# A Mass Spectrometry-based Reference Method for the Analysis of Lysozyme in Wine and the Production of Certified Reference Materials

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Food allergy is a significant public health problem in the developed world. In susceptible individuals an immunological response is formed to specific proteins that are contained in certain foods[1,2,3]. The effects of the immunological response can range from mild lip tingling to life threatening anaphylaxis and has serious social consequences. Legislation has been introduced to address the problem and the labelling of foods that are formulated to contain any of the 14 major allergens defined in the Directive 2000/13/EC is legally required[4]. The development of techniques that allow absolute quantification of allergens in food matrices has the potential to provide an effective route to allergen control standardisation. Current methods for the quantification of allergenic proteins detect either the allergenic protein (ELISA or MS) or DNA specific to that species (PCR). For the purpose of quantification the indirect nature of the detection by PCR has been criticised. Immunochemical detection such as ELISA exhibits variable and manufacturer specific sensitivities and lack of detection due to structural changes induced in the target protein during manufacturing processes.

Mass spectrometry is a powerful technique for identification of proteins in complex matrices and it is also considered applicable for quantification of proteins leading to results traceable to the International System of Units (SI) and the production of certified reference materials (CRM). The assignment of the SI traceable concentration of allergenic proteins in food matrices and the production of CRM would be a major advantage in facilitating standardisation of current analytical techniques. To obtain reliable and comparative results by mass spectrometry the bias of methods must be understood. Homologies in protein sequences have to be considered as do post-translational modifications induced by the manufacturing process. Furthermore for a mass spectrometry platform to be used correctly for quantification of allergens leading to results traceable to the SI, appropriate selection of internal standards, their stability and equilibration in the matrix must be evaluated[5,6,7,8].

The feasibility of developing mass spectrometry based reference methods which can be used for the production of CRM has been demonstrated herein by quantification of 1 mg $kg^{-1}$ of lysozyme spiked into white wine. Lysozyme is one of the allergenic proteins contained in eggs and it is used as a fining agent and anti-microbial stabiliser/additive in wine and cheese and if used it must be declared on the wine label[9].

## Method

One mg $kg^{-1}$ of lysozyme in white wine was quantified by enzymatic digestion and exact matching isotope dilution mass spectrometry[5,6,7,8]. Three peptides were selected as internal standards T5 (HGLDNYR), T7 (FESNFNTQATNR) and T16 (GTDVQAWIR). Because of the instability of the T16 peptide in the stock solution, only T5 and T7 were used for quantification; T16 was used to check for consistency of enzymatic digestion. Two series of

three "*sample*" blends containing the protein to be quantified and equimolar amounts of labelled peptides and two series of "*calibration*" blends containing equimolar amounts of unlabelled peptide and labelled peptides were concentrated and subjected to tryptic digestion. Sample clean-up was performed by using reverse phase STRATA solid phase extraction material. Samples were finally analysed by selected reaction monitoring experiments on a liquid chromatography QQQ instrument (Quattro Ultima, Waters). Standard peptides were quantified by amino acid analysis to ensure results were traceable to the SI. The peptides were hydrolysed by acid hydrolysis on an Ethos EZ Microwave digestion system (Milestone) and analysed by gas chromatography-mass spectrometry in selected-ion recording mode (Finnigan Trace DSQ mass spectrometer, ThermoFisher Scientific).

# Results

Wine spiked with 1 mg $kg^{-1}$ lysozyme was quantified by exact-matching isotope dilution mass spectrometry (IDMS) with an uncertainty of 3%. The three peptides used as internal standards were unique to avian lysozyme and all criteria for the selection of the peptides to be used as internal standards were met[8]. The sample preparation and sample clean-up were optimized to assure peptides were stable before and during tryptic digestion; tryptic digestion was monitored to assure complete release of the peptides of interest from the protein was achieved. Finally the recovery of the peptides from the sample clean-up step was maximized. The characterization of the aspecific binding of the peptides with tannins in the wine played a major role in the optimization of the recovery of the peptides after tryptic digestion and in the development of the reverse phase sample clean up step.

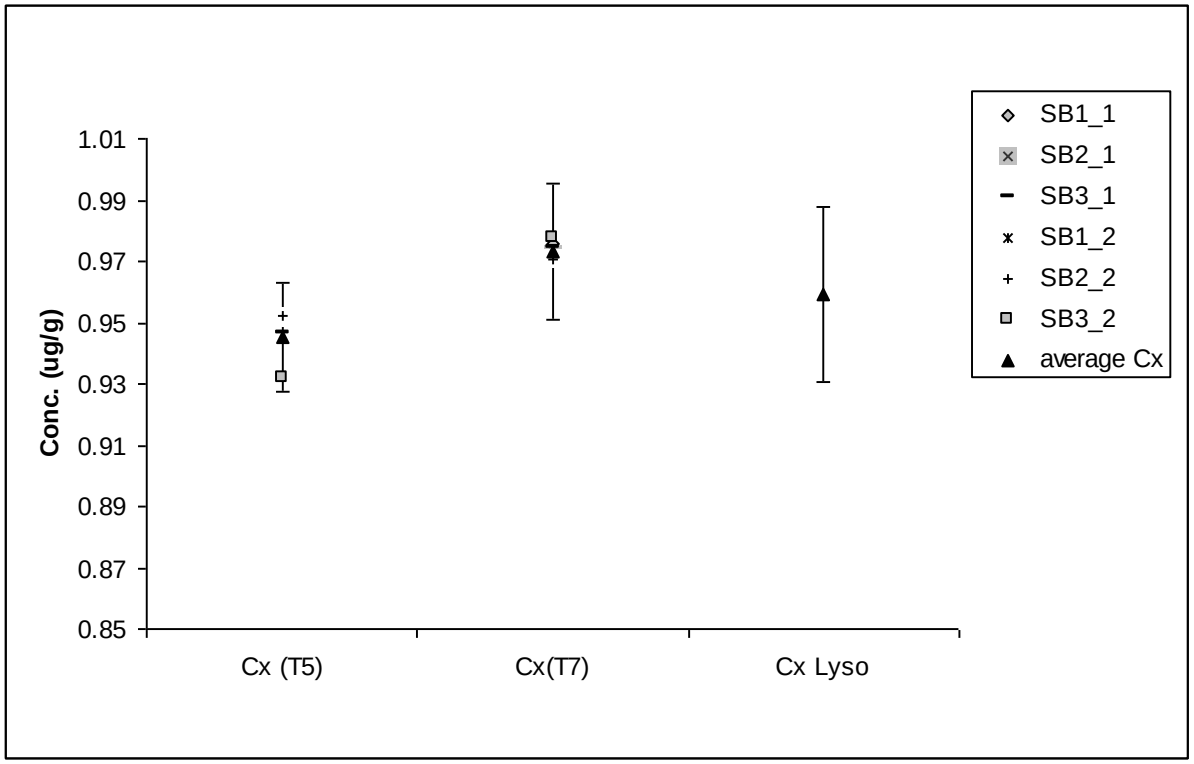
The results of the quantification of lysozyme spiked in white wine at 1 mg $kg^{-1}$ are reported in Figure 1. Table 1 shows the concentration of lysozyme obtained from each peptide.

# Conclusion

Exact matching IDMS has been successfully applied for the development of a reference method which can be used for the production of CRMs for the quantification of lysozyme in wine. This is a major step forward in the production of biological reference materials in the food area which in turn can improve robustness, confidence and comparability of the results obtained by immunoassay and other techniques. The IDMA method development is obviously expensive and time consuming however the knowledge gained of the effects of the sample preparation and of the matrix on the final results supports understanding the bias of the techniques routinely used and will improve measurement confidence. Additionally, confirmation of the feasibility of mass spectrometry methods for quantifying an allergenic protein in a food matrix with results traceable to the SI is a further step towards the production of certified reference materials to support standardisation in quality control and in risk assessment and risk management of food allergens.

# Figure 1
# Results obtained by Exact-Matching IDMS of Lysozome spiked in Wine at 1 g $kg^{-1}$.

In transparent are reported the results obtained from the release of the T5 and T7 peptides in two separate digestion of three samples and in black is reported the average of the results. Error bars are the uncertainty calculated in accordance with Eurachem 38 and with the ISO Guide to the Expression of Uncertainty Measurements.




# Table 1
# Concentration of T5 and T7 Calculated in Six Sample Blends from two sets of Tryptic Digestion (a and b)

| | T5 | T7 |
|---|---|---|
| SB1a | 0.953 | 0.975 |
| SB2a | 0.952 | 0.973 |
| SB3a | 0.946 | 0.974 |
| SB4b | 0.951 | 0.971 |
| SB5b | 0.952 | 0.970 |
| SB6b | 0.932 | 0.978 |
| | | |
| Average | 0.948 | 0.974 |
| Standard deviation | 0.008 | 0.003 |
| RSD% | 0.854 | 0.277 |

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