JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Development of a Method for the Quantification of Caseinate Traces in Italian Commercial White Wines Based on Liquid Chromatography−Electrospray Ionization−Ion Trap−Mass Spectrometry

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S Supporting Information

ABSTRACT: A method using the combination of size exclusion−solid phase extraction and ultrafiltration, followed by tryptic digestion and analysis of the protein digest by liquid chromatography−electrospray ionization−3D ion trap−mass spectrometry (LC-ESI-3D IT-MS), was developed for the detection and quantification of caseinate traces potentially resulting from fining processes in white wines. In particular, several tryptic peptides generated from the main proteins constituting caseinate (β-, α_{S1}-, and α_{S2}-caseins) were used as markers of its presence in the wine matrices; among them, the β-casein peptide GPFPIIV was found to be the best marker for quantification purposes. Method linearity and sensitivity were assessed on a series of Italian commercial white wines, first checked for the absence of any peptide signal attributable to caseins introduced during their production and subsequently spiked with increasing concentrations of caseinate, to provide samples for matrix-matched calibrations. Limits of detection ranging between 0.09 and 0.29 mg/L (S/N = 3), according to the wine, were achieved using a 10 mL sample volume and the MS signal of GPFPIIV as the response related to the caseinate concentration. Such levels are comparable or even lower than the one (0.25 mg/L) recently adopted as a threshold by European Union legislation concerning the indication of milk- and egg-derived fining agents on wine labels, that is, the most restrictive one among those currently proposed in the world.

KEYWORDS: *milk allergens, wine fining, caseins and caseinate, peptide markers, liquid chromatography−3D ion trap−mass spectrometry*

INTRODUCTION

The risk posed to allergic consumers by the possible presence of milk-, egg-, and fish-derived protein residues in wines has been the object of a very active debate in recent years. In fact, products containing milk- (caseinates or milk powders), egg- (ovalbumin or egg-white powders), or fish-derived (isinglass or fish gelatin) proteins are often used during wine fining. In particular, such proteins are added to wine to promote interactions with undesirable compounds, especially (poly)-phenolic ones, aiming at their partial removal through precipitation.[1] Unfortunately, the presence of protein traces in the final product cannot be excluded.

To protect allergic consumers, the Australia and New Zealand Food Standards Code has required wines (as well as all other food products) to carry a declaration on their labels about the presence of renown protein allergens since 2002.[2] After some deferments, the declaration of milk- and egg-derived products on wine labels has become mandatory also in the European Union since July 1, 2012.[3] Similar regulations have also come into force in Canada since August 4, 2012.[4] Despite these legislative developments a remarkable degree of uncertainty still exists on the actual need for indicating potentially allergenic fining agents on wine labels. As an example, one of the key aspects of the new Canadian regulation is represented by the classification of a wine fining agent as either a *food additive* or a *processing aid*. In the latter case the final concentration of the agent would be, by definition, negligible; thus, no significant risk would exist for protein-allergic wine consumers and its indication on the product label would not be mandatory. However, no concentration threshold has been indicated by the Canadian authorities to enable such a distinction. On the contrary, the implementing regulation 579/2012 issued by the European Commission[3] provides, although not explicitly, a quantitative limit to decide about the indication of fining agents on wine labels. The limit is related to the detection of fining agents in the final product using the analytical methods referred to in Article 120g of Regulation 1234/2007,[5] that is, the methods issued by the Organisation Internationale de la Vigne et du Vin (OIV). Following an interlaboratory trial based on an enzyme-linked immunosorbent assay (ELISA),[6] the *Criteria for the Methods of Quantification of Potentially Allergenic Residues of Fining Agents Proteins in Wines* have been published by OIV in 2012.[7] In this

Received: August 6, 2013
Revised: October 22, 2013
Accepted: November 26, 2013
Published: November 26, 2013

© 2013 American Chemical Society

dx.doi.org/10.1021/jf4034909 | J. Agric. Food Chem. 2013, 61, 12436−12444

document detection/quantification limits ≤0.25/0.50 mg/L are indicated among method performance criteria. Such values represent the most restrictive thresholds currently posed for the indication of fining agents on wine labels. Actually, several applications of ELISA or other immunochemical methods for the detection of residual fining agents in wine have been reported in the literature in recent years,[8−14] with detection limits ranging between a few micrograms per liter and several milligrams per liter, according to the fining agent, the wine matrix, and the experimental conditions adopted. Very recently, two international collaborative tests have been performed using ELISA methods to quantify caseins and egg white powders in white and red wines, respectively.[15] LOD values ranging between 0.15 and 0.35 mg/L have been reported, thus confirming the performance criteria issued by OIV, although problems related to outliers and low recoveries have been occasionally encountered.

As an alternative to immunochemical approaches, mass spectrometry (MS) has been applied to the detection and quantification of residual caseins or egg white proteins in white or red wines in the past three years. In particular, the possibility of detecting and quantifying by MS residual caseins or ovalbumin/lysozime resulting from the addition of caseinate or egg-white powders to raw or commercial white wines has been demonstrated in our laboratories,[16−19] with the lowest detection limits ranging between 0.4 and 1 mg/L. In these investigations mass spectrometry represented the last step of a procedure including, in chronological order, a preliminary extraction of proteins from the wine matrix based on ultrafiltration, their digestion with trypsin, and the separation, detection, and quantification of selected tryptic peptides, used as quantitative markers, by liquid chromatography coupled to single (MS) and tandem (MS/MS) mass spectrometry. A method based on the recovery of proteins by potassium dodecyl sulfate-mediated precipitation, followed by their tryptic digestion and the liquid chromatography−tandem MS analysis of the digests, has been developed by other authors to detect egg proteins in red wines purposely spiked with a commercial egg white preparation, often used as a fining agent.[14] Subsequently, the presence of residues of caseins and egg-white proteins in a series of commercial wines has been ascertained using the same method, although no quantification has been performed.[20] The cited studies were all based on high-sensitivity/resolution mass spectrometry instrumentations, such as quadrupole time-of-flight (Q-ToF) and Orbitrap mass spectrometers. In other papers[21,22] liquid chromatography−mass spectrometry was applied to protein digests to assess the identity of residual fining proteins, extracted from the wine matrices by a complex approach based on combinatorial peptide ligand libraries (CPPLs), then separated and quantified using gel electrophoresis. Limits of detection down to the micrograms per liter scale were reported in this case, although the risk of interferences on fining agent quantification due to grape proteins or even wine polyphenolic compounds, coextracted with the proteins of interest and sometimes comigrating with them during the electrophoretic separation, could not be excluded.

Although promising from the point of view of sensitivity, the methods cited so far can hardly make mass spectrometry a realistic alternative, in terms of sharpness of the procedure and/or availability of instrumentation, to ELISA or similar approaches. From the perspective of a large-scale application to the analysis of residual proteins in wines, MS-based methods characterized by a suitable sensitivity but also implying a more accessible instrumentation could represent a good compromise between cost containment and reliability of the analysis. Work in this direction has been thus undertaken in our laboratory. In the present paper the development of a method for the analysis of residual caseinate in commercial white wines, based on a 3D ion trap mass spectrometer and characterized by LOD values close to or even lower than the ELISA-related values indicated by OIV, will be described.

■ MATERIALS AND METHODS

Chemicals and Wine Samples. LC-MS grade acetonitrile and water, used as solvents for the HPLC mobile phase, analytical grade formic acid, used as a mobile phase additive, ammonium hydrogen−carbonate, tris(hydroxymethyl)aminomethane (TRIS), hydrochloric acid, iodoacetamide, and dithiothreitol (DTT) were purchased from Sigma-Aldrich (Milan, Italy). Trypsin (proteomic grade) was purchased from Promega (Madison, WI, USA). The Rapigest surfactant, used during tryptic digestions as a protein denaturating agent, was purchased from Waters-Micromass (Manchester, UK). α-Casein (actually a mixture of α_{S1}- and α_{S2}-caseins, ≥70% purity, vendor code C6780), β-casein (≥98% purity, vendor code C6905), κ-casein (≥70% purity, vendor code C0406), and sodium caseinate (vendor code C8654) were purchased from Fluka/Sigma-Aldrich and used without further purification. Stock solutions of single caseins or caseinate were freshly prepared, at different concentrations, in 50 mM NH_4HCO_3 and used as such or, in the case of caseinate, also exploited to spike wine samples.

Eight commercial Italian white wines, produced using the grape varieties Trebbiano d'Abruzzo, Pinot Grigio, Moscato Bianco (sparkling wine), Greco di Tufo, Müller-Thurgau, Chardonnay, Vermentino di Sardegna, and Garganega-Trebbiano, were considered for method preliminary testing and/or for screening purposes. All wines were purchased from local stores.

Extraction of Residual Caseins from White Wines as Such or Spiked with Caseinate. To separate residual caseins from potentially interfering wine phenolic compounds, a two-step procedure, based on a combination between size exclusion−solid phase extraction (SE-SPE) and ultrafiltration (UF), was finally devised.

The white wines under study were subjected to protein extraction either as such or after being spiked with appropriate caseinate concentrations. In the second case, after the addition of aliquots of the caseinate stock solution, the wines were kept under stirring for 15 min at room temperature and then were left quiescent for a further 15 min. Afterward, a centrifugation at 3900*g* and at room temperature for 10 min was performed to check for the eventual presence of a precipitate.

The SE-SPE step was undertaken by using PD-10 desalting columns manufactured by GE Healthcare (Little Chalfont, UK). Such devices, originally developed for protein or oligosaccharide desalting, could be applied also to casein (and, eventually, grape protein) separation from wine phenolic compounds, due to the 5 kDa exclusion limit provided by the Sephadex G25 medium, employed as stationary phase. Before proceeding with wine loading, the SPE columns were emptied of the storage solution and equilibrated four times with water; afterward, a 2.5 mL wine aliquot was loaded on top of the column and left to enter its packed bed slowly, by gravity force; the flow-through was then discarded. A 3.5 mL volume of LC-MS grade water was subsequently added on top of the column to elute caseins (and, eventually, other wine-contained proteins) still located in the column, although not retained in the pores of its stationary phase. According to the SPE column manufacturer, a percentage ranging between 70 and 95% of proteins characterized by molecular weights of some tens of kilodaltons should be recovered in this fraction. To evaluate the eventual loss of caseins during sample loading or their incomplete removal in the first eluted fraction, both the original flow-through and an additional eluted fraction, obtained by loading a further 3.5 mL water aliquot, were collected during an ad hoc experiment, performed on a caseinate 500 mg/L standard solution. The absence of caseins was checked in both cases by subsequent tryptic digestion and LC-MS

analysis of the digests. When a 10 mL total volume of wine was subjected to protein extraction to enhance method sensitivity (see later), the described SE-SPE step was performed using four columns in a parallel configuration, each loaded with a 2.5 mL aliquot of the same wine.

The coupling between SE-SPE and ultrafiltration (UF) was accomplished by transferring the first 3.5 mL fraction eluting from the desalting column directly into an ultrafiltration tube having a 5 mL capacity and equipped with a 10 kDa cutoff membrane, produced by Merck-Millipore (Cork, Ireland). When 10 mL was used as the initial volume of wine samples, the 3.5 mL fractions obtained from each of the four columns adopted during the SE-SPE procedure were pooled and the resulting 14 mL volume was loaded into a Merck-Millipore 10 kDa cutoff UF tube having a 15 mL capacity. After loading the SE-SPE fraction(s), the UF tubes were subjected to centrifugation at 3900*g* for 40 min; a 1 or 3 mL volume of LC-MS water, according to the UF tube capacity, was subsequently added, and the tube was centrifuged for a further 15 min to wash off the UF retentate and get a cleaner extract. A final volume of nearly 300 μL was collected from the UF filter. Afterward, three different approaches were investigated to prepare the SE-SPE/UF protein extract for the subsequent tryptic digestion, all implying a change of solvent and a further preconcentration. In the first and second cases the SE-SPE/UF extract solvent was evaporated completely by heating at 40 °C or by using a gentle nitrogen stream at room temperature, respectively. In the third case the solvent was only partially evaporated under the nitrogen stream so that a 50 μL residual volume was obtained.

Tryptic Digestion of Protein Extracts. A tryptic digestion protocol based on the use of Rapigest as denaturing agent was adopted during the present investigation. In particular, Rapigest solutions were prepared in 50 mM NH_4HCO_3 and used to dissolve both the proteins to be digested and the reactants required for tryptic digestion. In the case of standard casein or caseinate solutions appropriate aliquots of the corresponding stock solutions were diluted to 50 μL of 0.1% (w/v) Rapigest to reach the final desired concentrations. The protein pellets resulting from the complete evaporation of solvent from the SE-SPE/UF extracts obtained from wine samples were redissolved into 50 μL of the 0.1% Rapigest solution. When the solvent of the SE-SPE/UF extract was evaporated to a residual 50 μL volume, only 25 μL was withdrawn and diluted with the same volume of a 0.2% (w/v) Rapigest solution, to keep both the final volume and the surfactant concentration constant with respect to the other two approaches. Obviously, only half of the amount of extracted proteins could be subjected to tryptic digestion in the latter case (see later).

The digestion procedure was started with a protein reduction step, performed by adding 5 μL of 50 mM DTT solution and incubating the mixture in a thermoshaker at 60 °C for 30 min. After cooling the sample at room temperature, 10 μL of 100 mM iodoacetamide solution was added, and the mixture was left to react in the dark for 30 min. As a final step, 2 μL of trypsin solution (concentration 1 μg/μL in acetic acid 50 mM) was added to the mixture and the tube was gently flicked to mix. The digestion was performed by incubation at 37 °C, under shaking, overnight. At the end of this stage, 5 μL of a 1 M HCl solution was added to the digest and an incubation at 37 °C for 30 min was performed to quench the trypsin activity and hydrolyze Rapigest. Because a precipitation of the Rapigest hydrolysis byproducts occurred after acidification, the digestion mixture was centrifuged at 16000 rpm for 10 min. Subsequently, the pellet was discarded, and the supernatant was carefully transferred into microvials prior to LC-MS analysis.

LC-ESI-MS Instrumentation. The HPLC-ESI-3D ion trap-MS instrumentation used for the analysis of protein tryptic digests consisted of a P680 chromatographic pump (Dionex-Thermo Fisher, San Donato Milanese, Milan, Italy) connected to an LCQ Classic 3D ion-trap mass spectrometer (Thermo-Electron, San Jose, CA, USA) through its ESI interface. HPLC separations of tryptic peptides were carried out at 25 °C and at a flow rate of 0.2 mL/min using a Discovery Bio wide-pore C18 column (250 × 2.1 mm, packing particles size = 5 μm), purchased from Supelco (Bellefonte, PA, USA). A binary gradient elution, based on water and acetonitrile, both containing 0.1% (v/v) formic acid (solvents A and B, respectively), was adopted. The elution program was as follows: from 90 to 30% A in 50 min, return to 90% A in 5 min; column reconditioning time was 20 min. The divert/inject valve embedded in the LCQ mass spectrometer was used for sample injection (sample loop = 20 μL). The electrospray interface and ion optics parameters adopted for peptide analysis were the following: spray voltage, 4.5 kV; sheath gas (nitrogen) flow rate, 1.0 L min^{-1} ; capillary voltage, 14.0 V; heated capillary temperature, 190 °C; tube lens offset voltage, 35.0 V; octapole 1 offset, −3.0 V; octapole 2 offset, −5.0 V; lens voltage, −16.0 V; octapole RF amplitude, 400.0 V; trap DC offset, −10.0 V.

The LCQ Classic spectrometer, entirely controlled by Xcalibur (Thermo-Electron) software, was operated in positive ion mode. Two types of acquisitions were performed for each sample (run time = 60 min). The first was a MS *full scan* in the m/z range 50−2000. The resulting LC-MS total ion current (TIC) trace was subsequently processed by IntelliXtract software (ACD Laboratories, Toronto, Canada) to obtain information on m/z ratios related to well-defined peaks in the digest chromatographic traces, potentially corresponding to tryptic peptides. The m/z ratios thus retrieved were first confirmed by generating the relevant extracted ion chromatograms (XIC) from the TIC traces. Subsequently, their correlation with the m/z values of ions relevant to tryptic or semitryptic peptides of the main bovine caseins was assessed using *FindPept* software, available on the Expasy portal.[23] In this case cysteine carbamidomethylation was set as a fixed modification, whereas methionine oxidation and serine/threonine/tyrosine phosphorylations were set as variable modifications; a 0.5 m/z unit tolerance was adopted for the matching between experimental and predicted m/z ratios of peptides. Subsequently, MS/MS acquisitions were performed, divided into several runs, for all of the experimental m/z ratios that had been found to correspond potentially to casein peptides, to get a final confirmation of the proposed sequences. In particular, the precursor ions were isolated in the 3D ion trap using 4 m/z unit wide isolation windows, centered on the m/z value of the M + 1 isotopologue of the ion of interest, so that the entire isotopic pattern was isolated in the ion trap; a collisional energy of 35% was applied to promote their fragmentation. The predicted fragmentation of each hypothesized peptide sequence was generated using the MS Product program, available in the ProteinProspector portal,[24] and compared with the experimental one. Only sequences accounting for all of the MS/MS peaks having a relative abundance ≥10% were confirmed definitely.

RESULTS AND DISCUSSION

Tryptic Digestion of Single Casein/Caseinate Standard Solutions. To assess the protein sequence coverage achievable through tryptic digestion in Rapigest solution and subsequent LC-MS analysis by the 3D ion trap spectrometer, standard solutions of single caseins (α_{S1}, α_{S2}, β, and κ, each at a 100 mg/L concentration) or caseinate in water (concentrations = 500 mg/L) were first considered. The LC-ESI-MS TIC trace relevant to the tryptic digest of a caseinate 500 mg/L solution is reported in the top panel of Figure 1. Several peaks observed in the trace could be related to tryptic peptides of caseins through the procedure described under Materials and Methods. For the sake of example, the XIC traces relevant to four of these peptides are shown in the central panel of Figure 1 (the traces are scaled for the respective normalization intensity, NL). The MS/MS spectrum relevant to the m/z 742.3 ion is shown in the bottom panel of Figure 1. Product ion assignments leading to its identification as the $[M + H]^+$ ion of the GPFPIIV peptide, located at the carboxylic end of the β-casein sequence, are shown with the MS/MS spectrum. Ions belonging to four (b, y, x, a) of the six types of product ions usually observed in the 3D ion trap-MS/MS spectra of peptides were detected in this case. Moreover, b-type internal ions, all bearing a proline residue at the aminic terminus, as usually expected, were observed. A

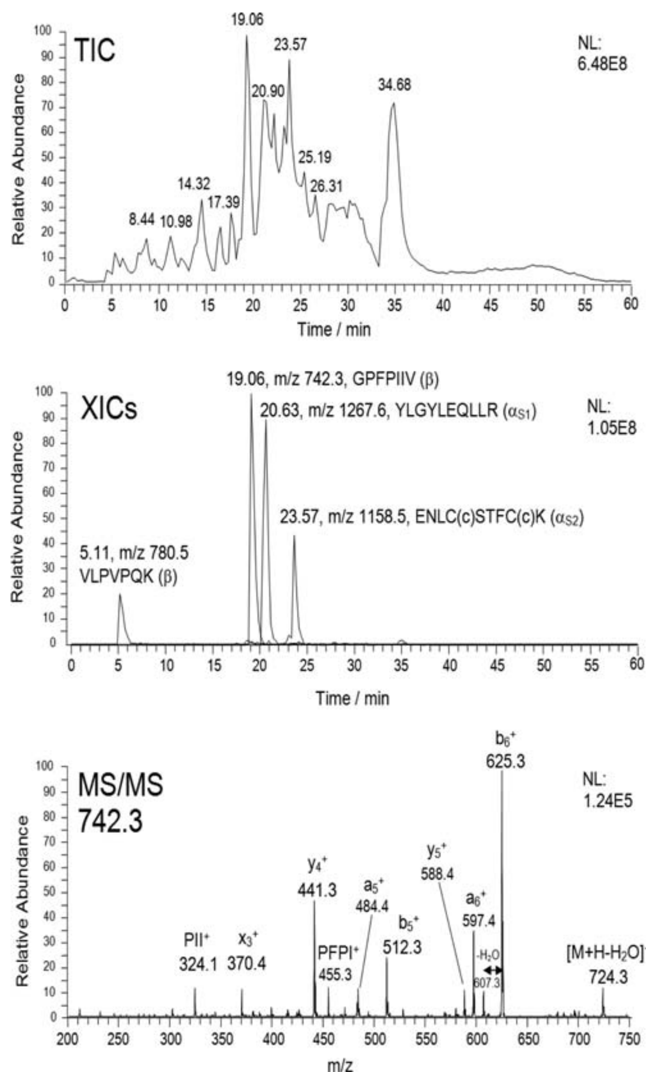

Figure 1. (Top) LC-MS total ion current (TIC) trace relevant to the tryptic digest of a caseinate 500 mg/L aqueous solution. (Center) Extracted ion current (XIC) chromatograms relevant to four m/z ratios corresponding to tryptic peptides of caseins (indicated in parentheses) detected in the digest. (Bottom) MS/MS spectrum referred to the m/z 742.3 ion. The product ion assignments leading to its identification as the $[M + H]^+$ ion of β-casein peptide GPFPIIV are reported.

summary of the peptides identified after each of three replicated tryptic digestions of a 500 mg/L caseinate or single caseins solution is provided in Table S1 in the Supporting Information. Interestingly, some peptides were systematically detected only in one of the two systems but not in the other; this finding suggests that differences in aggregations between caseins in the two systems could influence the yield of digestion on specific protein subsequences. The effect of intermolecular aggregation was particularly evident for κ-casein, for which only one marker could be identified when the single protein was digested. Actually, the high level of glycosylation occurring typically on κ-casein could play a role in the missed detection of unmodified tryptic peptides. In the case of caseinate the low concentration of κ-casein (due to its low abundance in milk) represented an additional negative factor; indeed, no tryptic peptide of the protein could be detected.

An evaluation of the quantitative capabilities of the procedure was performed as a further step, using caseinate standard solutions. In particular, the tryptic digests of caseinate at concentrations of 10, 25, 100, 250, and 500 mg/L were prepared in parallel experiments (three replicates for each concentration). Before the LC-MS analysis was begun, each digest was spiked with the synthetic peptide MRFA, a sequence not belonging to any casein, at a concentration of 100 mg/L. The caseinate concentration dependence of the MS responses for the peptide markers reported in Table S1 in the Supporting Information was evaluated. In particular, the peak areas arising from the corresponding XIC traces were used as responses for calibration purposes after being normalized to the peak area obtained for the MRFA peptide during the same chromatographic run. This normalization was accomplished to compensate for eventual fluctuations in the ionization efficiency of the ESI interface. Actually, only four peptides, belonging to different caseins, could be detected down to the lowest (10 mg/L) caseinate concentration: GPFPIIV from β-casein, FFVAPFPEVFGK and YLGYLEQLLR from α_{S1}-casein, and ENLC(c)STFC(c)K from α_{S2}-casein. Although the GPFPIIV peptide's response was characterized by the best linearity and *between-digest* reproducibility in the 10−500 mg/L range of dissolved caseinate concentration, as shown in Figure 2, the MS

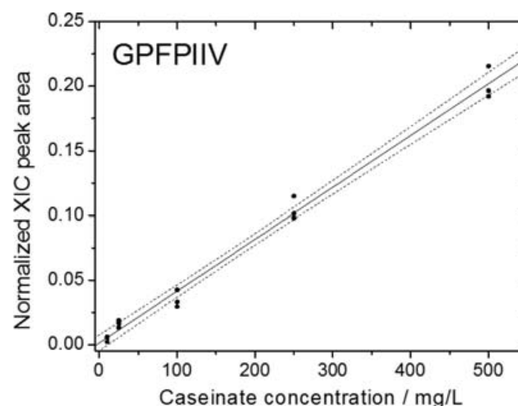

Figure 2. Calibration plot obtained for the β-casein marker GPFPIIV detected in the tryptic digests of standard aqueous solutions of caseinate. The linear regression line (continuous) and the 95% confidence bands (dotted) are shown. The responses correspond to peak areas obtained from the XIC trace of the marker and normalized by the XIC peak area of the internal standard MRFA (100 mg/L concentration).

responses of all the cited peptides were found to be linearly correlated with the concentration of dissolved caseinate. This result was thus considered as a promising starting point toward the analysis of caseinate dissolved into white wine matrices, which was investigated afterward.

Analysis of Commercial White Wines Spiked with Caseinate: Choice of the Best Strategy for Caseinate Extraction and Enrichment. As recently pointed out in the literature,[15,25] the best approach to assess the sensitivity of a method for the analysis of residual fining proteins in wines can be considered spiking wines virtually free of those proteins with different concentrations of the actual fining agents used by winemakers, such as caseinates and egg-white powders, rather than single proteins. Indeed, the eventual interactions occurring between the different proteins contained in those milk- or egg-related fining agents when dissolved into wine matrices may influence both the antibody response, in the case of immunochemical methods, and the recovery and/or tryptic

digestion yield of proteins, in the case of MS-based methods. Such important effects can be reasonably reproduced only by the contemporary presence of all the fining agent-related proteins in the wine matrix. During the present investigation caseinate was thus used to spike commercial white wines and the potential of single casein peptides as quantitative markers not of their proteins of origin but of caseinate itself (as already demonstrated for caseinate dissolved in water) was evaluated.

It is worth noting that ultrafiltration was chosen as the first approach for the extraction of caseins from caseinate-spiked white wines before tryptic digestion and LC-MS analysis of digests, because, despite a relatively low recovery, it had proved to be successful in the detection of spiked caseinate concentrations even of a few milligrams per liter when sensitive MS instrumentation, such as Q-ToF or HCD-Orbitrap mass spectrometers, had been employed for the LC-MS analysis of extracted protein digests.[16−19] Unfortunately, this was not the case when the 3D ion trap spectrometer was adopted; in fact, no response was obtained from any of the possible peptide markers of caseins, including GPFPIIV from β-casein, when four different commercial Italian white wines (Trebbiano d'Abruzzo, Chardonnay, Vermentino di Sardegna, Garganega-Trebbiano) were spiked with caseinate up to 500 mg/L concentrations. As an example, the TIC and the GPFPIIV-related (m/z 742.3) XIC chromatographic traces obtained for the tryptic digest arising from the UF extract of a Trebbiano d'Abruzzo wine spiked with 500 mg/L of caseinate are shown in Figure 3 (first and second panels from the top, respectively). In this case a complex chromatographic band is clearly visible in

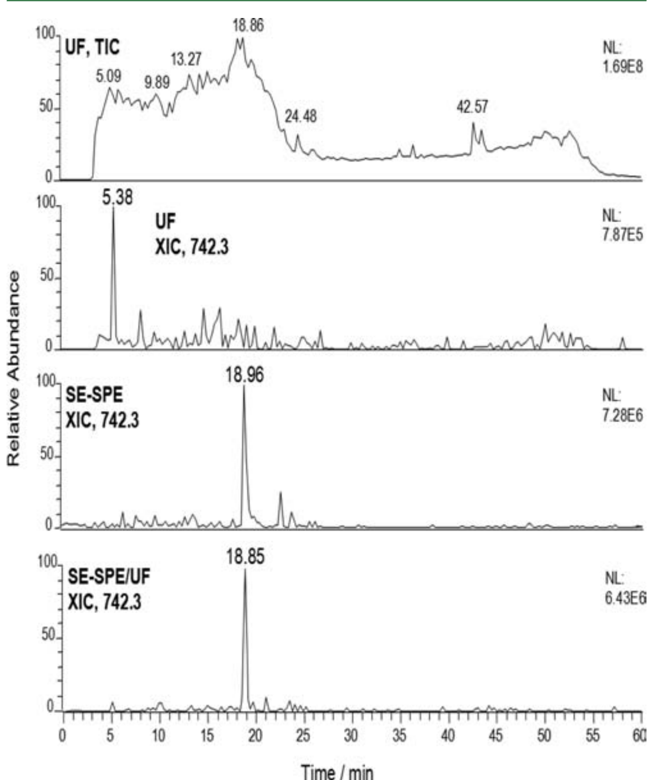

Figure 3. (First and second panels) TIC and GPFPIIV-related XIC traces obtained for the tryptic digest relevant to the UF extract of a Trebbiano d'Abruzzo wine spiked with 500 mg/L of caseinate. (Third and fourth panels) GPFPIIV-related (m/z 742.3) XIC traces obtained for the tryptic digests relevant to the SE-SPE and the SE-SPE/UF extracts of other aliquots of the same spiked wine.

the TIC trace in a time range including the retention time of the β-casein peptide marker (ca. 19 min, see Figure 1). This result suggests that matrix components, not removed by ultrafiltration, can interfere with the detection of caseinate markers when only UF is adopted as the extraction strategy; the effect seems to be overwhelming when a low-sensitivity mass spectrometer is adopted.

To overcome the problem, a different protein extraction strategy was investigated. In particular, SE-SPE was considered as an approach potentially able to improve the cleanup of casein extracts obtained from commercial white wines spiked with caseinate. In fact, the SE-SPE principle of separation of low-to-medium molecular weight matrix interferents from proteins is quite different from that of ultrafiltration. In the latter case protein molecules collected over the 10 kDa cutoff filter in the first stages of ultracentrifugation can hamper the removal of additional low molecular weight molecules, simply because the membrane pores are partly obstructed by protein molecules. When SE-SPE columns are employed, proteins or, more generally, compounds with a molecular weight higher than the cutoff value (5 kDa in this case), and thus not retained at all on the stationary phase, are removed from the column in the first fraction eluted. On the contrary, species having a molecular weight lower than the cutoff value, that is, most of wine phenolic compounds, are entrapped in the stationary phase and can be eluted only in a subsequent fraction. This mechanism was confirmed by a visual inspection of the SPE columns, because the upper portion of the their packing was always found to turn from white to a yellow-light green color after an aliquot of caseinate-spiked white wine was loaded. Moreover, the color did not disappear when the first fraction, that is, the one potentially containing proteins, was eluted.

The possibility of either an incomplete removal of proteins from the SPE column after the first elution or their partial loss already in the flow-through was checked experimentally. In particular, the first and second fractions eluted by a SPE column loaded with the Trebbiano d'Abruzzo wine spiked with caseinate at a 500 mg/L concentration were collected and slowly led to dryness under mild heating (40 °C). The residues were redissolved into a 0.1% w/v Rapigest solution (final volume = 50 μL) and then the tryptic digestion and the LC-MS analysis of the digests were performed. As a result, β-, α_{S1}-, and α_{S2}-casein markers were detected in the tryptic digest of the first fraction, whereas no signal was found for the second fraction. No signal was detected also when the flow-through obtained after loading the wine aliquot on the SPE column was subjected to digestion.

These experiments confirmed that the loss of caseins in the flow-through and in the second fraction obtained from the SPE procedure is negligible and, at the same time, that caseinate added to a commercial wine can be detected through peptide markers originated from its caseins using SE-SPE to extract the proteins. For the sake of example, the XIC trace obtained for the peptide GPFPIIV of β-casein after the LC-MS analysis of the tryptic digest of the first fraction eluted during the SE-SPE extraction of Trebbiano d'Abruzzo wine spiked with 500 mg/L of caseinate is reported in Figure 3 (third panel from the top). The improvement with respect to the UF procedure is apparent.

The MS response of selected peptide markers (i.e., the area of the corresponding peaks in XIC traces like the one shown in Figure 3) could be exploited also to estimate the caseinate recovery provided by the SE-SPE columns. In particular, an

aliquot of Trebbiano d'Abruzzo wine was spiked with caseinate at a 500 mg/L concentration and then loaded in a SE-SPE column; the first fraction eluted was collected and subjected to solvent evaporation and tryptic digestion/LC-MS analysis as described before. In a parallel experiment another aliquot of the same wine was loaded as such (i.e., without added caseinate) in a SE-SPE column and, before proceeding with solvent evaporation and tryptic digestion/LC-MS analysis, the first fraction eluted was spiked with the same amount of caseinate dissolved in the first aliquot of wine. Because the final volumes obtained for the tryptic digests were the same in the two cases, the MS responses of two selected tryptic peptides, that is, GPFPIIV from β-casein and YLGYLEQLLR from α_{S1}-casein, were used to estimate the recovery for the two main proteins of caseinate. The calculation was based on the consideration that the MS response obtained for those peptides was proportional to their final concentration in the digests and then to the concentration of their proteins of origin before the digestion, provided the digestion yields were comparable in the two type of samples, which is a reasonable assumption. As a result, recoveries of 85 ± 8 and 80 ± 6% (mean ± sd calculated for three replicated experiments) were obtained for β-casein and α_{S1}-casein, respectively. These results confirm that the indications provided by the manufacturer about the SE-SPE recovery of proteins are applicable also when caseinate proteins are dissolved into a complex matrix such as white wine.

Altough appearing quite promising in terms of caseinate detection, the SE-SPE procedure now described showed a significant drawback from a practical point of view. In fact, a long evaporation step (almost 12 h, due to the use of a mild heating temperature, 40 °C, to minimize protein thermal degradation) was required to eliminate the aqueous solvent from the protein-containing 3.5 mL fraction eluted from the SPE column, before proceeding with the redissolution in Rapigest solution, in which the tryptic digestion was accomplished. To overcome this drawback, a hybrid strategy was devised for casein extraction from wines, that is, collecting the first fraction eluted from the SPE column directly into a 10 kDa cutoff UF tube and then proceeding to ultrafiltration. A remarkable decrease of the final volume of the protein extract (from 3.5 to <0.5 mL) and, consequently, of the time required for the subsequent solvent evaporation, was actually accomplished, by implementing ultrafiltration. Another potential advantage of this hybrid approach was that eventual matrix interferents having a molecular weight between 5 and 10 kDa, certainly collected in the first fraction eluted from the SPE column, could be eliminated through ultrafiltration on 10 kDa cutoff tubes. Interestingly, a very low response difference was observed between the SE-SPE and SE-SPE/UF approaches when applied to wine aliquots spiked with the same caseinate concentrations, as shown by the normalization levels relevant to the XIC traces of the GPFPIIV peptide in Figure 3 (third and fourth panels from the top). This finding indicates that the protein recovery provided by the UF step is comparable to that obtained by the SE-SPE approach. The SE-SPE/UF approach was thus chosen as the best compromise between complexity and duration of the extraction procedure.

Subsequently, attention was focused on the preliminary step required for protein tryptic digestion, that is, the replacement of the protein extract solvent with a basic solution of Rapigest. In particular, a comparison was made between three different strategies applied to the SE-SPE/UF extract arising from a Trebbiano d'Abruzzo wine spiked with 5 mg/L of caseinate. In the first two cases the extract solvent was completely evaporated either by mild heating (40 °C) or by a nitrogen stream, and the protein pellet was subsequently redissolved into 50 μL of 0.1% (w/v) Rapigest. As shown in Figure 4, a

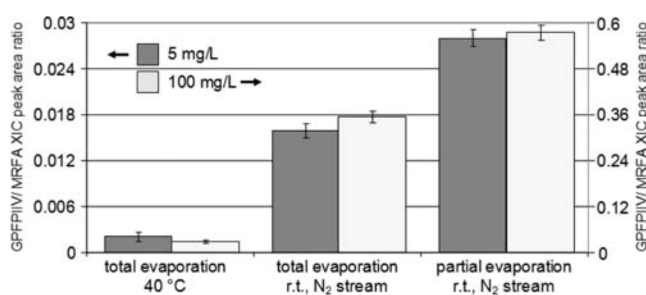

Figure 4. Comparison of XIC peak areas, normalized to the XIC peak area of MRFA (100 mg/L), obtained for the GPFPIIV marker in the tryptic digests of SE-SPE/UF extracts arising from 2.5 mL of Trebbiano d'Abruzzo wine spiked with 5 (left axis) or 100 (right axis) mg/L of caseinate and related to different strategies of extraction solvent removal before digestion. Error bars represent the standard deviations obtained from three replicated experiments. See text for details.

significant increase (almost an order of magnitude) was achieved in the latter case for the MS response of the β-casein marker GPFPIIV. Even more surprisingly, a further response increase was obtained when the extract solvent was evaporated only partially, leaving a residual 50 μL volume, half of which was subsequently diluted to 50 μL with a 0.2% (w/v) Rapigest solution. Despite the fact that only half of the extracted caseinate amount could be actually subjected to tryptic digestion, in this case the GPFPIIV marker response was almost doubled (see Figure 4). These results suggest that aggregation phenomena occurring between caseinate proteins when the extraction solvent is eliminated completely can influence significantly the subsequent digestion yield. In particular, the interprotein aggregation could mask some of the trypsin cleavage sites located along the protein chains, with a consequent decrease in digestion yield. Additionally, the possibility that caseinate proteins could stick to the tube walls after the complete evaporation of solvent and not be completely redissolved into the Rapigest solution before digestion cannot be excluded.

As shown in Figure 4, the same trend in MS responses was observed when aliquots of Trebbiano d'Abruzzo spiked with 100 mg/L of caseinate were analyzed using the three procedures. Interestingly, as emphasized by the double-ordinate scale adopted in Figure 4, the 20-fold increase expected for the peptide response when passing from the 5 to the 100 mg/L spiked wine was observed in all cases, thus suggesting a good response linearity in the corresponding concentration range. However, a better *between-sample* reproducibility could be achieved when using a nitrogen stream rather than heating to promote extract solvent evaporation (see the error bars in Figure 4, referred to standard deviations for three replicated experiments in each case). It is worth noting that freeze-drying could represent a further possible approach to the reduction, or even the elimination, of solvent from the SE-SPE/UF extracts before redissolution in Rapigest. However, it was not considered in this case because, as shown in the following, partial solvent evaporation under nitrogen was able to provide the required levels of sensitivity through an easier and more

accessible procedure not requiring expensive instrumentation, thus fulfilling one of the main goals of the present investigation.

The sensitivity of the procedure implying the partial removal of the extraction solvent and the 1:1 dilution with the Rapigest solution before tryptic digestion was checked on progressively lower added concentrations of caseinate. In particular, three further added caseinate concentrations (0.5, 1, and 10 mg/L) were tested on the Trebbiano d'Abruzzo wine. A good linearity was observed in the 0.5−100 mg/L range (R^2 = 0.9944), yet the LOD value associated with the GPFPIIV response (calculated at S/N = 3, using the standard deviation of the response interpolation line intercept as an estimate of noise), 1.1 mg/L, was significantly higher than the 0.25 mg/L limit dictated by OIV.

To achieve the 4-fold increase in the LOD value required for compliance with the OIV limit, an increase of the protein preconcentration factor was attempted by using a higher wine volume. In particular, 10 mL of Trebbiano d'Abruzzo wine spiked with 5 mg/L of caseinate was loaded into four parallel SE-SPE columns, and the resulting 4 × 3.5 mL SE-SPE fractions were pooled and loaded into a high-capacity (15 mL) ultrafiltration tube, as described under Materials and Methods. A clear increase in the peptide marker response was obtained, compared to the analysis of 2.5 mL of wine spiked with caseinate at the same level, thus confirming that the higher amount of caseinate finally recovered from the SE-SPE/UF procedure was not detrimental in terms of tryptic digestion yield.

A new calibration was thus performed using 10 mL aliquots of Trebbiano d'Abruzzo wine spiked with lower caseinate concentrations: 0.1, 0.2, 1, 2, and 5 mg/L (three replicates for each concentration). A good linearity was observed also in this range (R^2 = 0.9976), and a LOD of 0.29 mg/L (S/N = 3) was obtained when using the MRFA-normalized XIC peak area of the GPFPIIV peptide as a response. The new LOD value corresponds to almost one-fourth of the previous LOD, as expected, and is very close to the target value, 0.25 mg/L. Interestingly, MS responses due to further tryptic peptides arising from caseins could be observed during the same calibration, even at low concentrations (0.1−0.2 mg/L), and were found to be linearly correlated with caseinate concentration, although the corresponding LOD values were higher than the one achieved using the GPFPIIV peptide. This result is emphasized by Figures S1 and S2 of the Supporting Information, in which calibration plots, linear regression parameters (95% confidence intervals for slope and intercept, correlation coefficients), and LOD values (S/N = 3) related to peptides GPFPIIV and VLPVPQK from β-casein, YLGYLEQLLR from α_{S1}-casein, and FALPQYLK from α_{S2}-casein are reported. It is worth noting that GPFPIIV did not show the highest sensitivity among the four peptides, yet the better reproducibility of its MS response, especially at higher caseinate concentrations, led to the best LOD value.

The XIC traces relevant to the four peptides and referred to the tryptic digest corresponding to the Trebbiano d'Abruzzo sample initially spiked with a 0.2 mg/L concentration of caseinate, that is, a value lower than the best LOD achievable, are shown in Figure 5, along with the XIC trace referred to the MRFA peptide (100 mg/L). As discussed previously, the latter was added to the protein digests only for MS response normalization purposes, that is, to compensate for the variations in ESI efficiency often experienced, over prolonged analysis sessions, with the LCQ Classic spectrometer; if not accounted for, such variations would have affected the method performances negatively. Indeed, the XIC peak areas obtained for MRFA during the calibration procedure relevant to the Trebbiano d'Abruzzo wine showed a 15% between-analysis variability (relative standard deviation calculated for 15 replicates, performed over a 2 day time range).

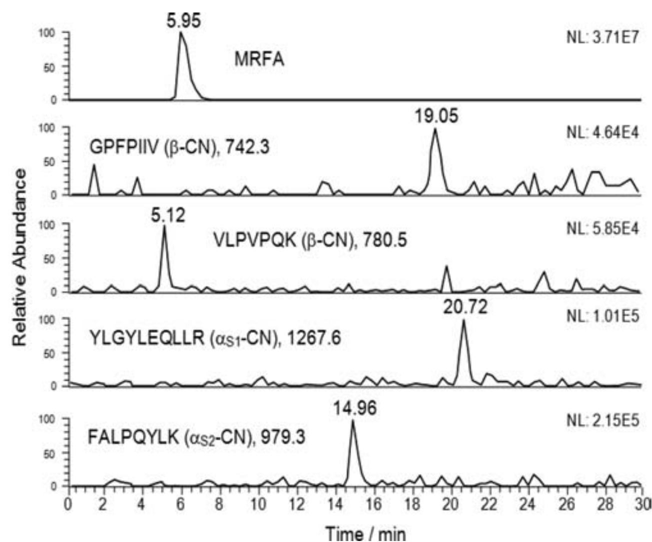

Figure 5. XIC traces relevant to four peptide markers of caseinate retrieved after analysis by LC-ESI-MS of the tryptic digest of the protein extract obtained from a Trebbiano d'Abruzzo wine aliquot (10 mL) spiked with 0.2 mg/L of caseinate. The XIC trace relevant to the MRFA peptide, added to the digest at a 100 mg/L concentration for MS response normalization purposes, is also reported.

Although noise is present in the XIC traces shown in Figure 5, the chromatographic peaks relevant to the casein peptide markers are clearly distinguished. Moreover, even at these concentration levels MS/MS spectra with an acceptable S/N ratio could be retrieved for the four peptides, and the observed fragmentation patterns enabled the unequivocal identification of peptide sequences, as shown in Figure S3 of the Supporting Information. These results indicate that the method described so far can provide clear evidence for the presence of residual caseinate in commercial white wines, even at concentration levels of a few tenths of milligrams per liter due to the detection of tryptic peptides arising from the main constituting proteins of caseinate. One or more of those peptides can be also used for caseinate quantitation, and the β-casein GPFPIIV was found to be the best marker, in terms of LOD value, in the case of the Trebbiano d'Abruzzo wine.

It is important to point out that no MS response assignable to the four casein peptides cited previously was observed when the method starting from 10 mL of wine was applied to the Trebbiano d'Abruzzo as such, that is, not spiked purposely with caseinate. Indeed, the XIC traces relevant to the four peptides, reported in Figure S4 of the Supporting Information, showed only ionic noise. This result was also suggested, although indirectly, by the calibration plots reported in Figures S1 and S2 of the Supporting Information, because the calibration line intercepts were never found to be significantly different from zero. In other terms, even if used by the manufacturer during production, caseinate was not present at concentration levels >0.29 mg/L in the final product.

Comparison of the Method Performance on Different Italian Commercial White Wines. In the last stage of the

present investigation the devised analytical method was applied to four further Italian commercial white wines (Pinot Grigio, Greco di Tufo, Müller-Thurgau, and a sparkling Moscato Bianco wine), all collected from local stores, to evaluate the eventual influence of different wine matrices on the method performance. Aliquots of each wine were first analyzed as such, and no response was obtained for any of the previously cited casein peptides or for different ones. Consequently, as in the case of Trebbiano d'Abruzzo, the four wines could be adopted as blank matrices to assess the method sensitivity through matrix-matched calibrations.

Calibration plots were obtained in the already cited 0.1−5 mg/L range of added caseinate concentration; GPFPIIV was always found to be the best marker, leading to LOD values ranging between 0.23 and 0.09 mg/L, as shown in Figure 6.

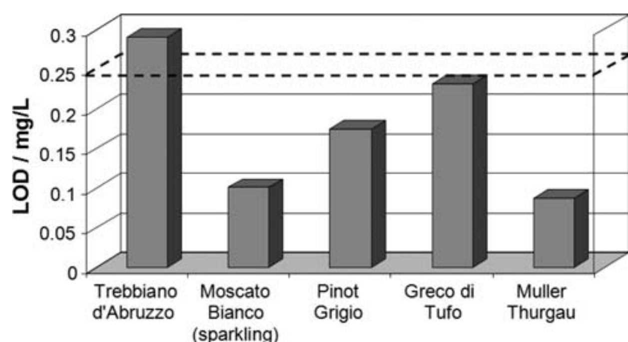

Figure 6. Comparison between LOD (S/N = 3) values obtained for caseinate added to different Italian commercial white wines when using a 10 mL sample volume, a SE-SPE/UF-based protein extraction, and the partial evaporation of the extraction solvent (final volume = 50 μL) by a nitrogen stream at room temperature before digestion. The reported LODs are based on the MS response of the tryptic peptide GPFPIIV from β-casein. The dotted lines emphasize the 0.25 mg/L LOD value recently indicated by the OIV as a method performance criterion and adopted as a reference in the current European legislation concerning fining agents declaration on wine labels.

The variability observed in the limits of detection for the five commercial wines considered during this investigation suggests that the matrix effect is not negligible in this case; thus, any quantitative application of the method to commercial wines should be based on a calibration performed using the real matrix as solvent, as during the present investigation. As expected from the tests performed on blank wines, the zero value was always included into the 95% confidence intervals obtained for the calibration line intercepts relevant to the four additional caseinate-spiked wines analyzed during the present work. The result confirms that the concentrations of residual caseinate, if present in the wines under consideration, are well below levels comprised between 0.1 and 0.3 mg/L.

Generally speaking, in the case of commercial wines showing a not negligible MS response due to caseinate peptide markers after being analyzed as such (due to the presence of residues of a caseinate-based fining process), the described calibration would become a standard addition procedure and the original caseinate concentration could be easily retrieved. In any case, for the sake of accuracy, the same caseinate originally adopted by the producer for wine fining should be used for calibration/quantification purposes.

As emphasized in Figure 6, the LOD values achieved for added caseinate in the five wines analyzed during the present investigation are generally lower than the 0.25 mg/L value indicated by OIV. This result suggests that if appropriate wine volumes and protein extraction/digestion strategies are devised, even a relatively accessible but less sensitive MS-MS/MS instrumentation, such as 3D ion trap spectrometers, can be exploited to quantify residual fining agents with limits of detection comparable to those obtained using immunochemical methods but with the advantage of an unequivocal recognition of the fining agent of interest. At least in principle the analyzed wine volume could be increased beyond 10 mL (using a higher number or higher capacity SE-SPE and UF tubes) in the case of wines exerting a severe matrix effect, that is, leading to inadequate LOD values, at least for the European legislation, when using a 10 mL sample volume.

Finally, because it is based on a non specific approach such as size exclusion, the protein extraction method described in this work could be potentially applied to the analysis of traces of other fining proteins in different wine matrices, for example, egg-white proteins in red wines, although the influence of phenolic compounds could be more severe in these matrices. Work in this direction is in progress in our laboratories.

■ ASSOCIATED CONTENT

Supporting Information

Additional table and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Ribereau-Gayon, P.; Glories, Y.; Maujean, A.; Dubordieu, D. The chemistry of wine stabilization and treatments. In *Handbook of Enology*; Wiley: Chichester, UK, 2006; Vol. 2, pp 301−333.
(2) Australia−New Zealand Food Standards Code (2002) Standard 1.2.3 – Mandatory Warning and Advisory Statements and Declaration; http://www.comlaw.gov.au/Details/F2011C00610 (accessed Aug 2, 2013).
(3) European Commission. . Commission implementing regulation 579/2012. *Off. J. Eur. Union* **2012**, *L171*, 4−7.
(4) Government of Canada, Food and Drugs Act. Regulations Amending the Food and Drug Regulations, 1220 — Enhanced Labelling for Food Allergen and Gluten Sources and Added Sulphites], 2011; http://gazette.gc.ca/rp-pr/p2/2011/2011-02-16/html/sor-dors28-eng.html (accessed Aug 2, 2013).
(5) European Commission. . Regulation 1234/2007. *Off. J. Eur. Union* **2007**, *L299*, 1−149. Regulation amended by Council Regulation 491/2009. *Off. J. Eur. Union* **2009**, *L154*, 1−56.
(6) Restani, P.; Uberti, F.; Tarantino, C.; Ballabio, C.; Gombac, F.; Bastiani, E.; Bolognini, L.; Pavanello, F.; Danzi, R. *Food Anal. Methods* **2012**, *5*, 480−486.
(7) Organisation Internationale de la Vigne et du Vin, Criteria OIV-MA-AS315-23, 2012; http://www.oiv.int/oiv/info/enmethodesinternationalesvin (accessed Aug 2, 2013).
(8) Weber, P.; Steinhart, H.; Paschke, A. *J. Agric. Food Chem.* **2007**, *55*, 3127−3133.
(9) Rolland, J. M.; Apostolou, E.; de Leon, M. P.; Stockley, C. S.; O'Hehir, R. E. *J. Agric. Food Chem.* **2008**, *56*, 349−354.
(10) Lifrani, A.; Dos Santos, J.; Dubarry, M.; Rautureau, M.; Blachier, F.; Tome, D. *J. Agric. Food Chem.* **2009**, *57*, 525−534.

(11) Weber, P.; Steinhart, H.; Paschke, A. *J. Agric. Food Chem.* **2009**, *57*, 8399−8405.
(12) Weber, P.; Steinhart, H.; Paschke, A. *Food Addit. Contam.* **2010**, *3*, 273−282.
(13) Lacorn, M.; Gößwein, C.; Immer, U. *Am. J. Enol. Vitic.* **2011**, *62*, 382−385.
(14) Tolin, S.; Pasini, G.; Curioni, A.; Arrigoni, G.; Masi, A.; Mainente, F.; Simonato, B. *Food Control* **2012**, *23*, 87−94.
(15) Lacorn, M.; Ristow, R.; Weiss, T.; Immer, U. *Food Anal. Methods* **2013**, DOI: 10.1007/s12161-013-9641-5.
(16) Monaci, L.; Losito, I.; Palmisano, F.; Visconti, A. *J. Chromatogr., A* **2010**, *1217*, 4300−4305.
(17) Monaci, L.; Losito, I.; Palmisano, F.; Visconti, A. *J. AOAC Int.* **2011**, *94*, 1034−1042.
(18) Monaci, L.; Losito, I.; Palmisano, F.; Godula, M.; Visconti, A. *Food Addit. Contam.* **2011**, *28*, 1304−1314.
(19) Monaci, L.; Losito, I.; De Angelis, E.; Pilolli, R.; Visconti, A. *Rapid Commun. Mass Spectrom.* **2013**, *27*, 2009−2018.
(20) Tolin, S.; Pasini, G.; Simonato, B.; Mainente, F.; Arrigoni, G. *Food Control* **2012**, *28*, 321−326.
(21) Cereda, A.; Kravchuk, A. V.; D'Amato, A.; Bachi, A.; Righetti, P. G. *J. Proteomics* **2010**, *73*, 1732−1739.
(22) D'Amato, A.; Kravchuk, A. V.; Bachi, A.; Righetti, P. G. *J. Proteomics* **2010**, *73*, 2370−2377.
(23) ExPASy, Bioinformatics Resource Portal, Swiss Institute of Bioinformatics (SIB). *FindPept* tool; http://www.expasy.ch/tools/findpept.html (accessed Aug 2, 2013).
(24) Protein Prospector, University of California, San Francisco. *MS-Product* tool; http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct (accessed Aug 2, 2013).
(25) Lacorn, M.; Weiss, T.; Immer, U. *Accred. Qual. Assur.* **2013**, *18*, 119−125.