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Towards quantification of residual milk allergens in caseinate-fined white wines using HPLC coupled with single-stage Orbitrap Mass Spectrometry

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Abstract:	<p>A method based on LC-ESI-High Resolution (HR)-MS analysis, using a single stage Orbitrap mass spectrometer, was developed for the quantification of casein allergens potentially present in white wines as a result of fining by caseinate. The method consists of i) extraction from the matrix by ultrafiltration, ii) digestion with trypsin and iii) detection/quantification of residual caseins, obtained by monitoring the LC-MS response of representative tryptic peptides (peak areas in extracted ion chromatograms). Method linearity was assessed first on caseinate solutions prepared either in water or in wine matrix (the ultrafiltration retentate of a protein-free white wine). Limits of detection (LOD) ranged from 0.1 to 0.3 µg/mL (S/N = 3) in water, and between 0.15 and 0.7 µg/mL in wine matrix, depending on the selected peptide. Method repeatability and reproducibility, meant as response variability (standard deviation) due to LC-MS analysis alone and to both enzymatic digestion and LC-MS analysis, were assessed on caseinate standard solutions in water and ranged from 5 to 12% and from 8 to 20%, respectively. A higher variability was usually observed for the peptide marker response in the case of matrix-matched samples, the only exception being peptide GPFPIIV from α-casein, the marker providing also the highest sensitivity. The method was finally applied to a casein-free white wine ("Greco di Tufo") fined with caseinate at different concentrations, after discarding the precipitate due to casein-wine components</p>

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4 aggregation. Minimum detectable added caseinate concentrations
5 (i.e. those corresponding to responses with $S/N = 3$) were
6 estimated between 39 and 51 $\mu\text{g/mL}$, according to the peptide
7 marker chosen. These limits are compatible with caseinate
8 concentrations typically adopted for wine fining purposes.
9 Moreover, a cross-check with the calibration performed in wine
10 matrix led to estimate the concentration of dissolved caseinate to
11 be in the low ng/mL range.
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3 **Towards quantification of residual milk allergens in caseinate-fined white wines**
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6 **using HPLC coupled with single-stage Orbitrap Mass Spectrometry**
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12 **Linda Monaci^{o*}, Ilario Losito^{1,2}, Francesco Palmisano^{1,2}, Michael Godula³ and Angelo**
13 **Visconti^o**
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60**Abstract**

A method based on LC-ESI-High Resolution (HR)-MS analysis, using a single stage Orbitrap mass spectrometer, was developed for the quantification of casein allergens potentially present in white wines as a result of fining by caseinate. The method consists of i) extraction from the matrix by ultrafiltration, ii) digestion with trypsin and iii) detection/quantification of residual caseins, obtained by monitoring the LC-MS response of representative tryptic peptides (peak areas in extracted ion chromatograms). Method linearity was assessed first on caseinate solutions prepared either in water or in wine matrix (the ultrafiltration residue of a protein-free white wine). Limits of detection (LOD) ranged from 0.1 to 0.3 $\mu\text{g/mL}$ ($S/N = 3$) in water, and between 0.15 and 0.7 $\mu\text{g/mL}$ in wine matrix, depending on the selected peptide. Method repeatability and reproducibility, measured as response variability (standard deviation) due to LC-MS analysis alone and to both enzymatic digestion and LC-MS analysis, were assessed on caseinate standard solutions in water and ranged from 5 to 12% and from 8 to 20%, respectively. A higher variability was usually observed for the peptide marker response in the case of matrix-matched samples, the only exception being peptide GPFPIIV from β -casein, the marker providing also the highest sensitivity. The method was finally applied to a casein-free white wine ("Greco di Tufo") fined with caseinate at different concentrations, after discarding the precipitate due to casein-wine components aggregation. Minimum detectable added caseinate concentrations (i.e. those corresponding to responses with $S/N = 3$) were estimated between 39 and 51 $\mu\text{g/mL}$, according to the peptide marker chosen. These limits are compatible with caseinate concentrations typically adopted for wine fining purposes. Moreover, a cross-check with the calibration performed in wine matrix led to estimate the concentration of dissolved caseinate to be in the low ng/mL range.

Keywords: food allergens, caseins, wine fining, ExactiveTM-Orbitrap mass spectrometry

Introduction

Due to their ability to bind and induce precipitation of phenolic and off-flavour compounds that might impair the organoleptic properties of commercial wine, caseins and caseinates derived from bovine milk are routinely used by wine makers as fining agents for clarification purposes (Castillo-Sánchez et al. 2006; Ferreira et al. 2001). The resulting complexes between proteins and phenolic compounds are usually removed from wine after filtration and/or decantation steps, depending on the practices adopted. However, the presence of casein residues in fined wine cannot be completely excluded; this might represent a hazard for allergic individuals since caseins are known as major food allergens of bovine milk (Monaci et al. 2006). The European Food Safety Authority (EFSA) has issued opinions on this matter stating that a real risk of caseins remaining in some wines might exist (EFSA 2007); moreover, the lack of good manufacturing practices represents a further issue for consumer's health concern. The most recent Directive on allergens issued by the European Commission, the 2007/68/EC (European Commission 2007), required that all milk products intentionally used for food or beverage (wine included) manufacturing have to be declared on the respective label. Actually, the deadline for mandatory labelling of egg and milk ingredients used as wine fining agents has been first postponed, to the end of December 2010 and then extended to the end of June 2012 (European Commission 2010). These decisions reflect both the debate on the real risk posed to allergic consumers by animal protein residues in fined wines and the fears of winemakers on the consequences of wine labelling new regulations from a commercial point of view. In spite of this legislative stalemate, several research groups are working on the development of analytical methods providing a clear indication of casein (and other fining proteins) concentrations in fined wines, thus enabling a real evaluation of the risks for allergic individuals.

The most widespread method used for the detection of caseins in wine is based on antibody recognition. In particular, several enzyme-linked-immunosorbent-assay (ELISA) formats have been

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3 recently developed (Rolland et al. 2008; Weber et al. 2007; Lifrani et al. 2009; Weber et al. 2009),
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5 thanks to their ease in execution and the possibility to be implemented along the wine making
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7 process. Weber et al. (2009) showed that α and β caseins, representing the most abundant fraction
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9 in caseinate powder, still remains in some analysed wines at detectable levels ≥ 0.2 mg/L. The same
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11 authors also proved that an additional treatment with bentonite, followed by a filtration step, could
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13 significantly contribute to reduce the presence of caseins in wine.
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20 As far as proteomics methods are concerned, although a number of MS-based approaches have been
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22 reported for the detection of allergens in food products (Monaci and Visconti 2009), only few
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24 papers have been published on milk allergens in fined wines (Wigand et al. 2009; Cereda et al.
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26 2010).
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32 In a preliminary investigation carried out in our laboratories using a capillary liquid
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34 chromatography (LC) separation combined with ESI-QTOF mass spectrometry (Monaci et al.
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36 2010), the possibility of detecting casein peptide markers in the tryptic digests of proteins extracted
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38 from caseinate-fined white wine has been demonstrated for the first time. Afterwards, Cereda et al.
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40 (2010) reported a method based on the extraction of standard caseins from wine by solid phase
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42 combinatorial peptide library technology, followed by separation and detection by gel
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44 electrophoresis. In that case the extraction was based on the interaction between proteins and
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46 peptide ligands synthesised by combinatorial chemistry and bound to beads that were added to the
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48 matrix. After elution from the beads the proteins were separated by 2D-PAGE and analysed by MS.
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52 Mass spectrometric analysis were carried out only to confirm, through tryptic digestion and peptide
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54 identification, the identity of proteins in the faint bands detected within gel. The authors reported
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56 the detection of concentrations of caseins in real wines, that resulted from previous fining
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58 processes, below 100 μ g/L, thanks to a challenging pre-concentration factor (estimated to be around
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60 5000, considering protein recovery). Although this limit of detection appears quite interesting, the

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3 reliability of such a method could suffer from quantification based on gel electrophoresis, whose
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5 reproducibility was not assessed. Moreover, the cost of employed materials and the complexity and
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7 length of the procedure make it hardly applicable on a large scale, in the perspective of a systematic
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9 screening of the casein content in fined wines before commercialization.
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15 The search for a simpler and less expensive method (at least in terms of sample treatment), in which
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17 both the identification and quantification of residual caseins in wine could be performed by mass
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19 spectrometry, has recently led us to assess the capabilities of High Resolution (HR) mass
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21 spectrometry based on a Orbitrap analyser (Monaci et al. 2011). In particular, we developed a LC-
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23 HR-MS method to identify peptide markers for residual caseins in white wine fined with caseinate.
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25 Due to the high mass accuracy provided by the Orbitrap analyser, liable peptide identification can
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27 be achieved just through a molecular mass determination. Confirmation of marker identity can be
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29 easily obtained by exploiting high resolution spectra resulting from the fragmentation of the
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31 protonated peptide in a HCD (Higher energy Collisional Dissociation) cell.
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39 In the present paper the quantitative capabilities of this method are discussed. In particular, the peak
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41 areas from extracted ion chromatograms relevant to four peptides already identified as reliable
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43 markers of caseins in wine have been exploited for calibration purposes, and the presence of matrix
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45 effects has been assessed using matrix-matched samples. Finally, the quantification of residual
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47 caseins in a protein-free white wine purposely fined with different levels of caseinate has been
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49 performed.
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Materials and methods

Chemicals and materials

Water and acetonitrile (both LC – MS grade), formic acid (MS grade), hydrochloric iodoacetamide, dithiothreitol (DTT), trypsin (proteomic grade), [Glu]-fibrinopeptide (GFP), sodium caseinate and ammonium bicarbonate were purchased from Sigma-Aldrich (Milan, Italy). RapiGest was purchased from Waters-Micromass (Manchester, UK). Ultrafiltration (UF) tubes with 10 kDa cut-off membranes were purchased from Millipore (Billerica, MA, USA).

LC-MS apparatus and operating conditions

An UHPLC pump equipped with an autosampler (Accela™, Thermo Fisher Scientific, San Jose, CA, USA,) was used for the chromatographic separation of protein tryptic digests. The separation was carried out by injecting 10 µL of sample onto a fused-core column (Ascentis Express C18, 100 mm length, 2.1 mm internal diameter, 2.7 µm packing; Supelco, Milan, Italy) and using a binary elution gradient, operated at a flow rate of 150 µL/min. The solvents used were: A) H₂O and B) CH₃CN, both containing 0.1% HCOOH (v/v). The linear gradient adopted for peptides elution was the following: linear from 93% to 50% solvent A in 40 min; from 50 to 30% A in 1 min and then 30% A for 2 min; back to 93% A in 1 min and re-equilibration for 10 min.

MS and HCD-MS analyses were performed using an Exactive™ non-hybrid single stage Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), equipped with an HESI II (heated electrospray interface). The spectrometer was operated at a resolving power as high as 50,000 (with ΔM estimated from the Full Width Half Maximum of peaks) and the relative mass deviation was typically less than 3 ppm without the use of internal mass axis correction.

A 5 µg/mL standard solution of GFP in acetonitrile-water (50/50 v/v) containing 0.1% formic acid was infused into the instrument to optimize the electrospray parameters towards peptides detection. Infusion experiments into the source were carried out by means of an automatic pump, delivering the standard solution at a flow rate of 5 µL/min. The mass accuracy of the instrument was assessed

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3 by comparing the experimental m/z with that expected for the protonated peptide molecule and was
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5 found to be always in accordance with the 3 ppm specification.
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8 The optimized values for ESI-MS parameters were the following. Scan range: 200-2000 Th,
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10 Resolution: High, Microscans: 1, AGC: Balanced $1 * 10^6$, Maximum IT: 100 ms, Sheath gas: 15
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12 arbitrary units, Auxiliary gas: 5 arbitrary units, Spray Voltage: 4 kV, Capillary Temperature: 250
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14 °C, Capillary Voltage: 32.50 V, Tube Lens Voltage: 130 V, Skimmer Voltage: 30 V, Heater
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16 Temperature: 30 °C.
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19 As for HCD-MS acquisitions, the Collisional Energy was set at 32 eV and the scan range for HCD-
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21 MS spectra was 200-2000 Th. All the other parameters were the same as those used for MS
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23 acquisitions.
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29 *Sample preparation*

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31 A white wine, produced in-house from “Greco di Tufo” grape variety using typical wine-making
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33 procedures but excluding fining processes, was used as a casein-free control wine throughout this
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35 investigation. The method for casein extraction/pre-concentration employed in the present work was
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37 the same described elsewhere (Monaci et al. 2010). Briefly, aliquots of 4 mL of wine, as such or
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39 preliminarily fined with caseinate, were first filtered through 0.2 μ m cellulose filters and then
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41 centrifuged for 40 min in ultrafiltration tubes with 10 kDa cut-off membranes (Millipore, Billerica,
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43 MA, USA). Afterwards, 1 mL of milliQ-water was applied to wash off the retentate, in order to
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45 obtain a cleaner extract. A final volume of nearly 200 μ L was collected, evaporated in a SpeedVac
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47 and successively re-suspended by vortexing 1 min with 100 μ L of 0.1% Rapigest solution (1 mg of
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49 RapidGest powder in 1000 μ L of 50 mM NH_4HCO_3). Tryptic digestion of wine extracts was
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51 performed as follows. Protein reduction was first performed by adding 5 μ L of 100 mM DTT
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53 solution and incubating the mixture in a thermoshaker at 60° C for 30 min. After cooling the sample
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55 to room temperature, 10 μ L of 200 mM iodoacetamide solution were added, and the mixture was
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57 left for 30 min under agitation in a thermoshaker (in the dark). In the final step, 20 μ L trypsin were
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3 added (final concentration 0.1 $\mu\text{g}/\mu\text{L}$) to the solution and the tube was gently flicked to mix. Tubes
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5 were incubated overnight at 37° C under shaking in order to ensure complete digestion. Ultimately,
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7 10 μL of 1 M HCl solution were added to the digest, that was then incubated for 30 min at 37° C
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9 and finally centrifuged at 16000 g for 10 min. After discarding the pellet the supernatant was
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11 transferred to a vial for LC-ESI-MS analysis.
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15 16 17 *Preparation of standard caseinate solutions and matrix-matched samples*

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19 Standard caseinate solutions were prepared, for calibration purposes, in milliQ-water, starting from
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21 a 10 $\mu\text{g}/\text{mL}$ stock solution. Aliquots corresponding to 10, 20, 50, 125 and 250 ng of caseinate were
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23 withdrawn from the latter, evaporated in Speed-Vac and re-dissolved in 100 μL of 0.1% Rapigest
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25 solution before undergoing enzymatic digestion (concentration range covered: 0.1 – 2.5 $\mu\text{g}/\text{mL}$).
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29 In order to check method sensitivity to caseinate in a wine matrix, matrix-matched samples were
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31 also prepared. At this aim aliquots of 4 mL of milk protein-free “Greco di Tufo” wine were
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33 subjected to ultrafiltration according to the procedure described in the previous paragraph.
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35 Afterwards, each ultrafiltration retentate was spiked with one of the caseinate amounts adopted for
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37 aqueous standard solutions, evaporated, re-dissolved in 100 μL of 0.1 % Rapigest solution and
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39 subjected to trypsin digestion.
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44 45 46 *Preparation of wine samples fined with caseinate*

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48 Wine fining experiments were performed by adding different amounts of caseinate to 10 mL
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50 aliquots of milk protein-free “Greco di Tufo”, in order to obtain nominal concentrations levels of
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52 10, 25, 100, 200, 500 and 1000 $\mu\text{g}/\text{mL}$. Samples were let to vortex for one minute and then
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54 decanted at room temperature for 15 minutes before filtration through 0.2 μm cellulose filters,
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56 required to discard caseinate aggregates eventually precipitated. A volume of 4 mL was withdrawn
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58 from each batch and submitted to the ultrafiltration/enzymatic digestion procedure previously
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60 described.

Results and discussion

Analysis of caseinate standard solutions

A recent investigation exploiting the ESI-based interfacing between LC and Orbitrap mass spectrometry (Monaci et al. 2011) has shown that four tryptic peptides, arising from the digestion of the two main caseins of caseinate, α S1 and β , can be used as markers to trace the presence of low concentrations of caseinate in complex matrices like wine. Their sequences (expressed using the one-letter code for amino acids) are: HQGLPQEVLENLLR, YLGYLEQLLR and FFVAPFPEVFGK (detected as doubly-charged ions, with experimental m/z 880.4771, 634.3568 and 692.8695, respectively), arising from α S1-casein, and GPFPIIV (m/z 742.4490 for the singly charged ion) from β -casein, the latter corresponding to the carboxylic terminal of the protein. These peptides match most criteria recently suggested for protein peptide markers, such as good ionization features, absence of post-translational modification sites, reproducible retention times, good fragmentation pattern and suitable size (comprised between 6 and 12 residues) (Johnson et al. 2011).

In order to check the suitability of the four peptides for caseinate quantification, in the present investigation a preliminary calibration was performed with caseinate standard solutions in a 0.1 – 2.5 $\mu\text{g/mL}$ concentration range. Extracted ion chromatograms were obtained for each peptide marker from the TIC chromatographic trace relevant to a tryptic digest. At this aim 0.04 Th wide m/z windows, centered on the experimental m/z of the protonated peptide molecule, were adopted. For the sake of example, the extracted ion chromatograms obtained for the m/z 742.4490, relevant to peptide GPFPIIV from β -casein, at different caseinate concentrations, are shown in Figure 1 together with the TIC trace relevant to a 0.5 $\mu\text{g/mL}$ caseinate concentration.

Calibration plots were obtained by reporting the peak area of each peptide marker, resulting from the extracted ion chromatogram, versus the concentration of caseinate. Calibration-related

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3 parameters, obtained from data interpolation by linear least squares, are reported, along with LOD
4 and LOQ values, in Table 1 (upper panel). In particular, correlation coefficients, calibration line
5 slopes (mean \pm standard deviation) and relative sensitivities, expressed as ratios between the latter
6 and referred to the GPFPIIV peptide, are shown. The LOD and LOQ values were calculated as the
7 concentrations corresponding to responses that differ from that of the blank by, respectively, 3
8 (Kaiser criterion) and 10 times the standard deviation on the blank response. The latter was
9 estimated from the corresponding value of the calibration line intercept (Vial et al. 1999).

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22 A good linearity was observed for all the peptide markers, with correlation coefficients always
23 better than 0.98; and LOD values equal to or lower than 0.3 $\mu\text{g/mL}$ were calculated. Interestingly, a
24 significant difference in the ESI-MS response was found between markers, with peptide GPFPIIV
25 from β -casein being the best one in terms of sensitivity.

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34 The peculiar behavior observed for this peptide could depend on a better ionization yield during the
35 ESI process, yet the relative amount of its protein of origin in the caseinate and the evolution of
36 enzymatic digestion could also play a significant role.

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44 It is worth noting that β - and αS1 -caseins are the most abundant caseins in caseinate and have
45 similar concentrations, thus it seems that the tryptic digestion follows a preferential route towards
46 the generation of the GPFPIIV peptide, which is located at the carboxylic end of β -casein.

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Once all the intrinsic parameters (incubation time and temperature, protein-enzyme ratio, pH) have
been standardized, as in the present investigation, the digestion yield may depend on several factors.
Among the latter, aggregation phenomena occurring on proteins to be digested, significant changes
in their tridimensional conformation and interactions with other matrix components, that might
mask the trypsin cleavage sites, can be envisaged in the system under study. Such aspects, that will

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3 be further discussed throughout the text, are quite relevant in the perspective of a quantitative
4 analysis of residual caseinate in wine based on tryptic peptide quantification.
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10 An evaluation of the quantification repeatability and reproducibility was also performed on standard
11 caseinate solutions. In this context repeatability is meant as a measure of the ESI-MS response
12 variability for a specific peptide in the same tryptic digest (i.e. a within sample variability, a sample
13 being represented by a specific tryptic digest). The parameter was assessed by analyzing, in
14 triplicate, during the same day, the same digest of caseinate standard solutions at concentration
15 levels of 0.5, 2.5 and 10 $\mu\text{g/mL}$. Depending on the peptide marker, relative standard deviations
16 (RSD) ranging from 5 to 12% were observed on peak areas obtained from extracted ion
17 chromatograms. It is worth noting that no significant differences in RSD values were observed for
18 the same marker between the three concentrations investigated. The values obtained are in
19 accordance with the variability observed for the ESI-MS response of the GFP peptide throughout
20 the calibration procedure. Indeed, a 2.5 $\mu\text{g/mL}$ solution of the peptide was periodically injected,
21 between calibration runs, in the LC-ESI-MS system and a relative standard deviation always lower
22 than 5% was observed for the peak area calculated from the corresponding extracted ion
23 chromatogram.
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46 Tests were made also to assess the within-sample/between-day variability. In particular, the same
47 tryptic digest of a 2.5 $\mu\text{g/mL}$ caseinate solution was analyzed daily by LC-MS up to four days after
48 the completion of the tryptic digestion. The sample was stored at $-25\text{ }^{\circ}\text{C}$ between each analysis and
49 the resulting between-day variability on the peptide marker responses was not significantly different
50 from the within-day one.
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The reproducibility of the procedure, meant as the combination between response variabilities due
to the combination of two stages, i.e. enzymatic digestion and LC-MS analysis, was assessed by

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3 analyzing, during the same day, three different digests of the 10 µg/mL caseinate obtained in
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5 parallel experiments. In this case relative standard deviations were found to range between 8 and
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7 20%, values slightly higher than those arising from repeatability tests, as expected, but acceptable
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9 for a quantitative application of the method.
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12 13 14 15 *Analysis of caseinate in matrix-matched samples*

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17 As a further step towards the development of a quantitative method for residual caseinate in wine,
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19 the ESI-MS response for the cited casein markers was assessed also in wine matrix. Indeed, the
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21 presence of matrix components in the ultrafiltration retentate obtained from fined wine could
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23 influence both the digestion yield and the marker peptide detection, thus modifying method
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25 sensitivity and reproducibility, compared to that observed with standard caseinate solutions.
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29 During this investigation white wine certainly free of caseins was prepared in-house from “Greco di
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31 Tufo” grape variety and used to prepare matrix-matched samples, as described in the Experimental
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33 section. A calibration plot was then obtained for each peptide marker.
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38 For the sake of comparison with data relevant to caseinate standard solutions, the corresponding
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40 linear regression parameters and LOD/LOQ values are reported in the lower panel of Table 1.
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44 Interesting information can be inferred from this comparison. First, the presence of residual wine
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46 matrix (i.e. wine components not eliminated by ultrafiltration) seems to have a significant effect on
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48 sensitivity, since the calibration line slope decreased for all peptides when matrix-matched samples
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50 were considered. On the other hand peptide GPFPIIV from β-casein resulted less affected by
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52 matrix effects, the ratio between its calibration line slopes in matrix-matched samples and standard
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54 solutions being 0.32, compared to values ranging between 0.15 and 0.16 calculated for the other
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56 peptides. As a result, the relative sensitivities of the latter, already low in standard caseinate
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58 solutions, were almost halved in matrix-matched samples. Peptide GPFPIIV was also not
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60 significantly affected by the increase in response variability generally occurring in this kind of

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3 samples, as evidenced by the increase in the standard deviations of calibration line slopes and the
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5 decrease of squared correlation coefficients (see the lower panel in Table 1).
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8 As also shown in Table 1, the combination between the factors now discussed led to an increase of
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10 LOD and LOQ values, in the presence of wine matrix components, for peptide markers different
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12 from GPFPIIV, although LOD values were always lower than 1 $\mu\text{g/mL}$.
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17 A tentative explanation for data reported in the lower part of Table 1 could be the presence of
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19 peculiar interactions between residual wine components, reasonably characterized by molecular
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21 masses higher than 10 kDa (i.e. the cut-off of ultrafiltration membranes), and integral caseins or
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23 their peptide markers. Such interactions do not induce casein precipitation, at least at the
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25 concentration levels investigated, since no evidence of this phenomenon was obtained when
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27 caseinate was added to the ultrafiltration retentate arising from caseinate-free wine. However, in
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29 spite of the denaturation action exerted by Rapigest, aggregates between wine components and
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31 caseins could persist and hinder the subsequent tryptic digestion, leading to a decrease of casein
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33 peptide concentration and, consequently, of sensitivity, compared to that observed for aqueous
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35 caseinate solutions. The exception observed for peptide GPFPIIV could be due to its position along
36
37 the β -casein chain. Indeed, its location at the carboxylic end of the protein structure could preserve
38
39 the corresponding trypsin cleavage site from being hindered even when an aggregation (without
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41 precipitation) with wine matrix components had occurred.
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51 The interaction between high molecular mass wine matrix components, present in the solutions
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53 subjected to tryptic digestion but not involved in the process, and casein tryptic peptides could also
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55 give rise to the higher variability generally observed for the LC-MS response of casein markers
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57 when matrix-matched samples were considered.
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3 The use of synthetic analogues of the selected casein markers could be very helpful in clarifying
4 both the aspects discussed in this section but it goes beyond the aim of the present investigation,
5 whose final stage was evaluating the suitability of the method for the quantification of caseinate
6 added directly to the “Greco di Tufo” casein-free wine or to commercial white wines.
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12 13 14 15 *Quantification of residual caseinate in fined white wine*

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17 In the last stage of the present investigation the raw “Greco di Tufo” wine was purposely spiked
18 with different concentrations of caseinate, in order to mimic primary fining treatments performed
19 during winemaking procedures.
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27 Six caseinate added concentrations ranging from 10 to 1000 μg per mL of wine, covering the values
28 typically used by manufacturers for wine fining purposes, were chosen. In particular, aliquots of 10
29 mL were withdrawn from the batch of wine and spiked with an appropriate volume of concentrated
30 (10 mg/mL) basic solution of caseinate. The formation of an opalescence, due to insoluble
31 aggregates potentially embedding caseins, was observed soon after caseinate addition at
32 concentration levels equal or higher than 100 $\mu\text{g}/\text{mL}$, whereas it was not visibly appreciable at
33 lower added concentrations. Subsequent decantation led to a well visible, brown-green precipitate in
34 the case of high concentrations of added caseinate; the colour of the precipitate was expectedly due
35 to wine coloured compounds embedded in it. Finally, 4 mL aliquots of the supernatant resulting
36 from wine decantation were subjected to the ultrafiltration/tryptic digestion procedure.
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50 For the sake of example, the TIC chromatogram obtained by LC-ESI-HR-MS analysis of the tryptic
51 digest relevant to wine spiked with 100 $\mu\text{g}/\text{mL}$ of caseinate is reported in Figure 2 along with the
52 extracted ion chromatograms obtained for the four casein peptides adopted as markers.
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It is apparent that, differently from standard caseinate solutions (see Figure 1), almost no evidence
for the presence of peaks related to the peptide markers was obtained from the TIC trace, the only

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3 exception being a peak of low intensity for peptide GPFPIIV, at $t_r = 21.81$ min. Although peptide
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5 peaks are well visible in the extracted ion chromatograms, this result suggests that the concentration
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7 of dissolved caseins in wine spiked with caseinate is quite lower than that added initially. It is worth
8
9 noting that the peak corresponding actually to peptide HQGLPQEVLENLLR from α S1-casein is
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11 the one at $t_r = 15.88$ min in the XIC trace for m/z 880.4770, whereas the higher peak at $t_r = 9.34$ min
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13 is due to a matrix interferent.
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20 Peak areas arising from extracted ion chromatograms were used to construct “MS response vs.
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22 added caseinate concentration” plots for the four peptide markers; in particular, three integral
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24 replicates of the extraction/digestion procedure were obtained for each concentration. The plots
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26 relevant to peptides GPFPIIV from β -casein and FFVAPFPEVFGK and YLGYLEQLLR from
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28 α S1-casein have been reported in Figure 3.
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35 A surprisingly linear dependence can be observed in the figure and R^2 values higher than 0.98 could
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37 be obtained after linear interpolation of data. In the case of peptide HQGLPQEVLENLLR from
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39 α S1-casein the correlation was poorer compared to the other peptides, likely due to its lower
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41 response; thus the marker was not used for further data elaboration.
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47 The linear dependence of the MS response for casein peptide markers would suggest that the
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49 concentration of dissolved caseinate is itself linearly dependent on the added caseinate
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51 concentration and, in turn, that casein precipitation is not due to the attainment of a solubility
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53 threshold, otherwise a plateau would have been expected at higher added concentration. This
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55 finding is in good agreement with studies performed by Mezdour et al. (2006; 2008) on the
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57 solubility of sodium caseinate in model water-ethanol solutions, in which different pH and/or the
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59 ethanol percentage were tested. Indeed, their data show that, for pH values lower than 4 and an
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ethanol content lower than 15% (v/v), i.e. in the conditions corresponding to the wine under study

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3 in the present investigation, caseinate solubility is close to 10 mg/mL, a value ten times higher than
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5 the maximum caseinate concentration added to wine in our case.
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10 It can be then inferred that caseins are removed from the solution, after caseinate addition, only as a
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12 result of aggregation with wine components (e.g. polyphenols), absent in the cited model solution.
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14 Moreover, the extent of the removal seems to be proportional to the amount of caseinate added
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16 initially to raw wine.
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21 Further interesting information on the process can be obtained from data in Table 2. First, some
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23 significant differences in terms of relative sensitivity for the peptide markers can be observed with
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25 respect to matrix-matched samples. Indeed, the response from α -S1 casein markers
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27 FFVAPFPEVFGK and YLGYLEQLLR appears to be systematically higher in spiked wine.
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31 It is worth noting that, besides caseinate concentration, the main difference between matrix-matched
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33 and spiked wine samples is the kind of wine components available for interaction with caseins. In
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35 fact, only species having relatively high molecular mass (virtually higher than 10 kDa) would be
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37 available for interaction with caseins in matrix-matched samples, whereas the entire population of
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39 matrix compounds is present in wine spiked with caseinate before ultrafiltration.
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45 This difference could influence the nature of interactions between wine matrix and dissolved
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47 caseins and affect the yields of the enzymatic digestions leading to each of the three peptide
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49 markers, e.g. by changing the accessibility of the involved cleavage sites.
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55 Starting from linear regression parameters, a calculation of the minimum added caseinate
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57 concentration detectable at $S/N = 3$ was performed, using the same approach adopted for previous
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59 LOD calculations. According to the peptide marker considered, values ranging between 39 and 51
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 μg of caseinate added per mL of wine were obtained (see Table 2); they are suitable for the

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3 quantification of concentrations of caseinate typically added to raw wines for primary fining
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5 purposes.
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10 On the other hand, due to precipitation, the concentration of caseinate actually dissolved in fined
11 wines is certainly lower than that added initially. The remarkable difference between the regression
12 line slopes relevant to spiked wine samples and matrix-matched ones seems to confirm this
13 hypothesis. Indeed, if the MS responses obtained for the peptide markers at the minimum detectable
14 added concentrations are used for an extrapolation on the corresponding matrix-matched regression
15 lines, concentrations ranging between 0.20 and 0.44 $\mu\text{g/mL}$ of caseinate dissolved in the Rapigest
16 solutions before digestion can be estimated. The 40-fold preconcentration factor potentially
17 achievable when passing from 4 mL of caseinate-spiked wine to 100 μL of Rapigest solution would
18 then lead to estimate residual caseinate concentrations of a few ng/mL in the raw wine obtained
19 after decantation of precipitated caseins.
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36 In order to check method capabilities also on commercial white wines, a “Pinot Grigio” and a
37 sparkling wine obtained from a “Moscato bianco” grape variety were also analyzed, as such or
38 spiked with caseinate at a 100 $\mu\text{g/mL}$ concentration level.
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46 The peak areas obtained from the XIC traces for the four peptides adopted as casein markers are
47 shown in Figure 4, where data relevant to the home-made “Greco di Tufo” wine, spiked at the same
48 concentration level, are also reported for comparison.
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55 As apparent, a significant increase in the MS response was obtained for each marker from the two
56 commercial wines. This result could be due to at least two reasons: i) a significant decrease of the
57 amount of precipitated caseins, ii) a change in sensitivity due to variation in the tryptic digestion
58 yield or to a different matrix effect, compared to the “Greco di Tufo” wine. As for the first aspect, it
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3 is worth noting that only a slight opalescence could be visibly appreciated when caseinate was
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5 added to the commercial wines and a very limited amount of precipitate was observed after
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7 decantation. This behavior is not surprising, since commercial white wines are usually subjected to
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9 fining processes before commercialization, thus a lower amount of wine phenolic compounds, i.e.
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11 those mainly responsible for casein precipitation, is expected, compared to a raw wine.
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17 As far as the second aspect is concerned, the wine matrix composition might influence both the
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19 yield of tryptic digestion, due to protein-matrix component interactions, and the MS response of
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21 peptide markers, due to competition effects during ionization. Both effects would lead to variations
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23 in the observed peak areas even in the presence of similar concentrations of dissolved caseins. An
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25 indirect confirmation to this hypothesis is provided by the significant variations observed for the
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27 relative responses of the four peptide markers. As an example, peptide FFVAPFPEVFGK from α -
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29 S1 casein becomes the leading marker, in terms of MS response, in the two commercial wines.
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34 The results now discussed suggest that a standard addition approach should be implemented if a
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36 reliable quantification of residual caseins, by a method based on tryptic digestion/MS analysis, like
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38 ours, is searched for.
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43 It is worth noting that no significant MS response was obtained for any of the four peptide markers
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45 of caseins when the two commercial wines were analyzed as such. Due to the generally higher
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47 sensitivity achieved by the method on these wines, concentration levels of few ng/mL can be then
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49 hypothesized for dissolved caseinate resulting from fining processes performed during wine
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51 production. Further work on the investigated raw wine and on commercial wines, spiked with
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53 caseinate concentrations directly in the ng/mL range, is in progress to confirm this hypothesis.
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Conclusions

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3 The combination of protein extraction by ultrafiltration, tryptic digestion and LC-ESI-HR-MS
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5 analysis of the resulting peptide markers has proved to be a reliable approach for the quantification
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7 of residual caseins in white wines subjected to caseinate addition at concentration levels comparable
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9 to those commonly adopted for fining purposes.
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15 In particular, detection limits of few tens of $\mu\text{g/mL}$, referred to the concentration of caseinate added
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17 to a protein-free wine, could be achieved by exploiting the MS response of selected tryptic peptides
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19 of major caseins (β and αS1). Moreover, a higher MS response was obtained for the same peptides
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21 when the method was applied to two commercial white wines spiked with caseinate at similar
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23 concentrations ($100 \mu\text{g/mL}$), thus suggesting that even lower LOD values could be achieved in the
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25 case of highly processed wines, e.g. wines resulting from a secondary fining treatment.
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29 A cross estimate, based on a comparison with the MS response obtained for caseinate markers in
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31 matrix-matched samples, led to predict concentrations of actually dissolved caseinate in the low
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33 ng/mL range. This finding makes then the method quite promising also for caseinate quantification
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35 in commercial white wines, in which such low residual concentrations have been recently reported.
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41 **Acknowledgments**

42
43 The authors are grateful to Elisabetta De Angelis and Barbara Introna for their valuable help in
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45 performing LC-MS analyses.
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Captions for figures

Figure 1. Total ion current (TIC) and extracted ion (XIC) chromatograms, relevant to the m/z (742.4490) of the singly charged ion of tryptic peptide GPFPIIV from β -casein, obtained by LC-ESI-HR-MS analysis of the tryptic digest of caseinate aqueous solutions at different concentrations.

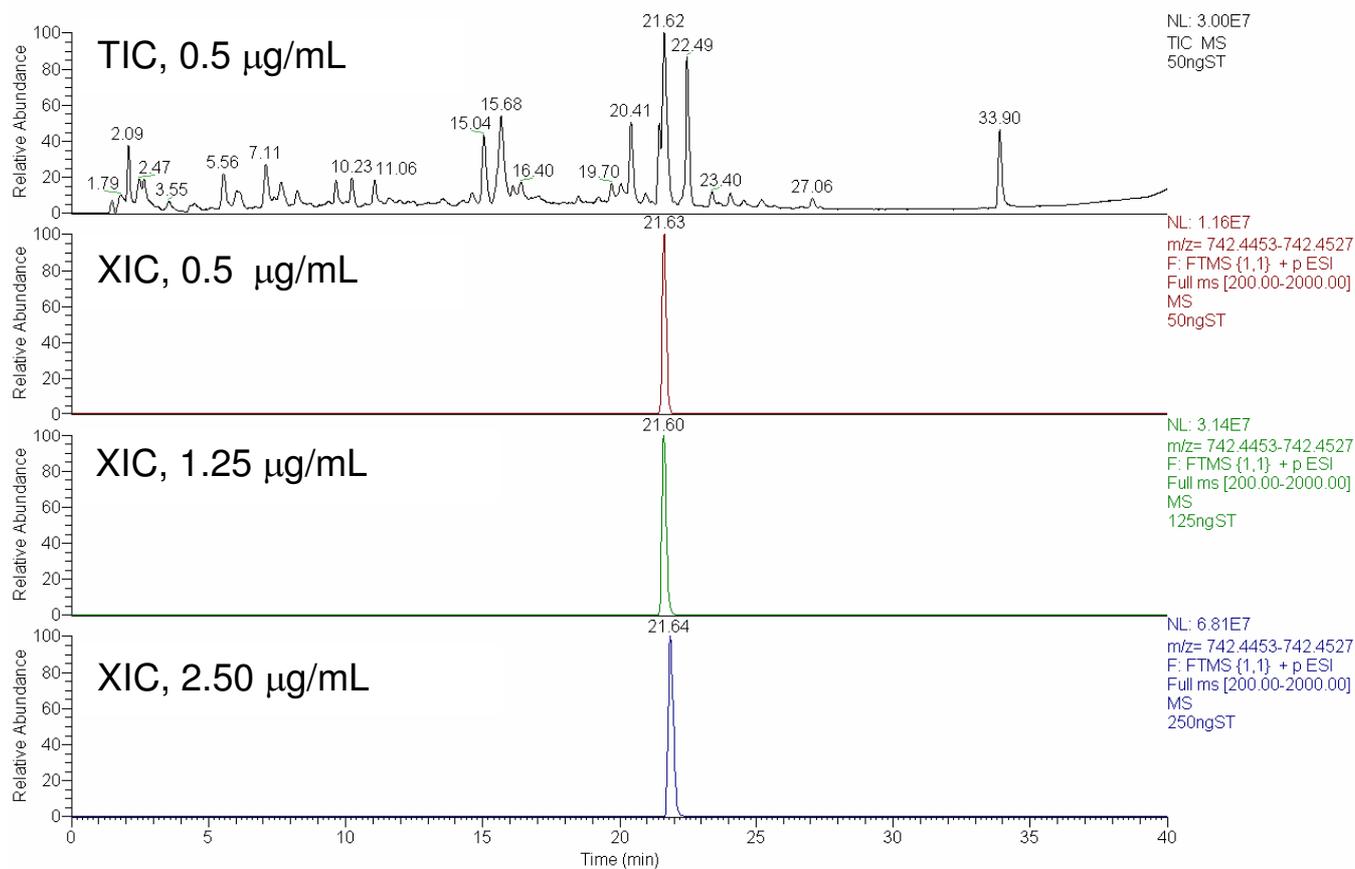
Figure 2. Total ion current (TIC) and extracted ion (XIC) chromatograms, in a time window 0-30 min, relevant to the m/z of four casein peptide markers, obtained by LC-ESI-HR-MS analysis of the tryptic digest of ultrafiltration retentate for “Greco di Tufo” wine spiked with 100 ($\mu\text{g}/\text{mL}$) of caseinate. The arrow indicates the peak actually corresponding to peptide HQGLPQEVLNENLLR in the XIC trace for the m/z ratio 880.4770.

Figure 3. Plots of “ESI-MS response vs added caseinate concentration” data obtained for three casein peptide markers after LC-ESI-HR-MS analysis of the tryptic digests of ultrafiltration retentate of “Greco di Tufo” wine spiked with caseinate. The line resulting from linear least squares interpolation is also reported.

Figure 4. Comparison of the MS responses obtained for the casein peptide markers after the LC-ESI-HR-MS analysis of the tryptic digest of ultrafiltration retentate from the “Greco di Tufo” wine and two commercial wines spiked with caseinate at the final concentration of 100 $\mu\text{g}/\text{mL}$

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For Peer Review Only



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Figure 1

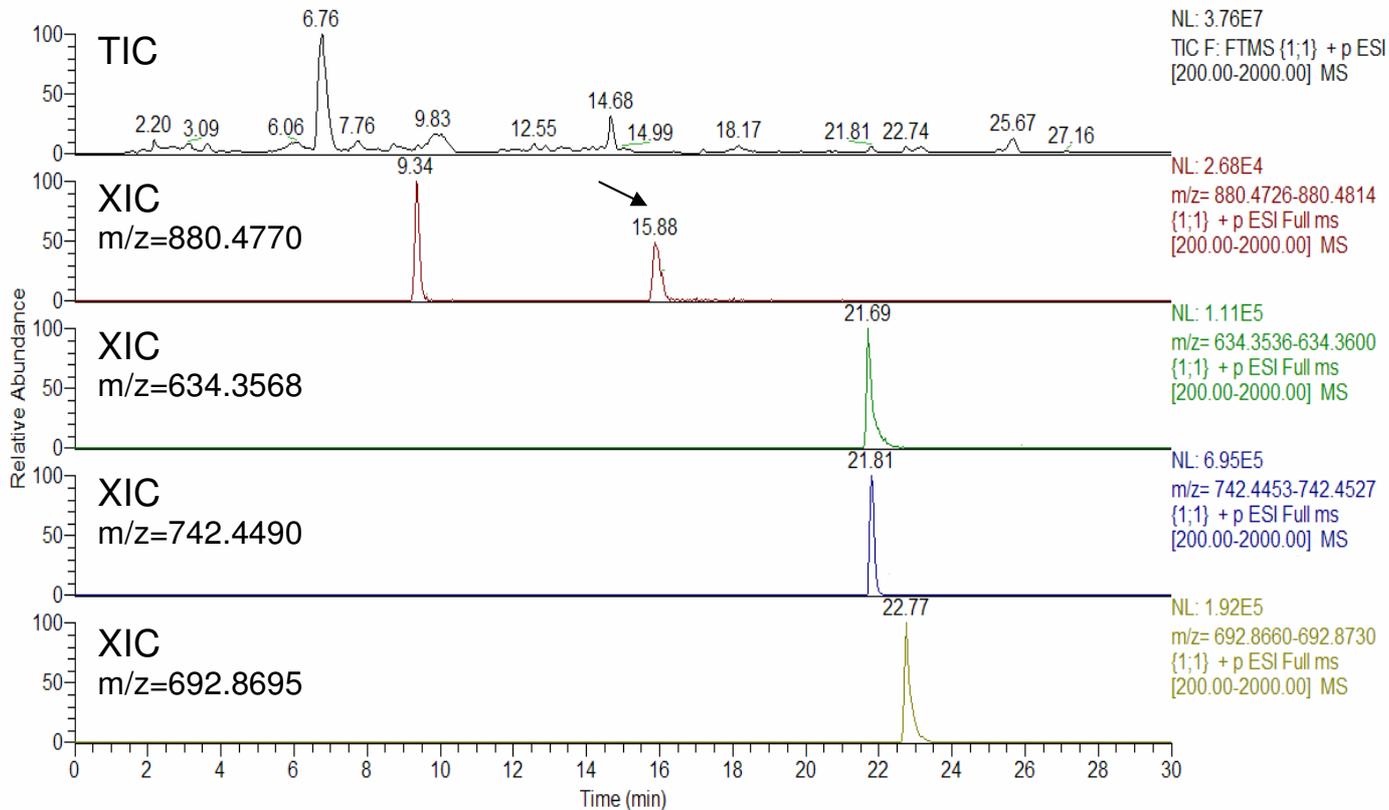


Figure 2

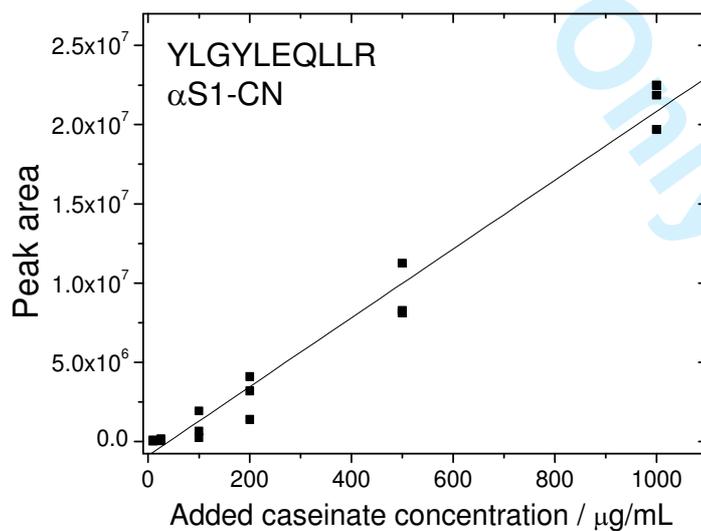
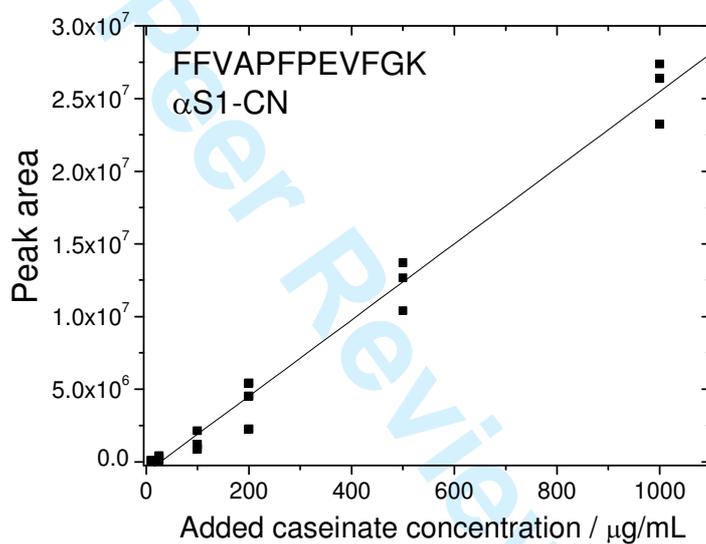
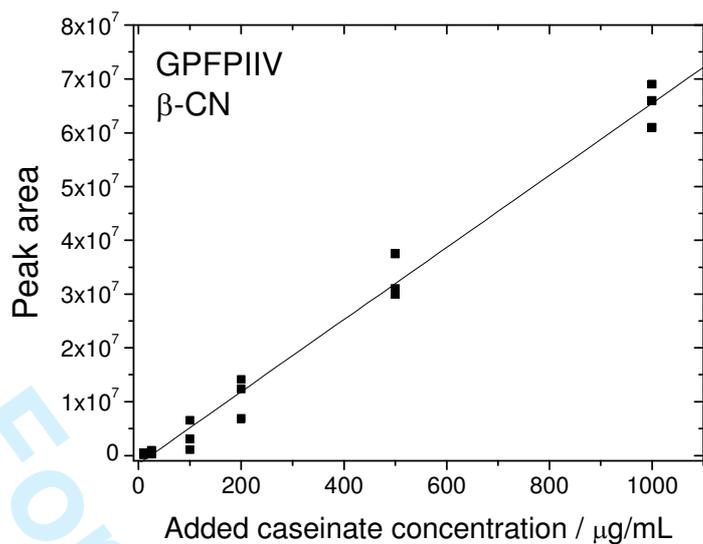
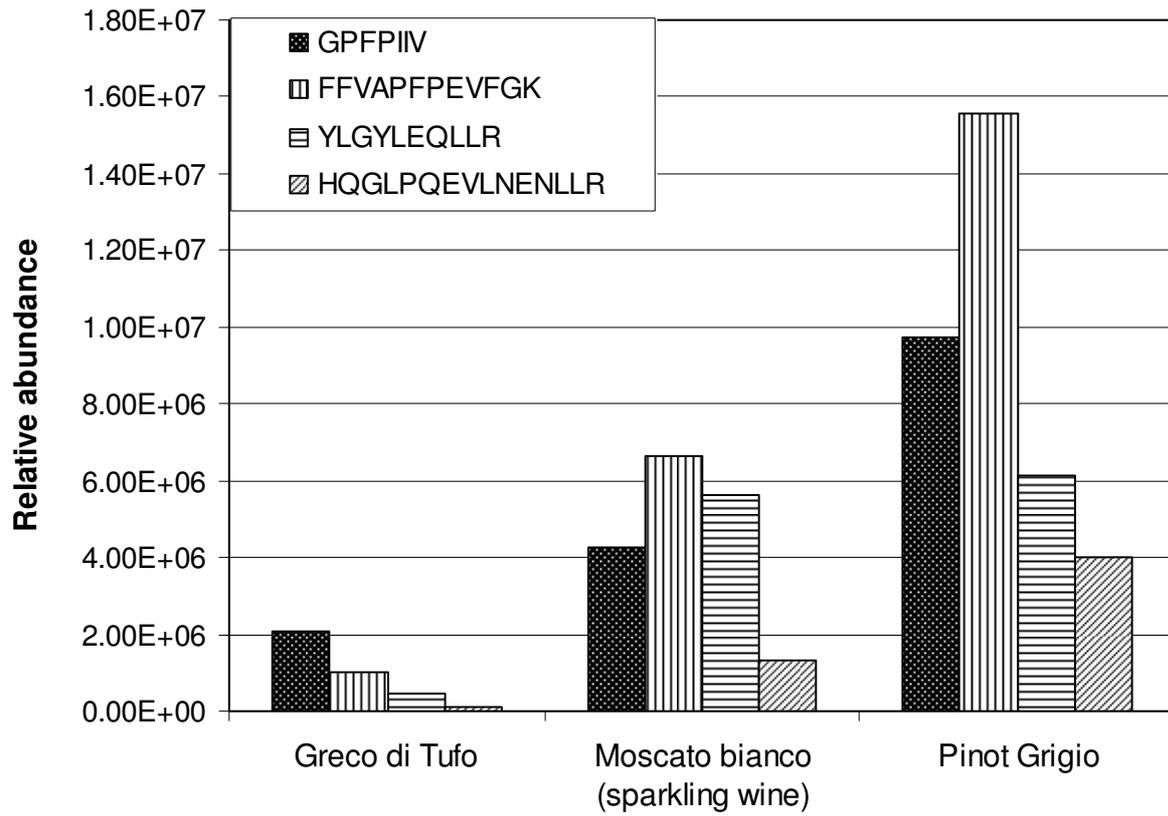


Figure 3



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Figure 4

Table 1. Calibration parameters for casein peptide markers relevant to standard caseinate solutions and wine matrix-matched samples (concentration range: 0.1-2.5 µg/mL).

Peptide, protein (charge state, m/z)	R ² Cal. line slope ± sd	standard solutions ^(a)		
		Rel. sens.	LOD (µg/mL)	LOQ (µg/mL)
GPFPIIV, β-CN (+1, 742.4490)	0.9979 (1.94 ± 0.09)*10 ⁸	1	0.13	0.4
FFVAPFPEVFGK, αS1-CN (+2, 692.8695)	0.9815 (3.8 ± 0.4) * 10 ⁷	0.19	0.3	1.0
HQGLPQEVLNENLLR, αS1-CN (+2, 880.4770)	0.9832 (4.6 ± 0.3) * 10 ⁷	0.24	0.3	1.0
YLGYLEQLLR, αS1-CN (+2, 634.3568)	0.9976 (2.69 ± 0.09) * 10 ⁷	0.14	0.10	0.3

Peptide, protein (charge state, m/z)	R ² Cal. line slope ±sd	matrix-matched samples ^(a)		
		Rel. sens.	LOD (µg/mL)	LOQ (µg/mL)
GPFPIIV, β-CN (+1, 742.4490)	0.9964 (6.1 ± 0.4) *10 ⁷	1	0.15	0.5
FFVAPFPEVFGK, αS1-CN (+2, 692.8695)	0.9516 (6.1 ± 1.4) * 10 ⁶	0.10	0.6	2
HQGLPQEVLNENLLR, αS1-CN (+2, 880.4770)	0.9504 (7.1 ± 1.1) * 10 ⁶	0.12	0.5	1.7
YLGYLEQLLR, αS1-CN (+2, 634.3568)	0.9476 (4.2 ± 1.1) * 10 ⁶	0.07	0.7	3

^(a) Relative sensitivities within the same sample type were calculated as ratios of calibration line slopes, using that of peptide GPFPIIV as reference. LOD and LOQ values are referred to S/N = 3 and 10, respectively, with noise estimated as the standard deviation on the calibration line intercept (see text for details).

Table 2. Calibration parameters calculated after linear interpolation of “ESI-MS response vs. added caseinate concentration” data relevant to casein peptide markers in the tryptic digests of ultra-filtration retentate obtained from “Greco di Tufo” wine spiked with caseinate.

Peptide, protein (charge state, m/z)	R ² Calibration line slope (±sd)	Rel. sens. ^(a)	Minimum detectable added conc. (µg/mL) ^(b)
GPFPIIV, β-CN (+1, 742.4490)	0.9874 (6.70 ± 0.19) *10 ⁴	1	39
FFVAPFPEVFGK, αS1-CN (+2, 692.8695)	0.9845 (2.62 ± 0.08) *10 ⁴	0.39	44
YLGYLEQLLR, αS1-CN (+2, 634.3568)	0.9803 (2.17 ± 0.08) * 10 ⁴	0.32	51

^(a) calculated as ratios of interpolation line slopes, using that of peptide GPFPIIV as reference.

^(b) calculated as LOD value at S/N = 3.