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Identification of allergenic milk proteins markers in fined white wines by capillary liquid chromatography–electrospray ionization–tandem mass spectrometry

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ABSTRACT

A method based on capillary liquid chromatography combined with electrospray ionization–tandem mass spectrometry (CapLC–ESI–MS–MS) for the detection and identification of casein deriving peptides in fined white wine is described. This is the first step towards the development of a liquid chromatography mass spectrometric method for the detection/identification of markers of potentially allergenic milk proteins used as wine fining agents. The method demonstrated to be capable of detecting some peptides arising from α and β casein (with the relative aminoacidic sequences elucidated) in extracts of white wine fined with casein at 100 and 1000 $\mu\text{g/mL}$. This MS based approach appears to be a useful tool for screening purposes as well as a confirmatory tool for the unequivocal identification of caseins in ELISA positive samples.

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1. Introduction

Proteins are commonly present in wines at low levels, most of them having a remarkable technological and economical relevance. The formation of tannin–protein aggregates or protein–polyphenols complexes has been widely described [1–3]. Typical proteins detected by spectrometric methods in white wines are chitinases and thaumatin-like proteins regarded as pathogenesis-related proteins originated from grapes [4]. As, in general, proteins can affect wine stability and clarity, a variety of procedures have been developed for their removal from wines [5]. Fining agents typically used for clarification of wine are milk proteins, egg proteins and/or fish gelatine. Among milk proteins, caseins are universally known as suitable agents for binding phenolic compounds and reducing off-flavour ingredients that may affect wine taste and colour. Although it is assumed that fining agents are nearly quantitatively removed during the manufacturing process, to date there is no evidence that the consumer ready product is truly free of residues. Rolland et al. [6] investigated whether wines fined with allergenic proteins (such as milk proteins, isin-

glass and egg proteins) can provoke significant clinical allergic reactions in sensitive patients. Although the consumption of milk protein-fined wine did not induce anaphylaxis, some mild reactions were observed. On the other hand due to the paucity of data collected, no statistical conclusion can be drawn from that study. Moreover, lack of good manufacturing practices represents a further issue for consumers health concern. Recent opinions issued by the scientific EFSA (European Food Safety Authority) panel on this matter address this concern and drew the conclusions that wines fined with milk, caseins or products thereof may trigger adverse reaction in susceptible individuals [7,8]. In this regard, the analysis of fining agents like caseins, that might be present as residues in wines, is of paramount importance to safeguard allergic consumers' health and to comply with the recent legislation issued on food allergens. The most recent Directive 2007/68/EC [9] (repealing Directive 2005/26/EC reporting a list of food ingredients provisionally excluded from the labelling requirements), requires that all milk products intentionally used for food or beverage manufacturing have to be declared in the relative label. The only exemptions are granted to whey used for making distillates or ethyl alcohol and lactitol. Moreover, as mentioned in Directive 2007/68/EC, Member States shall allow foodstuffs placed on the market (or labelled) before 31st May 2009, and complying with Directive 2005/26/EC, to be marketed until stocks are exhausted. However with the aim to help producers to facilitate the transition towards the new wine

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labelling requirements, the European Commission has agreed to extend the deadline for mandatory labelling of egg and milk ingredients used as fining agents in wines until December 2010. In order to comply with the current legislation reliable methods enabling to detect and/or confirm the presence of milk proteins in foods and beverages are urgently required. Concerning wine samples, the method of widespread use for the detection of caseins is based on antibody recognition. Several enzyme-linked-immunosorbent-assay (ELISA) formats have been recently developed for detection of casein residues in wine samples [10–13], with the lowest limit of detection as 8 ng/ml [11]. A quantitative ELISA method for determination of caseins in white and rose wines fortified within the range of 0.01–10 mg/L, has been recently reported by Weber et al. [13]. Nevertheless, drawbacks of ELISA methods are due to the strong matrix interferences often occurring in and to the use of monoclonal antibodies that limit their use to the detection of a specific protein. To overcome these issues confirmatory methods are required. As far as fined wines are concerned, although the potentials and features offered by tandem mass spectrometry make it suitable for the identification of allergenic proteins [14,15], there are not many methods so far available in the literature. The only exception regards the development of a CE–MS method for the detection of wine adulteration with egg lysozyme, not requiring any purification step [16]. To the best of our knowledge no mass spectrometric method has been developed for the detection of milk markers in wine. On the other hand, MS methodologies have been already employed to study protein profiles of seeds extract from different *Vitis vinifera* grape varieties [17] or to characterize protein composition in different wines [18]. Several proteins known to influence wine stability were identified while some pathogenesis-related proteins were detected in red wines.

In this paper a method using a capillary LC separation combined with ESI–Q–TOF mass spectrometry for the unequivocal identification of peptides from caseins is described. The method has been applied to white wine fined with caseinate where some peptides, arising from α and β caseins, present as residues in wine extracts could be detected and identified. The careful analysis of the fragmentation patterns provided by these peptides, that showed identical product ions in standard caseinate and in fined wine, provided the unequivocal identification of casein peptides. Peptides attribution and protein identification were both assessed by database searching. This preliminary study paves the way for further investigations aimed at evaluating the actual amount of caseins still detectable in fined wines. The method appears to be very useful for screening purposes as well as a confirmative method to corroborate positive results obtained by ELISA.

2. Materials and methods

2.1. Chemicals

Water and acetonitrile (LC–MS grade), formic acid, hydrochloric iodoacetamide, dithiothreitol (DTT) and trypsin (proteomic grade), were provided by Sigma–Aldrich (Milan, Italy). RapiGest was from Waters–Micromass (Manchester, UK). Skimmed milk powder and sodium caseinate were purchased from Sigma–Aldrich.

2.2. Sample pre-treatment prior to proteomic analysis

Blank white wine, produced in house from “Greco di tufo” grape variety, was used as “milk proteins free” control sample. 15 mL of the control sample was fined with caseinate at the final concentration of 100 and 1000 μ g/mL and left under stirring for 15 min at 40 °C to facilitate protein dissolution. The solution was then left decanted for 15 min at room temperature to allow protein precip-

itation. Aliquots of 4 mL were successively filtered through 0.2 μ m cellulose filters and centrifuged for 40 min in ultrafiltration tubes with 3 kDa cut-off membranes (Millipore, Billerica, MA, USA). An additional washing step of 1 mL of milliQ–water was applied to wash off the retentate leading to a cleaner extract. A final volume of nearly 300 μ L was collected and the membrane was double washed with 200 μ L water and shaken to increase protein recovery. The volumes collected were combined and evaporated under a gentle flow of nitrogen; the pellet obtained was re-suspended in 50 μ L of 0.1% RapiGest solution (1 mg of RapiGest powder in 1000 μ L of 50 mM NH_4HCO_3) and vortexed for 1 min. Protein reduction was performed by adding 5 μ L of 50 mM DTT solution and incubating the mixture in a thermoshaker at 60 °C for 30 min. After cooling down the sample at room temperature, 10 μ L of 100 mM iodoacetamide solution was added and the mixture was placed in the dark for 30 min. As final step, 25 μ L of trypsin was added (final concentration 0.1 μ g/ μ L) to the extract and the tube was gently flicked to mix. Incubation at 37 °C under shaking was then performed for 6 h, in order to ensure a nearly complete digestion. Ultimately, 10 μ L of 500 mM HCl solution was added to the digest, incubated for 30 min at 37 °C and then centrifuged at 16,000 $\times$ g for 10 min. The pellet was discarded and the supernatant was carefully transferred into micro-vials prior to LC–MS analysis.

2.3. Apparatus

Capillary LC–ESI–MS–MS analyses were performed on a capillary flow–liquid chromatography system equipped with an autosampler (XE System, Waters, Milford, MA, USA), operated with a binary elution gradient at a flow rate of 5 μ L/min. 1 μ L of peptide mixtures from trypsin digests was injected by the autosampler in a capillary Discovery Bio Wide Pore C18 analytical column (150 mm length, 320 μ m internal diameter, 5 μ m packing; Supelco, Milan, Italy). The eluents used were: (A) $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (90/10, v/v) + 0.1% HCOOH ; (B) $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (90/10, v/v) + 0.1% HCOOH . The linear gradient used for peptides elution was the following: 0–42 min, linear from 30% to 80% solvent B; 42–45 min isocratic; 45–47 min, from 80% to 30% solvent B; re-equilibration time: 15 min.

ESI–MS–MS experiments were carried out on a quadrupole–time-of-flight mass spectrometer (Q–TOF Ultima Global, Waters, Manchester, UK) equipped with a Z sprayTM source. The instrument was operated in the positive ion mode. The values adopted for the main parameters of the interface were as follows: capillary voltage: 3.2 kV; sample cone voltage: 100 V; source temperature: 80 °C; desolvation temperature: 150 °C; cone gas flow: 120 L/h; desolvation gas flow: 200 L/h.

Time-of-flight (TOF) measurements were performed in a continuous extraction mode and with one reflectron active (V mode). The TOF analyser was calibrated on a daily basis in MS/MS mode, using the Glu¹–fibrinopeptide B (Sigma–Aldrich) main product ions enclosed in the m/z range of 100–1700.

Full scans (MS mode) were performed over the m/z range of 100–1500 with a scan time of 0.9 s and inter scan time of 0.1 s. MS/MS acquisitions were obtained using the “MS survey scan” mode, i.e. a data-dependent approach. In particular, fragmentation of ions was effected only on the three most abundant ions among those, detected in the MS full scan, that overcame a user-specified intensity threshold. MS to MS/MS switch criteria were as follows: threshold intensity 6 counts/s, charge states: +1, +2, +3. MS/MS to MS switch was set to occur when the total ion current (TIC) was falling below 4 counts/s or after a defined time of 6 s. As for precursor ion isolation within the quadrupole analyser, low mass (LM) and high mass (HM) resolution settings of 10 were chosen. The cone voltage was set at 40 V.

The product ion spectra obtained from the “MS survey” mode were processed using ProteinLynx Global Server v2.1 (Waters),

Table 1

Protein hits, peptides detected and relative sequence assigned by interrogating the SwissProt database with MS data relevant to aqueous solutions of caseinate standard (100 µg/ml) digested with trypsin after being subjected to the same extraction procedure adopted for wine samples.

Protein assignment	Ion score ^a	Peptide detected (z)	Mr calculated	Delta mass	Sequence
αS1 CN	77	880.57 (+2)	1758.93	0.2	HQGLPQEVLENLLR (23–37)
	50	692.95 (+2)	1383.72	0.16	FFVAPFPEVFGK (38–49)
	44	634.43 (+2)	1266.69	0.15	YLGYLEQLLR (106–115)
αS2 CN	41	626.43 (+2)	1250.86	0.15	YIPIQYVLSR (46–55)
	31	598.42 (+2)	1194.67	0.15	NAVPIPTPLNR (130–140)
β CN	55	1093.72 (+2)	2185.16	0.27	DMPIQAFLLYQEPVLGPVR (199–217)
	36	780.59 (+1)	779.49	0.09	VLPVPQK (185–191)
	22	742.49 (+1)	741.44	0.04	GPFPIIV (218–224)
κ CN	121	990.54 (+2)	1979.08	0.01	SPAQLQWQVLSNTVPAK (69–86)

^a Individual ion score higher than 48 indicates identity or extensive homology ($p < 0.05$).

a software converting LC–MS–MS raw data into peak lists for database searching. The generated lists were then searched against the SwissProt database using Mascot Peptide Mass Fingerprint and MS/MS Ion Search (Matrix Science Ltd., London, UK) as a bioinformatic tool. The search was restricted to the Mammals taxonomy and the following stringent criteria were adopted for database searching. Fixed modification: cystein carboamidomethylation; variable modifications: methionine oxidation, and serine, threonine and tyrosine phosphorylations. Peptide MS and MS/MS tolerance: 0.8 and 0.4 Da, respectively. Only one missed cleavage was allowed for trypsin digestion.

3. Results and discussion

In the first part of the investigation an aqueous solution of caseinate, at a concentration of 100 µg/mL, was submitted to the same extraction procedure as that adopted for wine samples, digested with trypsin and finally analysed by capLC–ESI–Q–TOF–MS, with the aim to identify the most abundant and stable peptides. At a first glance, several peptides were displayed in the chromatographic runs. The most intense ions identified by Mascot software in caseinate standard digests are reported in Table 1 along with the identified protein, the ions score and the assigned sequence. Among the MS acquisition modes investigated, the “survey” mode demonstrated to be the most suitable for our purposes since it allowed to automatically switch from MS full scan to MS/MS mode, thereby

providing information both on molecular weights and on fragmentation patterns, at least for peptides whose molecular ions intensity overcame the threshold set (*vide infra*).

To target the concentration range of casein typically utilised by wine manufacturers for clarification purposes (comprised in the range of 10–100 g/hL), white wine samples were fortified with caseinate at the two level of 100 and 1000 µg/mL. These fined wines were successively submitted to MS analysis and a subgroup of peptides within the list of potential markers selected (in Table 1) was highlighted. As shown in Table 2 that also reports number and type of peptide fragments matching with those predicted *in silico* for the candidate sequence, αS1 and β caseins were identified with the best hits after database search against SwissProt. In particular, peptides with m/z 634.2, 692.6 and 742.2, accounting for these two proteins and observed in the tryptic digests of caseinate standard, were detected in fined wine extracts ($n = 3$) at both concentrations tested (100 and 1000 µg/mL). On the other hand, the peptide ion with m/z 729.2, found only in wine samples fined with caseinate, corresponds to the mono-protonated form of peptide DMPIQAFLLYQEPVLGPVR from β casein, that was detected as doubly charged ion in the tryptic digest of the caseinate standard (see Table 1). It is worth noting that no peptide related to milk proteins could be found when the tryptic digest of blank wine was considered.

A relevant role in assigning peptide sequences (and then the corresponding proteins) was played by fragmentation patterns

Table 2

Proteins identified in white wine fined with caseinate at 100 µg/mL. Assigned peptide sequences and product ions matching with those predicted for each sequence are also reported.

Protein identified	Ions observed (z)	Ion score ^a	Aminoacidic sequence	Matching b-ions	Matching y-ions
α S1 CN	634.2 (+2);	46	YLGYLEQLLR	b: 334.18 (b3), 277.15 (b2), b ₄ ²⁺ : 249.12 (b4)	y: 1104.64 (y9), 991.56 (y8), 934.53 (y7), 771.47 (y6), 658.38 (y5), 529.34 (y4), 288.20 (y3)
	692.6 (+2)	34	FFVAPFPEVFGK	b: 465.24 (b4), 394.21 (b3), 295.14 (b2)	y: 1090.50 (y10), 991.52 (y9), 920.49 (y8), 823.43 (y7), 676.37 (y6), 579.31 (y5), 450.27 (y4), 351.20 (y3), 204.13 (y2) y ₉ ²⁺ : 496.27 (y9)
β CN	729.2 (+3)	40	DMPIQAFLLYQEPVLGPVR	b: 1449.70 (b12), 1192.60 (b10), 1029.54 (b9), 457.21 (b4),	y = 1270.71 (y11), 1157.63 (y10), 994.57 (y9), 866.51 (y8), 737.47 (y7), 541.34 (y6), 428.26 (y4) y ₇ ²⁺ : 369.23 (y7)
	742.2 (+1)	27	GPFPIIV	b: 625.37 (b6), 512.29 (b5), 399.20 (b4), 302.15 (b3), 155.08 (b2)	y = 441.30 (y4), 231.17 (y2)

^a Individual ions scores >38 indicate identity or extensive homology ($p < 0.05$).

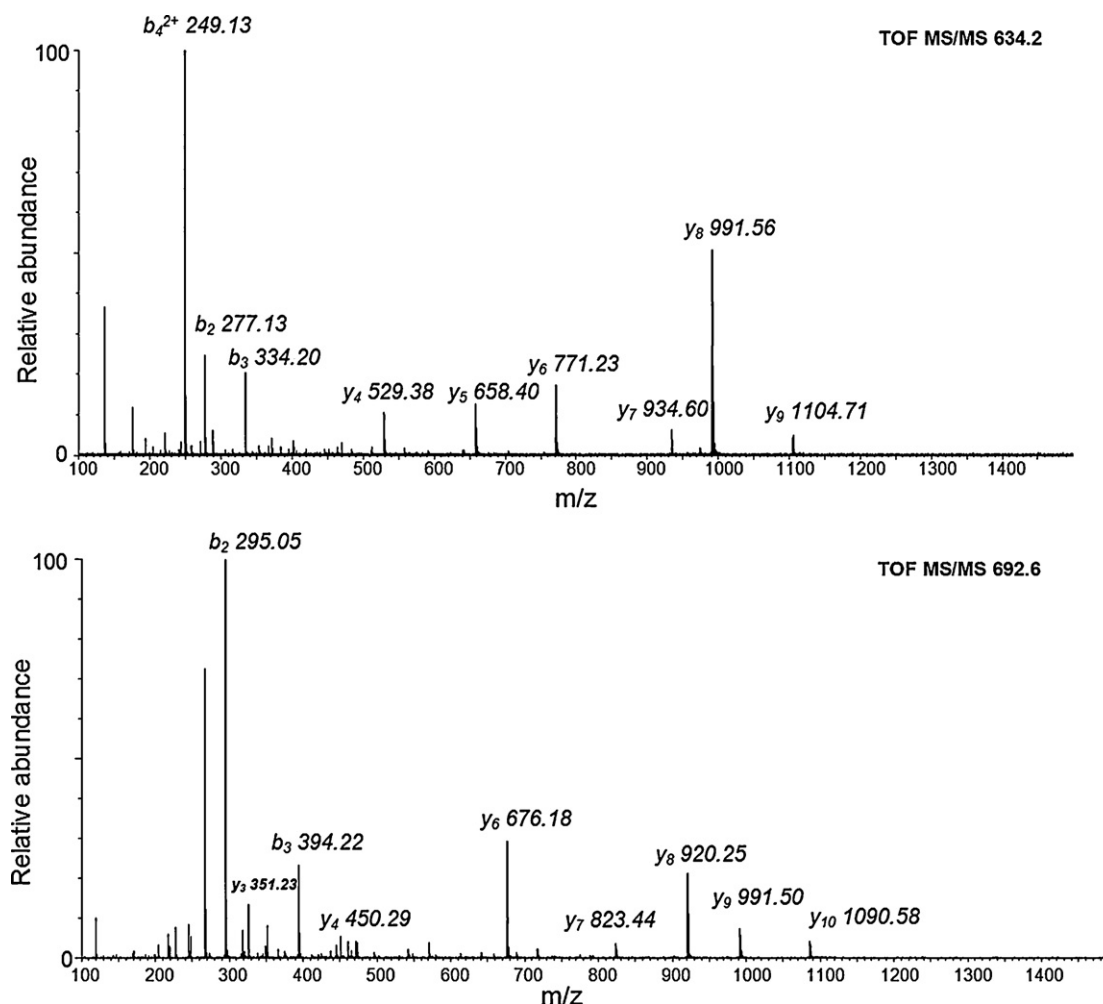


Fig. 1. MS/MS spectra for peptide ions with m/z 634.2 and 692.6, arising from α S1 casein, detected in fined white wine; the main matching product ions are indicated.

recovered from the MS/MS spectra acquired in the “survey” mode. Examples of spectra relative to peptide ions with m/z 634.2 and 692.6, assigned to α S1 casein, as observed in the tryptic digest of fined wine, are reported in Fig. 1.

As apparent, a good coverage of the main mass peaks in tandem MS spectra is assured by b - and y -type ions arising from the proposed aminoacidic sequences. A cross-check of the reliability of assignment for peptide sequences found in the tryptic digests of fined wine samples could be made in this case. Indeed, a comparison of retention times exhibited by a virtually identical peptide in the capLC traces relevant to the tryptic digest of caseinate standard and of fined wine could be performed.

As an example, a comparison of three chromatograms obtained by extracting the peptide ion current at m/z 742.2 from TIC chromatograms relative to the tryptic digests of fined white wine, caseinate standard solution (both at the final concentration of 100 $\mu\text{g/mL}$) and blank white wine, respectively, is depicted in Fig. 2.

A peak eluting near 17 min can be easily observed in the first two samples, whereas no significant signal is found, at the same retention time, in the case of the tryptic digest of blank white wine.

The MS/MS spectra averaged under the cited peak in the first two cases are compared in Fig. 3 and the relative fragments detected were assigned.

As highlighted in the figure, the two spectra show an identical fragmentation pattern, confirming the identity of the relevant peptide, GPFPIIV, i.e. the 218–224 sequence of β casein. It is worth noting that the “sequence query search”, performed by entering

the peptide mass-to-charge ratio and the experimentally observed fragments, allowed to identify β CN at the top with 59 as total score.

Further considerations, important from a quantitative point of view, arise from a comparison between peak intensities and shapes in extracted ion chromatograms and between MS/MS spectral intensities for the m/z 742.2 ion detected in the tryptic digests of fined white wine and of caseinate standard solution.

First, the peptide peak appears slightly higher and significantly larger in the second case, its area being almost 5 times larger than the one observed in the case of fined white wine. Moreover, ESI-MS spectra averaged under the two peaks show signals due to singly charged ions with m/z 1483.5 and to doubly charged ion with m/z 742.2 (together with singly charged ions having the same m/z ratio) in the case of caseinate tryptic digest. These evidences suggest the presence, in the latter case, of $[2M+H]^+$ and $[2M+2H]^{2+}$ cluster ions related to the GPFPIIV peptide. This phenomenon is likely due to the peptide higher concentration, compared to the tryptic digest of fined white wine.

It is worth noting that, when present contemporarily, singly and doubly charged ions having m/z ratio 742.2 are isolated and fragmented during the same MS/MS acquisition. It is reasonable to hypothesize that, among them $[2M+2H]^{2+}$ ions are rapidly split into two $[M+H]^+$ ions and subsequently fragmented inside the Q-TOF collision cell. As confirmed by Fig. 3, their fragmentation pattern is then identical to that observed for peptide ions originally present as $[M+H]^+$, like in the case of wine samples. However, the ratio of MS/MS spectral intensity for the two samples is quite higher than

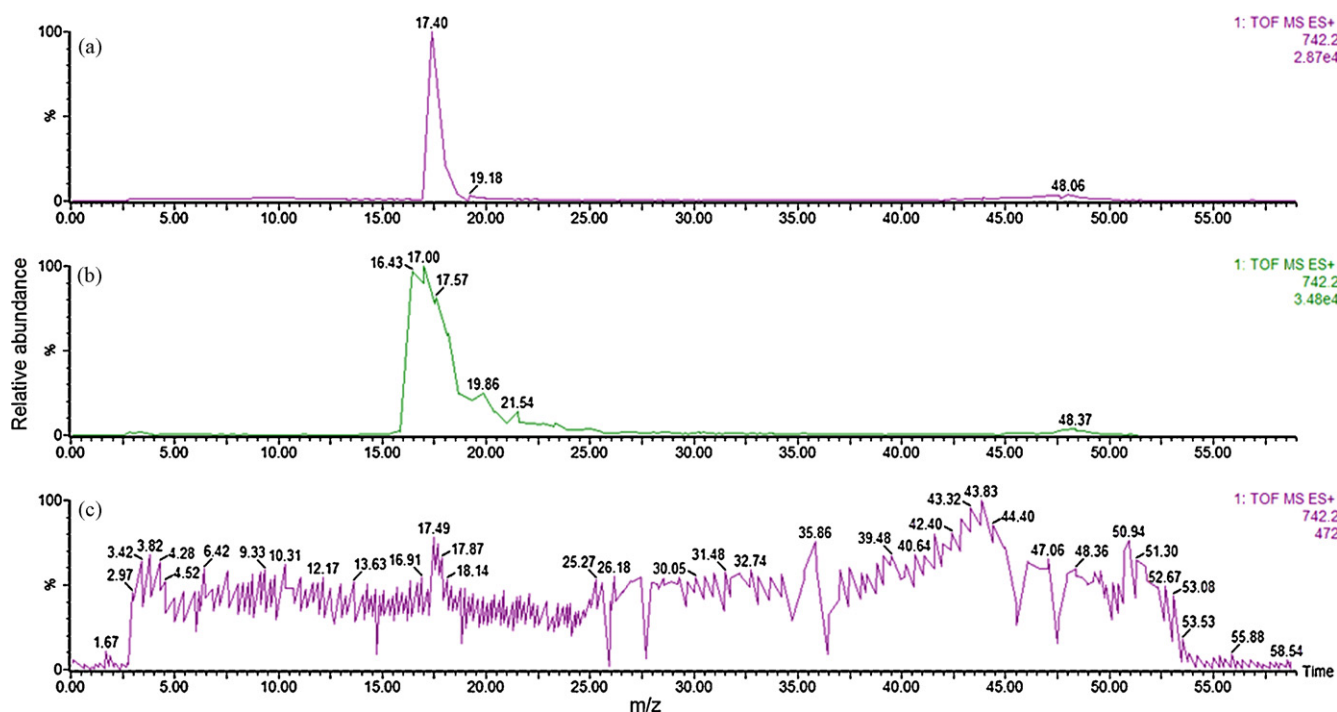


Fig. 2. Extracted ion chromatograms relevant to the m/z 742.2 peptide ion detected in the tryptic digests of (a) white wine fined with caseinate at 100 $\mu\text{g/mL}$, (b) caseinate standard 100 $\mu\text{g/mL}$ and (c) milk protein-free white wine.

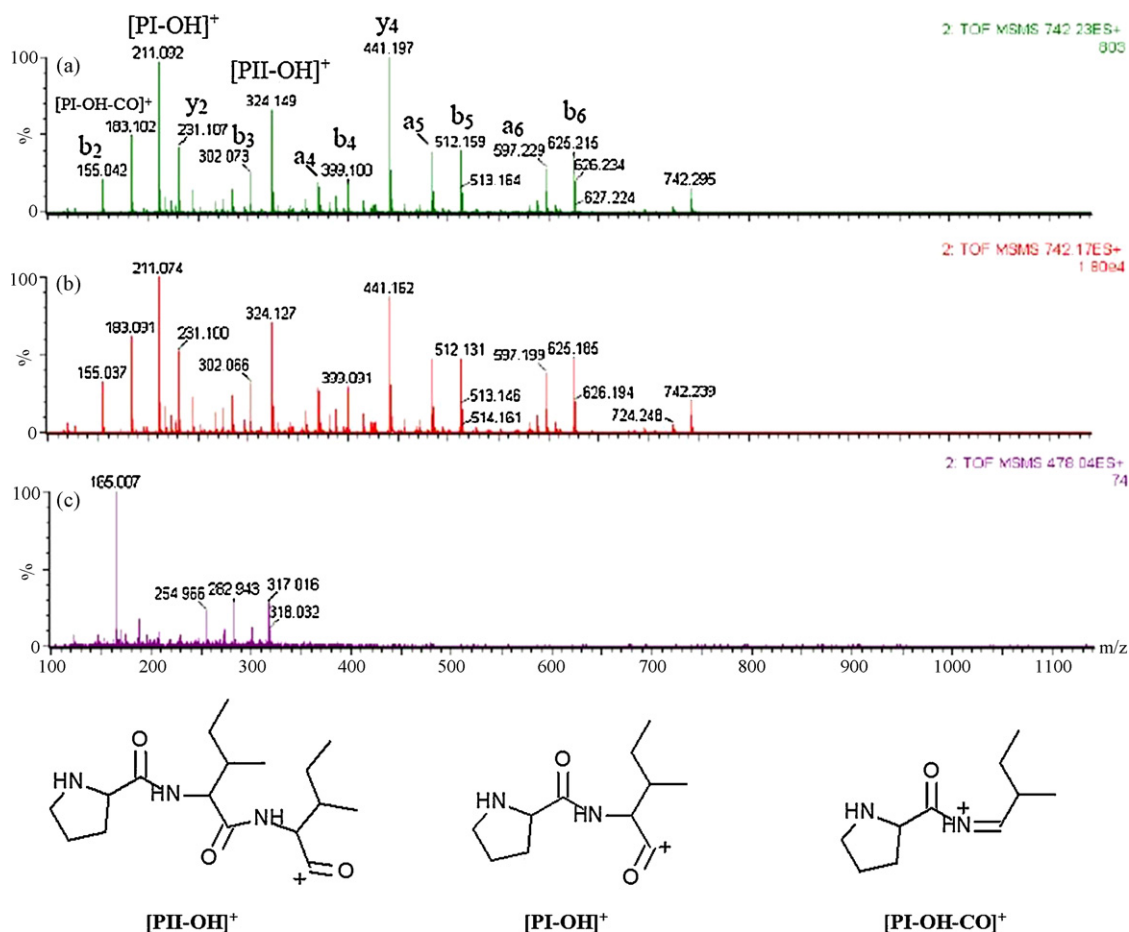


Fig. 3. MS/MS spectra averaged under the chromatographic peak eluting at 17 min in CapLC traces relevant to the tryptic digest of an extract of (a) white wine fined with caseinate at 100 µg/mL, (b) caseinate standard 100 µg/mL and (c) milk free white wine.

that observed for the peak areas in extracted ion chromatograms, as each $[2M+2H]^{2+}$ ion contributes as a single $[M+H]^+$ ion in a MS spectrum but as two $[M+H]^+$ ions in a MS/MS acquisition.

MS and MS/MS data presented so far for the m/z 742.2 ion contributed to unequivocally identify the presence of β casein in wine fined with caseinate. On the other hand, the absence of the peptide in milk free wine samples confirmed that it can be used to trace caseinate added to wine.

To the best of our knowledge, it is the first time that mass spectrometric detection is used to confirm the presence of caseins in wine, as residues of fining procedures, although the pH conditions of the medium are not optimal for their solubilisation.

As for quantitative aspects, the m/z 742.2 ion was used as a caseinate marker for a preliminary estimate of the limits of detection for the developed CapLC–MS method. In the case of caseinate standard, the marker could be detected in MS mode and confirmed by MS/MS data down to a $1 \mu\text{g/mL}$ concentration. When fined wine samples were considered, the lowest added concentration for which the peptide marker could be detected was $50 \mu\text{g/mL}$. Improvements of such limit could be potentially achieved by implementing peptide pre-concentration approaches and by using more sensitive MS acquisition modes (e.g. the Selective Ion Monitoring mode). Work is in progress in this direction.

4. Conclusions

Specific peptide ions (m/z 742.2, 729.2, 634.2 and 692.6), arising from the tryptic digestion of caseins in white wine samples fined with a caseinate standard, could be identified unequivocally by capLC–ESI–MS/MS analysis followed by bioinformatic search. Consequently, the four peptides can be proposed as candidate markers for the presence of caseinate added to white wines for processing aids.

The results obtained in this preliminary investigation suggest that a capLC–MS protocol targeted on specific peptides, supported by MS–MS analysis and fragmentation pattern interpretation, can be a successful approach to the task of milk proteins detection in wine samples.

As far as MS detection is concerned, the use of selective ion monitoring, rather than a typical full scan MS approach, should simplify the chromatographic traces and lower the limits of detection, that are currently of $50 \mu\text{g/mL}$ for caseinate added to wine, when the

m/z 742.2 ion (i.e. the GPFPIIV peptide from β casein) is used as a caseinate marker. Future efforts will be devoted also to improving the overall sensitivity of the method developed by introducing an on-line pre-concentration step by an enrichment column. The findings obtained in the present investigation appear important also from the consumer health point of view. Indeed, it was demonstrated that, in spite of their low solubility in the medium, caseins may dissolve in wine at concentration levels enabling subsequent detection of some of their tryptic peptides. Since caseins may trigger allergic reactions in sensitive consumers, it is of paramount importance to check for their presence also in wine.

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