methods is recognized by both regulators and industry stakeholders, and is needed to support legislation enforcement and food allergen risk management. This project represents the first step towards the development of CRMs in the food allergen area by using lysozyme in wine as a model system. The principle of EM-ID and the steps to be optimized during sample cleanup can be easily applied to other allergen proteins in diverse matrixes. However, the effects of food processing on the primary and tertiary structure of the allergen proteins have to be fully understood for the development of standards that can be used for both ELISA and MS-based measurements.

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