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Multi-allergen quantification of fining-related egg and milk proteins in white wines by high-resolution mass spectrometry

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RATIONALE: A method based on High-Resolution Mass Spectrometry was developed for the simultaneous determination of fining agents containing potentially allergenic milk (casein) and egg-white (lysozyme and ovalbumin) proteins, added to commercial white wines at sub-ppm levels. Selected tryptic peptides were used as quantitative markers. An evaluation of protein digestion yields was also performed by implementing the ¹⁵N-valine-labelled analogues of the best peptide markers identified for α_{S1} -casein and ovalbumin.

METHODS: The method was based on the combination of ultrafiltration (UF) of protein-containing wines, tryptic digestion of the dialyzed wine extracts and liquid chromatography/high resolution mass spectrometry (LC/HRMS) analysis of tryptic digests. Peptides providing the most intense electrospray ionization (ESI)-MS response were chosen as quantitative markers of the proteins under investigation.

RESULTS: Six-point calibrations were performed by adding caseinate and egg-white powder in the concentration range between 0.25 and 10 $\mu\text{g/mL}$, to an allergen-free white wine. The following three peptide markers, LTEWTSSNVMEER, GGLEPINFQTAADQAR and ELINSWVESQTNGIIR, were highlighted as best markers for ovalbumin, while GTDVQAWIR and NTDGSTDYGLQINSR for lysozyme and YLGYLEQLLR, GPFPIIV and FFVAPFPEVFGK for caseinate. Limits of detection (LODs) ranged from 0.4 to 1.1 $\mu\text{g/mL}$.

CONCLUSIONS: The developed method is suited for assessing the contemporary presence of allergenic milk and egg proteins characterizing egg white and caseinate, fining agents typically employed for wine clarification. The LODs of the method enable the detection of sub-ppm concentrations of residual fining agents, that could represent a potential risk for allergic consumers. Copyright © 2013 John Wiley & Sons, Ltd.

In the last decade, mass spectrometry (MS)-based methods have been applied extensively to tackle diverse issues in the field of protein analysis, including the determination of the molecular weight and, through enzymatic hydrolysis, of the amino acid sequence.^[1] Mass spectrometry can be considered the only non-immunological approach for high-specificity and high-sensitivity detection of proteins acting as food allergens.^[2] MS-based methods for detection of food allergens generally rely on the enzymatic digestion of the protein of interest, typically using trypsin, followed by a liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis to elucidate the amino acid sequences of individual peptides in the final digest. This is the *bottom-up* approach quite common in the field of proteomics.^[3] As in other contexts, the reliability of protein allergens identification

can be increased by monitoring many target peptides within the same run (multi-analyte method).^[4,5] Some guidelines have been recently issued in this regard to help analysts in designing suitable peptide markers to be used for the MS-based monitoring of food allergens in the food supply chain.^[6]

Milk and egg proteins, well known to be among the most important protein allergens, also play an important role as processing aids in winemaking. Indeed, they act as fining agents by interacting with wine polyphenols to form complexes that can be further removed by decantation or filtration steps, thus reducing the concentration of those endogenous components that might be responsible for undesirable phenomena (presence of haze, generation of off-flavors or deposits, etc.).^[7] Residual fining proteins might also be removed by a secondary fining procedure, using inorganic agents like bentonite.^[8] Some investigations seem to prove that the implementation of such removal steps for excess fining proteins can lead to non-allergenic end-products.^[9] However, the risk for wine consumers allergic to milk or egg proteins cannot be completely excluded. This consideration is clearly evidenced by several legislations issued in the last decade about food/beverage labelling for allergens.

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As far as the European Union is concerned, Directive 2007/68/EC^[10] regulates the general labelling of 14 allergenic food ingredients, whose intentional addition to food and beverages must be obligatorily indicated on the label. As from July 1 2012 European wine producers are obliged, likewise those of Australia and New Zealand where the compulsory indication started in 2002,^[11] to indicate egg (albumin and lysozyme)- and milk (caseins)-derived products on the wine labels whenever they are used for fining purposes. Within the new European legislative frame the development of reliable and sensitive methods enabling the detection and/or confirmation of milk and egg proteins in foods and beverages might open new perspectives for the producers. Indeed, the methods could help in the quantification of residual amounts of fining agents in the finished and ready-to-use products. Some works published on this topic and using enzyme-linked immunosorbent assay (ELISA) methods for allergen detection reported the absence of residual milk allergens in white wine subjected to clarification.^[11–14] On the other hand, Weber *et al.* documented the detection of α - and β -caseins in fined wines at levels higher than 0.2 $\mu\text{g}/\text{mL}$.^[15] As far as the fining with egg-white proteins is concerned, it was also demonstrated that, depending on the color of the wine and on the secondary fining with bentonite, different results could be obtained in dependence of the amount of egg protein added to the original wine.^[13] Although ELISA methods generally enable a good sensitivity and ease in execution, they often suffer from limitations due to the complexity of the matrix under study and the presence of interfering compounds that might compromise the outcome of the final tests. In order to overcome these drawbacks, mass spectrometry has recently entered the arena of residual fining agents analysis. In particular, it has been reported that residual milk or egg proteins can be identified by a MS-based analysis of their tryptic peptides once they are extracted, by different approaches, from white or red wines, either as such or after being purposely spiked with typical milk- or egg-related fining agents, like caseinate or egg-white powders.^[16–18]

As far as MS-based quantitation is concerned, the application of a method based on high-resolution mass spectrometry (HRMS) to a raw white wine subjected to fining with caseinate has been reported in a work performed in our laboratories.^[19] Limit of detection (LOD) values ranging from 39 to 51 $\mu\text{g}/\text{mL}$, referred to the amount of protein initially added to the raw wine, were achieved. Actually, due to partial precipitation, a quantification of the amount of milk protein remaining dissolved in wine after fining was not accomplished in that case. In the already cited work by Tolin *et al.*, a nanospray high-performance liquid chromatography/electrospray ionization quadrupole time-of-flight (nano-HPLC/ESI-QTOF) system has been exploited for the detection of residual egg proteins in red wines, at levels of 50 g/hL or higher.^[17] In a more recent paper the same authors reported the detection of milk and/or egg proteins in 8 out of 25 commercial red wines.^[20] Although no quantitative data were provided, this was the first time that traces of allergenic proteins were found in commercial wines by using MS-based analysis.

The MS-based quantitation of residual fining agents in wines is generally complicated by the interplay between protein recovery from the matrix, tryptic digestion yield and ESI-MS response of the resulting peptides. The use of

isotopically labelled peptides could prove quite useful to overcome this limitation, as demonstrated in a work by Lutter *et al.*,^[21] in which isotopically labelled peptides were used to quantify residual milk allergens in infant foods. More recently, the use of $^{13}\text{C}^{15}\text{N}$ -labelled α_{S1} -casein and of the relevant ^{15}N -labelled peptides has been described by Newsome and Scholl to study the effect of sample matrix on the selected reaction monitoring (SRM) acquisition mode.^[22]

Starting from the considerations made so far, HRMS implemented by the use of labelled peptides could represent a promising approach for the contemporary quantitative screening of milk and egg allergenic proteins in commercial wines, particularly useful when multiple agents are used for fining. However, to the best of our knowledge, this possibility has not been explored yet.

In the present paper the development of a HRMS-based method able to detect simultaneously milk and egg proteins potentially contaminating a white wine fined with caseinate or egg-white powder will then be described. The utility of isotopically labelled (^{15}N -valine-containing) peptides of ovalbumin and α_{S1} -casein in the evaluation of the yield of tryptic digestion of milk and egg proteins extracted from wine, as such or combined with ultrafiltration recovery, will also be discussed.

EXPERIMENTAL

Chemicals

Acetonitrile and water (both LC/MS grade), formic acid, hydrochloric acid, iodoacetamide, dithiothreitol (DTT), ovalbumin (purity higher than 98%), α -casein (purity higher than 70%), [Glu]-fibrinopeptide (GFP), egg-white powder and sodium caseinate were provided by Sigma-Aldrich (Milan, Italy). AlbuclatTM powder, a commercial fining agent obtained from egg white, was purchased from Enolapi (Verona, Italy). Trypsin (proteomic grade) was purchased from Promega (Milan, Italy); the RapigestTM surfactant was purchased from Waters (Milford, MA, USA). Ultrafiltration (UF) tubes with 10 kDa cut-off membranes were purchased from Millipore (Billerica, MA, USA). Peptides labelled with ^{15}N on the valine residue and arising from ovalbumin or α_{S1} -casein proteins were obtained from Proteogenix SAS (Oberhausbergen, France). The DC Protein assay kit utilized for protein quantification was purchased from Bio-Rad (Segrate, MI, Italy).

Standard/stock solutions

Standard solutions of the ^{15}N -valine-peptide LIEWTSSNVMEER (13 aa) from ovalbumin and ^{15}N -valine-peptide FFVAPFPEFVGK (12 aa) from α_{S1} -casein (both peptides with purity higher than 98%) were prepared by dissolving 1 mg of each peptide standard in 1 mL of Milli-Q water. Stock solutions were then stored at -20°C before their use.

Stock solutions of egg-white powder, caseinate and Albuclat were prepared in ammonium bicarbonate 50 mM at the concentration of 10 000 $\mu\text{g}/\text{mL}$ and were used for wine fortification.

Wine sample pre-treatment and protein tryptic digestion

Three different white wines, produced from *Falanghina*, *Pinot grigio* and *Greco di Tufo* grape varieties, were purchased from a local retailer and analyzed to check the applicability of the

developed method. For calibration purposes each type of wine was fortified with caseinate and egg-white powder to reach the following concentration levels: 0.25, 0.5, 1, 2, 5, and 10 µg/mL. Typically, 10 mL wine aliquots were collected and fortified with caseinate and egg-white powder. The fortified wine was left under stirring for 15 min and then was kept quiescent for 20 min at room temperature; no precipitation was observed, thus assuring that the added proteins had completely dissolved into the wine matrix. Aliquots of 4 mL were successively withdrawn and centrifuged for 40 min, at room temperature, in ultrafiltration tubes with 10 kDa cut-off membranes (Millipore, Billerica, MA, USA). The retained part of the sample was subsequently dialyzed against 1 mL of water to remove residual low molecular weight interferences eventually present in the extract. A final volume of 100 µL was collected from the filter and 50 µL of it were combined with 50 µL of Rapigest solution (0.2% w/v in ammonium bicarbonate 100 mM) and submitted to tryptic digestion. Protein content was estimated in the final ultrafiltered ready-to-digest wine extracts by using the DC Protein assay kit (absolute protein amount ranging from 8 to 25 µg) so that an enzyme/substrate ratio lower than 1:20 could be always adopted for the enzymatic reactions. Before digestion protein reduction was performed by adding 5 µL of 50 mM DTT solution followed by 30 min incubation in a thermoshaker at 60 °C. After cooling down the sample at room temperature, 10 µL of 100 mM iodoacetamide solution were added and the mixture was placed in the dark for 30 min. As the final step, 2 µL of trypsin solution (concentration 1 µg/µL in acetic acid 50 mM) were added to the extract and the tube was gently flicked to mix. Incubation at 37 °C under shaking was accomplished for about 14 h, in order to ensure a nearly complete digestion. In order to stop the trypsin activity and induce the precipitation of Rapigest hydrolysis products, 5 µL of a 1 M HCl solution were finally added to the digest, that was then incubated for 30 min at 37 °C and centrifuged at 16 000 g for 10 min. The pellet was discarded and the supernatant carefully transferred into micro-vials prior to LC/MS analysis.

LC/MS instrumentation and operating conditions

A system composed of an UHPLC pump equipped with an autosampler (Accela, Thermo Fisher Scientific, San José, CA, USA) and an Exactive ESI high-resolution mass spectrometer (Thermo Electron, San José, CA, USA), including an Orbitrap mass analyzer and a Higher energy Collisional Dissociation (HCD) cell, was used for all the LC/HRMS analyses. The separation was carried out by injecting 10 µL of sample in a C12 Jupiter Proteo 90 Å column (150 mm × 2 mm × 4 µm), purchased from Phenomenex (Castel Maggiore, Bo, Italy), and using a binary elution gradient operating at a flow rate of 150 µL/min. The eluents were: (A) H₂O + 0.1% HCOOH and (B) CH₃CN + 0.1% HCOOH. Peptide elution was accomplished by using the following linear gradient: 0–23 min, linear from 90 to 65% solvent A; 23–26 min, isocratic; 26–28 min, from 65 to 50%; 28–33 min, isocratic; 33–40 min, from 50 to 5% solvent A; 40–55 min, isocratic. In the following 5 min solvent A was raised to 90% and kept for 5 min to allow equilibration.

MS acquisitions, performed in positive polarity, were based on a single *segment*/two *events* acquisition scheme. The segment corresponded to the entire duration of the LC run;

the two events were represented, respectively, by a MS *full scan* and a HCD-MS *full ion fragmentation* acquisition, both accomplished in a 200–2000 *m/z* range. An optimization of the HCD collision energy, in terms of quality of fragmentation patterns for tryptic peptides, was performed preliminarily. In particular, a value of 40 eV was found to be the best compromise between number of detected product ions and signal-to-noise (S/N) ratio of the HCD-MS spectra.

The ESI settings were as follows: sheath gas flow (arbitrary units), 25; auxiliary gas flow (arbitrary units), 5; spray voltage, 4 kV; capillary temperature, 250 °C; capillary voltage, 32.5 V; tube lens voltage, 130 V; skimmer voltage, 30 V; heater temperature, 30 °C. The mass spectrometer was operated at a resolving power 50 000 (high-resolution) with a maximum injection time of 100 ms and 1 ms long microscans. Calibration of the Orbitrap analyzer was accomplished by infusing into the spectrometer ESI source the standard Exactive calibration mixture, containing caffeine, peptide MRFA and Ultramark 1600 (provided from Thermo Electron), dissolved in a 25:25:49:1 (v/v/v/v) mixture of water/methanol/acetonitrile/acetic acid solution.

Mass accuracy was assessed by infusing into the ESI source, at a flow rate of 5 µL/min, a 5 µg/mL standard solution of GFP dissolved in acetonitrile/water (50:50%, v/v) containing 0.1% of formic acid and comparing the experimental *m/z* value with the one calculated for the mentioned peptide (*m/z* 785.8412); it was found to be always better than 3 ppm. Mass spectral data were acquired and processed by using the Xcalibur software (Thermo Electron).

RESULTS AND DISCUSSION

Identification of peptide markers in caseinate and egg-derived fining agents

A preliminary MS analysis was carried out on the tryptic digests obtained from caseinate and ovalbumin standard solutions (each at 10 µg/mL concentration) with the aim of identifying the most reliable peptide markers, respectively, for caseins contained in caseinate and for the protein expected to be the most abundant in egg-white-derived fining agents.

Peptide markers highlighted in the tryptic digest of the aqueous solution of ovalbumin were subsequently screened in the tryptic digests obtained from egg-white powder and AlbuclarTM, both dissolved in water at a 10 µg/mL concentration. An overview of the ovalbumin tryptic peptides identified in both products is reported in Table 1. The molecular weights of the peptides usually fell between 1200 and 2000 Da; moreover, some sequences were compatible with either natural (phosphorylation/oxidation) or artificial (carbamidomethylation, occurring during digestion) modifications, occurring on serine, methionine or cysteine residues, according to the case. Interestingly, additional peptides arising from lysozyme were also detected in the MS spectra relevant to the digests of egg-white-based products, although it represents a minor egg-white protein (about 3.4% w/w).^[23]

It is worth noting that each peptide sequence reported in Table 1 was identified by exploiting two different types of mass spectrometric data. First, the accurate value of the *m/z*

Table 1. Overview of MS data and sequences retrieved for chicken ovalbumin and lysozyme tryptic peptides detected in the digests of egg-white powder (Sigma-Aldrich) and Albuclar™, each at 10 µg/mL concentration

<i>m/z</i> ratio	Charge	Molecular weight	Sequence (one letter code)
OVALBUMIN			
647.4545	+1	646.4545	VYLPR
624.3153	+2	1246.6306	ADHPFLFCIK
673.3721	+2	1344.7442	HIATNAVLFGR
761.9021	+2	1521.8042	YPILPEYLQCVK
844.4231	+2	1686.8462	GGLEPINFQTAADQAR
791.3646	+2	1580.7292	LTEWTSSNVMEER
887.4114	+2	1772.8228	ISQAVHAAHAEINEAGR
1230.6607	+2	2459.3214	NVLQPSSVDSQTAMVLVNAIVFK
929.9859	+2	1857.9718	ELINSWVESQTNGIIR
1004.9759	+2	2007.9518	EVVGSAEAGVDAASVSEEF
1044.9590	+2	2087.9180	EVVGSAEAGVDAASVSEEF
1287.6779	+2	2574.3558	ADHPFLFCIKHIATNAVLFGR
LYSOZYME			
877.4212	+2	1753.8273	NTDGSTDYGILQINSR
714.8288	+2	1427.6424	FESNFNTQATNR
523.2749	+2	1044.5347	GTDVQAWIR
437.7119	+2	873.4088	HGLDNYR
663.3190	+2	1324.6229	GYSLGNWVCAAK

C = Carbamidomethylated cysteine; S = phosphorylated serine

ratio of the corresponding ion was retrieved from MS acquisitions. The *FindPept* software, available on the *ExPASy* portal,^[24] was used to find potential correlations between experimental *m/z* ratios and those predicted for the tryptic peptides of proteins expected to have a not-negligible abundance (at least 3% w/w) in chicken egg-white-derived fining agents, i.e. ovalbumin, ovotransferrin, ovomucoid, ovoglobulin, ovomucin and lysozyme.^[23] For this purpose a mass tolerance of 10 ppm was set for *m/z* matching. A further confirmation of the amino acid sequence for each peptide was achieved through the detection of product ions upon HCD-MS experiments. This approach is different from that adopted for a typical MS/MS experiment performed on precursor ions in space (like in triple quadrupoles or QTOF systems) or in time (like in 3D or linear ion traps).

Indeed, since the Exactive spectrometer is unable to perform the isolation of a precursor ion, all the ions arriving from the ESI source can be fragmented if introduced into the HCD cell. Usually, as in the present case, two chromatographic traces, referred to respectively as the *full scan* and *full ion fragmentation mode*, can be generated if a two-event acquisition scheme (MS and HCD-MS) is adopted. In the first case ions arriving from the source are directly diverted into the Orbitrap analyzer using a C-trap; in the second case ions are first driven into the HCD cell and then their eventual product ions are transferred into the Orbitrap analyzer using the C-trap. The association of a product ion to a specific precursor ion can be made by obtaining eXtracted Ion Chromatograms (XICs) for the corresponding *m/z* ratios (in this case using extraction windows compatible with a 10 ppm mass accuracy) and evaluating the alignment, in terms of retention times, of the peaks detected in the two XIC traces. Once an amino acid sequence is hypothesized

for a peptide, based on HRMS measurements, the *m/z* ratios for the corresponding product ions can be predicted, exploiting the typical fragmentation patterns of peptides. In the present case, the *MS-Product* software, included into the *Protein Prospector* proteomic portal,^[25] was used for this aim. Subsequently, the corresponding HCD-MS XIC traces can be retrieved and compared with that of the peptide precursor ion. Whenever the alignment between peaks in the MS and HCD-MS-derived XIC traces is verified, a new product ion can be associated with the precursor one and, finally, MS/MS-like information is retrieved. It is worth noting that the described approach has been already exploited successfully in our laboratories to confirm the sequences of caseinate-related tryptic peptides.^[16,19] In the present investigation the occurrence of at least three product ions belonging to the *y*-series (the prevailing one in HCD-MS spectra), well aligned with a specific peptide precursor ion detected in the MS spectrum, has been considered as the criterion to confirm the amino acid sequence of the peptide.

As an example of the described procedure, the XIC traces relevant to the precursor ion at *m/z* 791.3640, detected in the tryptic digest of ovalbumin in aqueous solution, and to three product ions generated during the corresponding HCD-MS measurement are compared on the left side of Fig. 1. The alignment between the different XIC traces is apparent and, after considering the fragmentation pattern predicted for the LTEWTSSNVMEER sequence from ovalbumin, compatible with the *m/z* ratio 791.3640, the three product ions are found to correspond to *y*-type ions of the peptide. The MS and HCD-MS spectra relevant to the same peptide are reported on the right side of Fig. 1. In the former, although the *m/z* 791.3640 ion is dominating, several other ions are detected, due to the fact that other species can be co-eluted from the

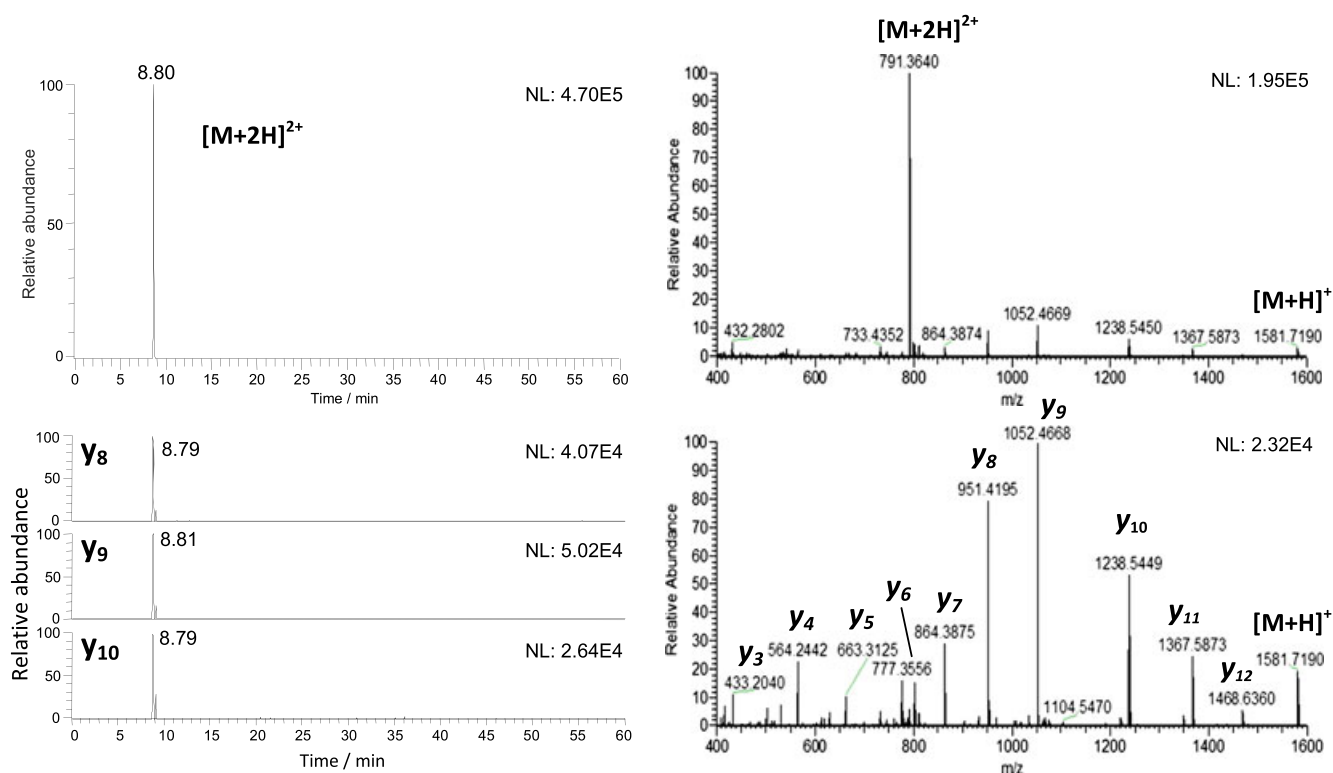


Figure 1. Left: eXtracted Ion Current (XIC) chromatograms obtained for the precursor ion of peptide LTEWTSSNVMEER (upper panel) and for three of its product ions generated during HCD-MS acquisitions (lower panel), referred to the tryptic digest of an ovalbumin standard solution (10 $\mu\text{g/mL}$). Right: MS Full scan (upper panel) and HCD-MS spectrum (lower panel) recalled at a retention time of 8.8 min.

column and then transferred into the Orbitrap analyzer from the ESI source. Among the further signals detected in the MS spectrum the one at m/z 1581.7190 corresponds to the singly charged ion of the LTEWTSSNVMEER peptide. When the HCD-MS experiment was performed, both ions relevant to the peptide could be fragmented and the resulting spectrum, shown in the bottom-right panel of Fig. 1, is actually indistinguishable from a typical MS/MS spectrum of the peptide, since the interference due to product ions arising from other species is minimal in this case.

Although a more complex data elaboration is required, the final result of the described HCD-MS approach can then be considered comparable to that of a LC/MS/MS experiment based on the fragmentation of user-selected precursor ions. However, HCD-MS has the unique feature of fragmenting, without a preliminary specification, virtually every ion generated inside the ionization source, which is an advantage also with respect to data-dependent MS/MS acquisitions. On the other hand, in particular situations, not encountered during the present investigation, e.g. if the precursor ion of interest is not abundant and enters the HCD cell almost at the same time as another, more abundant, ion (due to significant co-elution of the corresponding compounds), the attribution of product ions to the correct precursor can become a critical issue.

Interestingly, almost all the ions of the y-series are detected in the HCD-MS spectrum, whereas no significant signal is found for b-type ions, thus confirming the prevalence of the y-type fragmentation under HCD conditions. The same result

was observed systematically for the tryptic peptides analyzed in the present work.

The procedure now described was also used to identify tryptic peptides arising from the digestion of caseinate dissolved in water at 10 $\mu\text{g/mL}$ concentration. Their MS data and sequences are reported in Table 2. Interestingly, peptide markers from α_{S1} -, α_{S2} - and β -caseins were detected, whereas no peptide from κ -casein, the least abundant casein in caseinate, was identified.

Simultaneous quantification of caseinate and egg-white powder added to a white wine

Once the lists of tryptic peptides obtained for egg white-related products and caseinate were completed, peptides showing the highest MS response (meant as the area under the peptide peak observed in XIC traces) were selected as potential quantitative markers tracing for the related fining agent in wine. In order to assess the linearity of the method, aliquots of the *Falaghina* white wine were simultaneously fortified with both types of fining agents at six concentration levels, covering the 0.25–10 $\mu\text{g/mL}$ range. It is worth noting that no visible precipitation occurred for caseins or egg-white proteins when added to the wine under study.

Three protein extracts were prepared for each concentration point, starting from different aliquots of the same fortified wine; two replicated LC/MS runs were then performed on the tryptic digest obtained from each extract. As a result, three peptides were identified as the best markers, in terms of

Table 2. Overview of MS data and sequences retrieved for casein tryptic peptides detected in the digest of caseinate at 10 µg/mL concentration.

<i>m/z</i> ratio	Charge	Molecular weight	Sequence (one letter code)
<i>α_{s1}</i> -CASEIN			
748.3700	+1	747.3700	TTMPLW
634.3561	+2	1266.7122	YLGYLEQLLR
692.8688	+2	1383.7376	FFVAPFPEVFGK
880.4757	+2	1758.9514	HQGLPQEVLNENLLR
936.4977	+2	1870.9954	YKVPQLEIVPNSAEER
964.3496	+2	1926.6992	DIGSESTEDQAMEDIK
976.4798	+2	1950.9596	YKVPQLEIVPNSAEER
831.3920	+1	830.3920	EDVPSER
790.9169	+2	1579.8338	VPQLEIVPNSAEER
830.8999	+2	1659.7998	VPQLEIVPNSAEER
972.3464	+2	1942.6928	DIGSESTEDQAMEDIK
<i>α_{s2}</i> -CASEIN			
748.3700	+1	747.3700	LTEEEK
880.4754	+2	1758.9508	TVDMESTEVF ^T TKKTK
936.4950	+2	1870.9900	KTVDMESTEVF ^T TKKTK
976.4795	+2	1950.9590	KTVDMESTEVF ^T TKKTK
979.5610	+1	978.5610	FALPQYLK
1158.4900	+1	1157.4900	ENLCSTFCK
598.3428	+2	1194.6856	NAVPIPTLNR
684.3506	+2	1366.7012	ALNEINQFYQK
733.8092	+2	1465.6184	TVDMESTEVF ^T K
1047.4454	+2	2092.8908	NMAINP ^S KENLCSTFCK
<i>β</i> -CASEIN			
742.4500	+1	741.4500	GPFPPIV
748.3700	+1	747.3700	EMPF ^F PK
991.4345	+2	1980.8690	FQSEEQQQT ^E DELQDK
1031.4179	+2	2060.8358	FQSEEQQQT ^E DELQDK
1093.5877	+2	2185.1754	DMPIQAFLLYQEPVLGPVR
950.4926	+2	1898.9852	VKEAMAPKHKEMP ^F PK

C = Carbamidomethylated cysteine; M = oxidized methionine; S = phosphorylated serine

sensitivity, for caseins (YLGYLEQLLR, GPFPPIV and FFVAPFPEVFGK) as well as for ovalbumin (LTEWTSSNVM EER, GGLEPINFQTAADQAR and ELINSWVESQTNGIIR). Furthermore, two peptide markers were highlighted for lysozyme (GTDVQAWIR and NTDGSTDYGILQINSR). For the sake of example, the XIC traces of the eight peptide markers referred to a *Falanghina* white wine sample fortified with 1 µg/mL of caseinate and egg-white powder are reported in Fig. 2.

Calibration graphs could be obtained by plotting the XIC peak areas relevant to selected peptide markers versus the concentration of the fining agent containing the corresponding proteins. Data resulting from linear interpolations, i.e. calibration line equations (with standard deviations for slope and intercept) and correlation coefficients, are reported in Table 3 for the eight selected peptide markers and for three wine matrices investigated. As apparent from the table, a generally good linear correlation was observed between the MS response of a specific peptide marker and the added concentration of the fining agent including the peptide's protein of origin. This result is quite interesting for several

reasons. First, it implies that the proteins contained in each of the two fining agents under study do not change significantly their original quantitative distribution when dissolved into the wine matrices. In other words, no selective precipitation occurs, as expected, since a complete solubility was always observed at any of the concentration levels explored for the added fining agents. Moreover, two further parameters that clearly contribute to the peptide marker MS responses, i.e. the UF recovery and the digestion yield of proteins contained in caseinate or egg-white powders, seem to be not significantly influenced by the added concentration of the fining agent (and then of the protein itself) in each wine matrix. The most important consequence of these findings is that a specific tryptic peptide can be exploited, although indirectly, as a quantitative marker not only for its protein of origin, but also for the entire mixture of proteins that corresponds to caseinate or egg-white powders, according to the case. The LOD values reported in the last column of Table 3 are then referred to the minimum concentration of an added fining agent that can be detected, at S/N=3 (the standard deviation of the calibration line intercept was used in this case as an estimate of noise),

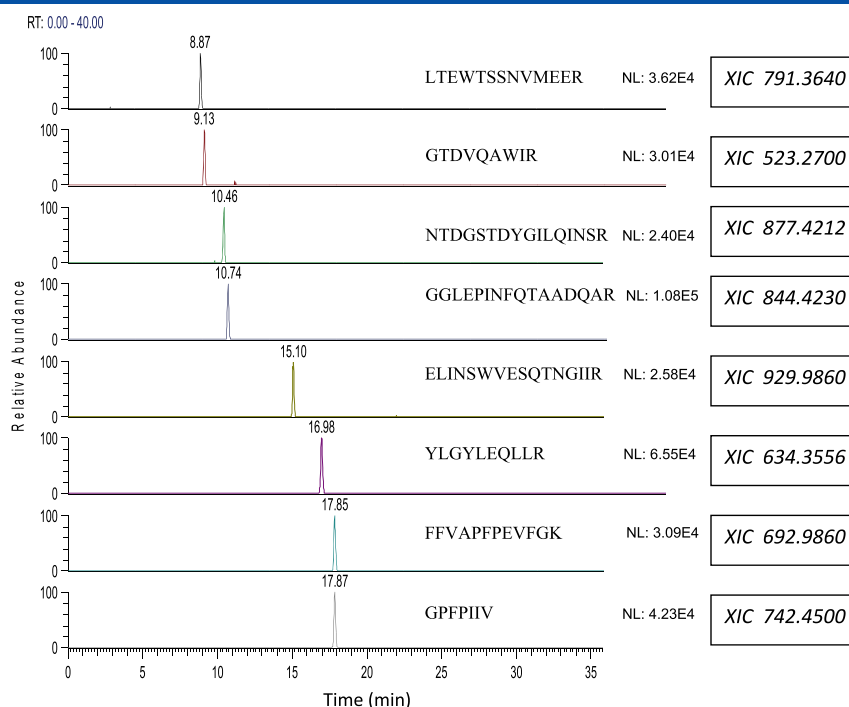


Figure 2. Overlay of eXtracted Ion Current (XIC) chromatograms obtained for the *m/z* values of selected peptide markers for ovalbumin, lysozyme and caseins and referred to a *Falanghina* wine sample fortified with caseinate and egg-white powder, each at 1 µg/mL concentration.

Table 3. Calibration data obtained for eight selected tryptic peptide markers, related to ovalbumin (OVA), lysozyme (LYS) and α_{s1} - or β -casein (CN), referred to *Falanghina*, *Pinot grigio* and *Greco di Tufo* white wine aliquots fortified with caseinate and egg-white powder in a 0.25–10 µg/mL concentration range. LOD values are referred to the added concentrations of fining agents

Peptide marker sequence (<i>m/z</i> ratio)/allergenic protein	Wine	Equation of the calibration line (mean $\pm$ sd)	Correlation coefficient	LOD (µg/mL)
Egg-white powder				
LTEWTSSNVMEER/OVA (<i>m/z</i> 791.3640)	FALANGHINA	$301 (\pm 4) \cdot 10^3 x - 77 (\pm 38) \cdot 10^3$	0.996	0.4
	PINOT GRIGIO	$212 (\pm 9) \cdot 10^3 x + 10 (\pm 5) \cdot 10^4$	0.991	0.7
	GRECO	$253 (\pm 9) \cdot 10^3 x - 2 (\pm 5) \cdot 10^4$	0.994	0.6
GGLEPINFQTAADQAR/OVA (<i>m/z</i> 844.4230)	FALANGHINA	$731 (\pm 5) \cdot 10^3 x - 120 (\pm 90) \cdot 10^3$	0.996	0.4
	PINOT GRIGIO	$342 (\pm 12) \cdot 10^3 x - 2 (\pm 70) \cdot 10^3$	0.994	0.6
	GRECO	$352 (\pm 12) \cdot 10^3 x - 8 (\pm 7) \cdot 10^4$	0.994	0.6
ELINSWVESQTNGIIR/OVA (<i>m/z</i> 929.9860)	FALANGHINA	$221 (\pm 2) \cdot 10^3 x - 43 (\pm 27) \cdot 10^3$	0.997	0.4
	PINOT GRIGIO	$164 (\pm 6) \cdot 10^3 x - 3 (\pm 4) \cdot 10^4$	0.993	0.7
	GRECO	$149 (\pm 5) \cdot 10^3 x - 3 (\pm 3) \cdot 10^4$	0.994	0.6
NTDGSTDYGILQINSR/LYS (<i>m/z</i> 877.4212)	FALANGHINA	$107 (\pm 6) \cdot 10^3 x - 7 (\pm 4) \cdot 10^4$	0.992	1.1
	PINOT GRIGIO	$96 (\pm 5) \cdot 10^3 x + 3 (\pm 3) \cdot 10^4$	0.989	0.9
	GRECO	$117 (\pm 5) \cdot 10^3 x - 3 (\pm 3) \cdot 10^4$	0.991	0.8
GTDVQAWIR/LYS (<i>m/z</i> 523.2700)	FALANGHINA	$85 (\pm 2) \cdot 10^3 x - 45 (\pm 24) \cdot 10^3$	0.994	0.8
	PINOT GRIGIO	$46 (\pm 2) \cdot 10^3 x + 24 (\pm 14) \cdot 10^3$	0.987	0.9
	GRECO	$58 (\pm 3) \cdot 10^3 x - 14 (\pm 16) \cdot 10^3$	0.990	0.8
Caseinate				
FFVAPFPEVFGK/ α_{s1} -CN (<i>m/z</i> 692.9860)	FALANGHINA	$98 (\pm 3) \cdot 10^4 x - 26 (\pm 14) \cdot 10^4$	0.995	0.4
	PINOT GRIGIO	$117 (\pm 4) \cdot 10^4 x - 5 (\pm 20) \cdot 10^5$	0.994	0.6
	GRECO	$79 (\pm 3) \cdot 10^4 x + 29 (\pm 19) \cdot 10^5$	0.992	0.7
GPFPIIV/ β -CN (<i>m/z</i> 742.4500)	FALANGHINA	$433 (\pm 5) \cdot 10^3 x - 80 (\pm 73) \cdot 10^3$	0.995	0.5
	PINOT GRIGIO	$107 (\pm 5) \cdot 10^4 x - 5 (\pm 3) \cdot 10^5$	0.990	0.8
	GRECO	$74 (\pm 4) \cdot 10^4 x + 2 (\pm 2) \cdot 10^5$	0.988	0.9
YLGYLEQLLR/ α_{s1} -CN (<i>m/z</i> 634.3556)	FALANGHINA	$721 (\pm 25) \cdot 10^3 x - 21 (\pm 15) \cdot 10^4$	0.992	0.6
	PINOT GRIGIO	$113 (\pm 5) \cdot 10^4 x - 5 (\pm 3) \cdot 10^5$	0.990	0.8
	GRECO	$81 (\pm 4) \cdot 10^4 x + 2 (\pm 2) \cdot 10^5$	0.988	0.9

using a tryptic peptide of one of its constituting proteins as a quantitative marker. As shown in the table, values always lower than 1.1 $\mu\text{g/mL}$ could be obtained for caseinate and egg-white powder in the three wines under study. Not unexpectedly, the worst LOD values for the egg-white powder were obtained when peptides arising from lysozyme were used as markers, since the abundance of that protein in egg white is quite low. On the contrary, peptides FFVAPFPEVFGK from α_{S1} -casein and LTEWTSSNWMEER from ovalbumin, i.e. peptides arising from the most abundant proteins in caseinate and egg-white powders, respectively, could be chosen as the best markers for tracing the presence of the two fining agents in white wines.

It is worth noting that the zero value was always included into the 95% confidence intervals obtained for all the calibration line intercepts. This is indirect evidence of the fact that, if present, residual egg-white proteins or caseins eventually introduced in the wine matrices during fining have concentrations well below the LOD values achieved. A direct confirmation of this indication is represented by the absence of response (either from the peptides indicated in Table 3 or from other possible tryptic peptides of ovalbumin, lysozyme and caseins) for the three wines under study when analyzed as such (see the Supporting Information for an example). This result is particularly interesting if the allergological significance of the achieved LODs, discussed later in this paper, is considered.

Evaluation of ultrafiltration recoveries and digestion yields for allergenic proteins

In a further development of the present investigation two important steps of the described analytical method, the recovery through ultrafiltration and the yield of their tryptic digestion, were studied in more detail for the most abundant proteins present in the two fining agents under study, i.e. α -casein and ovalbumin.

In order to evaluate the ultrafiltration recovery the MS responses of their best peptide markers, i.e. FFVAPFPEVFGK and LTEWTSSNWMEER, respectively, were compared for two samples. The first was prepared by digesting the UF extract obtained from a *Falanghina* wine aliquot fortified either with ovalbumin or with α_{S1} -casein at a concentration of 2 $\mu\text{g/mL}$. In the second case ultrafiltration was performed on a blank aliquot of the same wine, the resulting extract was fortified either with ovalbumin or with α_{S1} -casein, using an equal amount as that previously used for spiking the wine, and tryptic digestion was finally accomplished. It is worth noting that the choice of spiking the UF extract with the two proteins, in the second case, aimed at reproducing possible matrix effects during the subsequent digestion. Indeed, wine compounds not discarded by the UF procedure could influence the digestion yield or, eventually, interfere with the detection of tryptic peptides, thus influencing the marker response. The ratio between the XIC peak areas obtained for a specific peptide marker from the two samples previously described provides a direct estimate of the UF recovery, provided that the digestion yield does not change significantly in the sample types under comparison. As a result, recoveries of 36 and 20% were obtained for ovalbumin and α_{S1} -casein, respectively.

A more complex approach was required to estimate the protein digestion yields. In particular, the isotopically labelled analogues of the best markers of α_{S1} -casein and ovalbumin were employed: FFV*APFPEV*FGK and LTEWTSSNV*MEER, bearing two and one ^{15}N atoms, respectively, located on the valine residues. In a preliminary test, aimed at the assessment of their isotopical purity, both peptides were analyzed by infusing their standard solutions into the Exactive mass spectrometer. As the peptides were detected as doubly charged ions their m/z ratios were found to be shifted by 1 and 0.5 units, respectively, compared to those of their non-labelled analogues and no evidence for the presence of signals certainly related to unlabelled peptides (i.e. those related to the M isotopologues of such peptides) was obtained.

In order to calculate the digestion yield, the UF extract relevant to a blank *Falanghina* aliquot was spiked with a known amount of ovalbumin or α_{S1} -casein, according to the case. Subsequently, the concentration expected in the final digests for each of the two peptides if a 100% digestion had occurred (i.e. if one peptide molecule was generated from each protein molecule) was calculated. The amounts of the isotopically labelled peptides required to reproduce those concentrations were then added to the final digests, using small aliquots of their concentrated stock solutions. Generally speaking, the digestion yield could be estimated from the ratio between the peak areas obtained from the XIC traces relevant to the unlabelled and the isotopically labelled peptides, respectively, provided that no interference on the signal of the labelled peptides arose from specific isotopologues of the natural peptides. However, in this case the interference due to the M+2 and to the M+1 isotopologues of the unlabelled peptides FFVAPFPEVFGK and LTEWTSSNVMEER, respectively, had to be considered. In particular, once the peak areas for the M isotopologues of the non-labelled peptides were known, the contribution due to the cited isotopologues to the peak areas of the labelled peptides could be calculated, using the isotopic patterns of the unlabelled peptides.

The digestion yields calculated according to the present procedure were found to be 55% for ovalbumin and approximately 100% for α_{S1} -casein (considering the lowest purity indicated for this protein by its commercial source, i.e. 70%).

If the UF recovery and the digestion yield are multiplied an estimate of the method efficiency, i.e. the percentage of fining protein molecules that are successfully recovered from the wine matrix and subsequently digested by trypsin, can be predicted. As a result, method efficiency was calculated to be about 20% for ovalbumin and α_{S1} -casein.

With the aim of evaluating the method efficiency experimentally, a *Falanghina* wine sample fortified with ovalbumin at 2 $\mu\text{g/mL}$ concentration was processed according to the entire procedure but the final digest was spiked with the isotopically labelled marker of ovalbumin, the peptide LTEWTSSNV*MEER, at a concentration corresponding to that expected for the natural peptide by assuming 100% method efficiency. The isotopic pattern observed for the peptide $[\text{M}+2\text{H}]^{2+}$ ion in the MS spectrum averaged under the peptide chromatographic peak is depicted in Fig. 3. The contribution due to the isotope pattern relevant to the $[\text{M}+2\text{H}]^{2+}$ ion of the labelled peptide is apparent (see, for example, the peak for its M isotopologue, at m/z 791.8627), although the peculiar peak of the unlabelled

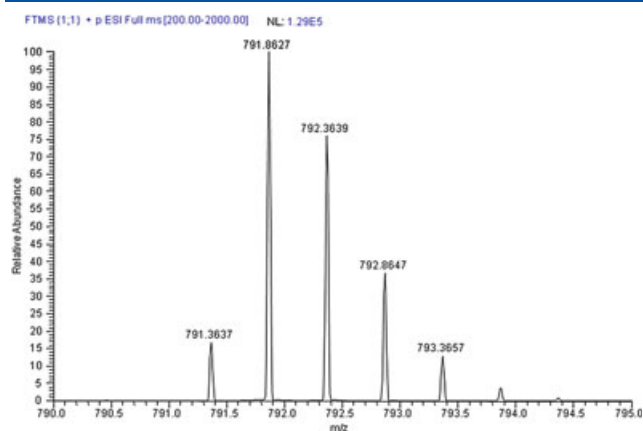


Figure 3. Isotopic pattern observed for the $[M+2H]^{2+}$ ion of the ovalbumin tryptic peptide LTEWTSSNVMEER in the high-resolution mass spectrum averaged under the corresponding chromatographic peak and relative to a white wine sample fortified with $2\text{ }\mu\text{g/mL}$ of ovalbumin. The digest was spiked with an appropriate amount of the ^{15}N -isotopically labelled analogue of the peptide to estimate the digestion yield. See text for details.

peptide (M isotopologue at m/z 791.3637) can be clearly observed too. The method efficiency was then easily estimated by rationing the XIC peak areas obtained for the M isotopologues of the natural and the isotopically labelled peptide after correcting the latter for the contribution due to the $[M+2H]^{2+}$ ion of the M+1 isotopologue of the natural peptide. The same procedure was adopted for a wine sample spiked with α_{S1} -casein at $2\text{ }\mu\text{g/mL}$ concentration; in this case the final digest was spiked with the peptide FFV*APFPEV*FGK, labelled on the two valine residues. As a result, method efficiencies identical to those previously calculated were estimated for ovalbumin and α_{S1} -casein.

Comparison between achieved LOD values and allergological thresholds

In the last step of this study the limits of detection (LODs) achieved with the described multi-allergen method for milk- or egg-derived fining products were evaluated in terms of compliance with threshold values estimated for milk and egg allergens.

A great deal of data regarding the human allergic response to food, usually based on oral challenge trials, has been gathered in the last years, showing that a high variability exists between the individual thresholds.^[26–28] Recently, the VITAL Scientific Expert Panel, born from a collaboration between the *Allergen Bureau* (Australia & New Zealand), the *Food Allergy Research & Resource Program* (FARRP) of the University of Nebraska (USA) & the *Netherlands Organization for Applied Scientific Research* (TNO), has actively worked at the establishment of "allergens action levels" in the VITAL grid. The panel recognized that sufficient threshold data, arising from clinical oral challenges, have been collected for the major food allergens to allow the development of dose-distribution relationships using statistical modeling, as described by Crevel *et al.*^[29] The dose-response curves enable the identification of an allergen dose at which a proportion of the allergic population would likely react, although they

cannot identify a dose below which no allergic individual would react. In the case of milk and egg allergens the panel recommended the reference doses to be set at 0.1 mg and 0.03 mg , respectively, corresponding to an elicit dose for 1% of the studied population of allergic individuals (ED01). If 200 mL are considered as the volume representative of a normal single consumption of wine by an individual, the two cited reference doses would correspond to 0.5 and $0.15\text{ }\mu\text{g/mL}$ threshold concentrations for milk and egg proteins dissolved in wines, respectively. On the other hand, data reported in the Informall Database on Food Allergens report different thresholds for milk and egg allergens, based on the LOAEL (*Lowest Observed Adverse Effects Level*) values available in the literature. Indeed, thresholds of 0.6 and 0.18 mg have been set for milk and egg allergens, respectively, in this database. These values would translate into threshold concentrations of 3 and $0.9\text{ }\mu\text{g/mL}$ for milk and egg proteins, respectively, by assuming the same single consumption of wine (200 mL) by allergic individuals. The LOD values achieved by the MS-based method described in the present investigation are thus comprised between the threshold concentrations estimated according to different approaches. This makes the method a promising approach for a multi-allergen screening, at allergologically relevant concentrations, of egg- and milk-related products added to wines for fining purposes.

CONCLUSIONS

The combination of ultrafiltration, tryptic digestion and LC/ESI-MS analysis based on a high-resolution mass spectrometer enabled the development of an analytical method capable of detecting and quantifying simultaneously traces of caseinate and egg-white powders potentially remaining in white wines upon fining. In particular, eight peptide markers arising from some of the proteins composing such fining agents, i.e. caseins, ovalbumin and lysozyme, were selected as possible quantitative markers, since their MS responses were linearly correlated with fining agent concentrations added to white wines in a $0.25\text{--}10\text{ }\mu\text{g/mL}$ range. The achieved LODs were found to range between 0.4 and $1.1\text{ }\mu\text{g/mL}$, according to the peptide marker and the fining agent considered. These values fall between the minimal concentrations of milk and egg proteins that, according to different sources of allergological data, could elicit a response in allergic individuals after a typical single consumption of wine (200 mL).

Targeted experiments, performed through the implementation of isotopically labelled variants of selected peptide markers, allowed the calculation of the digestion yields for the most abundant proteins of the two fining agents under study, ovalbumin and α_{S1} -casein, that were found to be 55 and 100% respectively. The total yield of the analytical procedure, meant as the product of ultrafiltration recovery and digestion yield, could also be estimated.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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REFERENCES

- [1] I.C. Guerrero, O. Kleiner. Application of mass spectrometry in proteomics. *Biosci. Rep.* **2005**, 25, 71.
- [2] G. Picariello, G. Mamone, F. Addeo, P. Ferranti. The frontiers of mass spectrometry-based techniques in food allergenomics. *J. Chromatogr. A* **2011**, 1218, 7386.
- [3] T. Wehr. Top-down versus bottom-up approaches in proteomics. *LC GC No. Am.* **2006**, 24, 1004.
- [4] C. Bignardi, L. Elviri, A. Penna, M. Careri, A. Mangi. Particle size column versus silica-based monolithic column for liquid chromatography-electrospray-linear ion trap-tandem mass spectrometry multiallergen trace analysis in foods. *J. Chromatogr. A* **2010**, 1217, 7579.
- [5] J. Heick, M. Fischer, B. Popping. First screening method for the simultaneous detection of seven allergens by chromatography mass spectrometry. *J. Chromatogr. A* **2011**, 1218, 938.
- [6] P. Johnson, S. Baumgartner, T. Aldick, C. Bessant, V. Giosafatto, J. Heick, G. Mamone, G. O'Connor, R. Poms, B. Popping, A. Reuter, F. Ulberth, A. Watson, L. Monaci, E. N. C. Mills. Current perspectives and recommendations for the development of mass spectrometry methods for the determination of allergens in foods. *J. AOAC Int.* **2011**, 94, 1026.
- [7] J.J. Castillo-Sánchez, J.C. Mejuto, J. Garrido, S. García-Falcón. Influence of wine-making protocol and fining agents on the evolution of the anthocyanin content; colour and general quality of Vinhão wines. *Food Chem.* **2006**, 97, 130.
- [8] F.X. Sauvage, B. Bach, M. Moutounet, A. Vernhet. Proteins in white wines: Thermo-sensitivity and differential adsorption by bentonite. *Food Chem.* **2010**, 118, 26.
- [9] S. Kirschner, B. Belloni, C. Kugler, J. Ring, K. Brockow. Allergenicity of wine containing processing aids: a double-blind; placebo-controlled food challenge. *J. Investig. Allergol. Clin. Immunol.* **2009**, 19, 210.
- [10] European Commission **2007**. Commission Directive 2007/68/EC. *Off. J. Eur. Union* L310:11.
- [11] P. Weber, H. Steinhart, A. Pascke. Investigation of the allergenic potential of wines fined with various proteinogenic agents by ELISA. *J. Agric. Food Chem.* **2007**, 55, 3127.
- [12] J. M. Rolland, E. Apostolou, M. P. de Leon, C. S. Stockley, R. E. O'Hehir. Specific and sensitive enzyme-linked immunosorbent assays for analysis of residual allergenic food proteins in commercial bottled wine fined with egg white, milk, nongrape derived tannins. *J. Agric. Food Chem.* **2008**, 56, 349.
- [13] A. Lifrani, J. Dos Santos, M. Dubarry, M. Rautureau, F. Blachier, D. Tome. Development of animal models and sandwich-ELISA tests to detect the allergenicity and antigenicity of fining agent residues in wines. *J. Agric. Food Chem.* **2009**, 57, 525.
- [14] P. Restani, F. Uberti, R. Danzi, C. Tarantino, C. Ballabio, F. Pavanello. Absence of allergenic residues in experimental and commercial wines fined with caseinates. *Food Chem.* **2012**, 134, 1438.
- [15] P. Weber, H. Steinhart, A. Pascke. Determination of the bovine food allergen casein in white wines by quantitative indirect ELISA, SDS-PAGE, Western blot and immunostaining. *J. Agric. Food Chem.* **2009**, 57, 8399.
- [16] L. Monaci, I. Losito, F. Palmisano, A. Visconti. Reliable detection of milk allergens in food using a high-resolution stand-alone mass spectrometer. *J. AOAC* **2011**, 94, 1034.
- [17] S. Tolini, G. Pasini, A. Curioni, G. Arrigoni, A. Masi, F. Mainente, B. Simonato. Mass spectrometry detection of egg proteins in red wines treated with egg white. *Food Control* **2012**, 23, 87.
- [18] A. Cereda, A.V. Kravchuk, A. D'Amato, A. Bachi, P.G. Righetti. Proteomics of wine additives: mining for the invisible via combinatorial peptide ligand libraries. *J. Proteomics* **2010**, 73, 1732.
- [19] L. Monaci, I. Losito, M. Godula, A. Visconti. Towards the quantification of milk allergens in caseinate fined white wines using HPLC coupled with single stage Orbitrap mass spectrometry. *Food Addit. Contam.* **2011**, 28, 1304.
- [20] S. Tolini, G. Pasini, G. Simonato, F. Mainente, G. Arrigoni. Analysis of commercial wines by LC-MS/MS reveals the presence of residual milk and egg white allergens. *Food Control* **2012**, 28, 321.
- [21] P. Lutter, V. Parisod, H. Weymuth. Development and Validation of a method for the quantification of milk proteins in food products based on liquid chromatography with mass spectrometric detection. *J. AOAC Int.* **2011**, 94, 1.
- [22] G.A. Newsome, P.F. Scholl. Quantification of allergenic bovine milk α -S1-casein in baked goods using an intact N-labeled protein internal standard. *J. Agric. Food Chem.* **2013**, 61, 5659.
- [23] H.D. Belitz, W. Grosch, P. Schieberle. *Food Chemistry*, (4th edn.). Springer-Verlag, Berlin, **2009**, pp. 548–549.
- [24] ExPASy Bioinformatics Resource Portal. Swiss Institute of Bioinformatics (SIB), **2012**. FindPept tool; <http://www.expasy.ch/tools/findpept.html>.
- [25] Protein Prospector Portal. University of California, San Francisco, **2012**. MS-Product tool; <http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct>.
- [26] S. L. Taylor, J. O. B. Hourihane. Food allergen thresholds of reactivity, in *Food Allergy – Adverse Reactions to Foods and Food Additives*, (4th edn.), (Eds: D. D. Metcalfe, H. A. Sampson, R. A. Simon), Blackwell Publishing, Hoboken, NJ, USA, **2008**, pp. 82–89.
- [27] S. L. Taylor, R. W. R. Crevel, D. Sheffield, J. Kabourek Baumert. Threshold dose for peanut: a risk assessment based upon results from challenges of peanut-allergic individuals. *Food Chem. Toxic.* **2009**, 47, 1198.
- [28] S. L. Taylor, S. M. Gendel, G. F. Houben, E. Julien. The key events dose-response framework: a foundation for examining variability in elicitation thresholds for food allergens. *Crit. Rev. Food Sci. Nutr.* **2009**, 49, 729.
- [29] R. W. R. Crevel, D. Briggs, S. L. Hefle, A. C. Knulst, S. L. Taylor. Hazard characterization in food allergen risk assessment: the application of statistical approaches and the use of clinical data. *Food Chem. Toxicol.* **2007**, 45, 691.

SUPPLEMENTAL MATERIAL

Figure S1. Example of a blank (non fortified) aliquot of *Falanghina* wine digested and analysed by HR-MS. The figure shows an overlay of eight chromatograms filtered on the accurate masses of the eight selected peptide ions (mass tolerance: 10 ppm).

Figure S1.

