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Investigation of different sample pre-treatment routes for liquid chromatography–tandem mass spectrometry detection of caseins and ovalbumin in fortified red wine

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ABSTRACT

Different sample treatment protocols for the liquid chromatography–electrospray-tandem mass spectrometry (LC–ESI-MS/MS) analysis of potential residuals of ovalbumin and caseins added to red wines were developed. In particular, attention was paid to the simultaneous detection and quantitation of fining agent residues, i.e. ovalbumin, α- and β-casein, in wine samples. The different sample treatment methods were compared in terms of protein recovery. The use of denaturing agents combined with size exclusion concentration and purification allowed to obtain a reproducible (RDS < 20%) analytical protocol with good recoveries (73(±2) – 109(±4)% range) for digested proteins from 12.5 mL of wine sample. Matrix-matched calibration from LC–ESI-MS/MS analysis indicated that the devised method allowed detection of target peptides in the 0.01–0.8 μg/mL range. Finally, method applicability and selectivity was demonstrated by using fining agents commonly exploited in winery industry and by analyzing 20 commercial red wine samples.

1. Introduction

The liquid chromatography–electrospray-tandem mass spectrometry (LC–ESI-MS/MS) determination of hidden allergens in foods is becoming of relevant importance because of the several advantages offered by this technique, including multi-tag detection, unambiguous allergen identification and accurate quantitative data (Faeste, Ronning, Christians, & Granum, 2011; Monaci & Visconti, 2009). The experimental workflow is generally based on the selection of targeted peptides and the use of selected reaction monitoring (SRM) acquisition mode for quantitative purposes (Ansari, Stoppacher, Rudolf, Schuhmacher, & Baumgartner, 2011; Bignardi, Elviri, Penna, Careri, & Mangia, 2010; Heick, Fischer, & Popping, 2011; Mattarozzi, Bignardi, Elviri, & Careri, 2012; Monaci, Losito, Palmisano, & Visconti, 2010). Basically, quality of the final analytical results is based on the sample preparation procedure, the performance of the whole analytical method and the selection of a suitable calibration mode. Different sample treatment methods have been proposed as a function of the investigated food matrices and protein allergens (Bignardi et al., 2012; Careri et al., 2008; Le Bourse, Jégou, Conreux, Villaume, & Jeandet, 2010; Monaci & van Hengel, 2008), representing a crucial step of the whole procedure. The sample preparation method is usually performed manually and it is expected to extract and purify the compounds of interest in an easy, quantitative and reproducible manner.

In this study our attention was focused on the LC-MS/MS analysis of potential residuals allergens in red wine. Red wine production in Italy plays an important role in the agricultural economy of several regions both at the local and international level. The organoleptic, antioxidants and anti-inflammatory properties of the red wine constituents are widely investigated and known. Red wine is an extremely complex matrix rich in polyphenols, tannins, anthocyanins and other molecules that can easily interact with proteins making challenging their quantitative analysis (Le Bourse et al., 2010; Moreno-Arribas, Pueyo, & Polo, 2002; Vincenzi et al., 2005). Recently, attention was paid to the putative presence of traces of exogenous proteins (i.e. caseins, albumins, lysozyme, gluten) added during wine fining process and removed before bottling. These proteins present allergen activity and the accurate

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determination of their residual concentration level is desiderable to ensure consumer safety (D'Amato, Kravchuk, Bachi, & Righetti, 2010; Tolin, Pasini, Curioni, et al., 2012; Tolin, Pasini, Simonato, Mainente, & Arrigoni, 2012; Weber, Steinhart, & Paschke, 2007).

Different analytical methods were proposed in the literature for quantitative purposes based both on immunoassay and mass spectrometry techniques (D'Amato et al., 2010; Lacorn, Gosswein, & Immer, 2011; Monaci, Losito, De Angelis, Pilolli, & Visconti, 2013; Monaci, Losito, Palmisano, Godula, & Visconti, 2011; Monaci et al., 2010; Restani et al., 2012; Rolland, Apostolou, De Leon, Stockley, & O'Hehir, 2008; Simonato, Mainente, Tolin, & Pasini, 2011; Tolin, Pasini, Curioni, et al., 2012; Tolin, Pasini, Simonato, et al., 2012; Weber et al., 2007; Weber, Steinhart, & Paschke, 2009). Immunoassays present unique advantages of simplicity and fastness, but they are usually performed on a single target and mainly suffer of cross-reactivity reactions and poor accuracy. In this work, an LC-MS/MS method for the simultaneous determination of ovalbumin, α- and β-casein in red wine is proposed. Different sample treatments for the detection of allergen residues in wine were evaluated and compared in terms of protein recovery. Generally, the investigated processes involved the use of cut-off filters, denaturing agents, protein precipitation or size exclusion purification cartridges. Finally, by using the most suitable and efficient sample treatment protocol, the LC–MS/MS method was validated and applied to commercial fining agents and red wine samples.

2. Material and methods

2.1. Chemicals

Urea (99.8%, purity) and thiourea were purchased from Carlo Erba (Milan, Italy). Acetonitrile (HPLC purity), formic acid (analytical reagent grade), trifluoroacetic acid (TFA, >98% purity), sodium dodecyl sulfate (SDS, 99% purity), trichloroacetic acid (TCA, >99% purity), ammonium hydrogen carbonate (99% purity), trypsin from bovine pancreas, iodoacetamide (IAM, >99% purity), DL-dithiothreitol (DTT, >99% purity), α-casein, β-casein, ovalbumin and Bradford reagent were from Sigma–Aldrich (St. Louis, Missouri, USA). Potassium caseinate (Protoclar®) and egg-white powder were purchased from a local enological store. Buffered solutions and mobile phases were obtained in HPLC-grade water prepared with a Milli-Q element A10 System (S. Francisco, CA, USA).

A total of 20 Italian commercial red still wine samples from different wine-producing regions and brands were purchased from local stores. The investigated wines were produced during 2007–2011 vintages (Table 1).

2.2. Bioinformatic analysis

For each targeted protein, peptides providing good ESI sensitivity and unequivocally identifying the target protein were selected. Thereby, a tryptic digest of a standard mixture of the three proteins (200 μg/mL) in NH_4HCO_3 50 mM pH 8 was analyzed by LC–MS/MS under data-dependent acquisition (DDA). Using this acquisition mode, the ion-trap was programmed to ignore any singly charged species acquired in the 300–1200 amu mass range and to perform MS/MS analysis (normalized collision energy: 30) only on eluting species that overcome a predefined threshold of 500 cps.

For each protein two marker peptides were selected (Table 2), considering different criteria such as quality of product ion spectra matches (Bioworks 3.3 database searching software, Thermo Electron Corporation, Marietta, Ohio) signal intensity of the most abundant product ion of MS/MS spectrum, no post translational modification sites and sequence specificity (BLAST search; algorithm: blastp; MATRIX PAM 30; GAP COSTS: existence 10, extension 1; DATABASE: non-redundant protein sequences).

Table 1
List of the analyzed red wine samples.

Sample N.	Red wine sample	Production Italian region	Vintage
1	Barbera	Piemonte	2010
2	Bonarda	Emilia Romagna	2010
3	Cannonau	Sardegna	2009
4	Cannonau	Sardegna	2010
5	Lambrusco	Emilia Romagna	2010
6	Lambrusco	Emila Romagna	2011
7	Lambrusco	Emilia Romagna	2011
8	Magliocco	Calabria	2009
9	Magliocco	Calabria	2011
10	Magliocco	Calabria	2011
11	Mamertino	Sicilia	2009
12	Montepulciano	Abruzzo	2011
13	Nebbiolo	Piemonte	2010
14	Nebbiolo	Piemonte	2011
15	Nero d'Avola	Sicilia	2007
16	Nero d'Avola	Sicilia	2008
17	Nero d'Avola	Sicilia	2009
18	Sangiovese	Emilia Romagna	2007
19	Syrah	Sicilia	2008
20	Syrah	Sicilia	2009

2.3. Sample treatment

A volume of 12.5 mL of red wine sample was fortified with the three proteins at different amounts and the mixture was homogenized before sample treatment. Five different sample treatments were investigated (procedure 1–5).

In procedure 1 the sample was centrifuged at 7000 rpm for 150 min in ultrafiltration tube with 5 kDa cut-off membrane (Sartorius Stedim Biotech, Goettingen, Germany), previously conditioned with distilled water, to obtain a concentrated final volume of 2.5 mL. Afterward, protein precipitation was performed by diluting the sample (1:8 ratio) with ethanol/TCA (15%, *w/v*) and keeping in ice for 2 h. The solution was centrifuged at 9000 rpm for 10 min at 4 °C. The pellet was then washed with ethanol and solubilized in 1 mL NH_4HCO_3 50 mM to pH 8. Tryptic digestion of wine extracts was performed after protein reduction and alkylation. Reduction was performed by addition of DDT to a final concentration of 10 mM and incubating the mixture at 30 °C for 40 min. For alkylation reaction IAM was added to a final concentration of 20 mM and the mixture was left for 40 min in the dark; then DTT was added to have a final concentration of 10 mM. In the final step,

Table 2
SRM transitions monitored for the target peptides from the allergen proteins investigated.

Protein	Precursor ion sequence (*m/z*; charge state)	Product ion sequence (*m/z*; charge state; fragment type)
α-casein	HQGLPQEVLNENLLR (*m/z* 587.2;+3)	HQGLPQEVLNENLL (*m/z* 793.6;+2; b_{14}^{+2})[a]
	YLGYLEQLLR (*m/z* 634.8;+2)	HQGLPQEV (*m/z* 445.2;+2; b_8^{+2})
		GYLEQLLR (*m/z* 991.8;+1; y_8^{+1})[a,b]
		LEQLLR (*m/z* 771.4;+1; y_6^{+1})
β-casein	VLPVPQK (*m/z* 390.9;+2)	PVPQK (*m/z* 568.4;+1; y_5^{+1})[a]
		PVPQK (*m/z* 284.6;+2; y_5^{+2})
	AVPYPQR (*m/z* 415.9;+2)	PYPQR (*m/z* 330.6;+2; y_5^{+2})[a,b]
		PYPQR (*m/z* 660.4;+1; y_5^{+1})
Ovalbumin	GGLEPINFQTAADQAR (*m/z* 844.9;+2)	PINFQTAADQAR (*m/z* 666.3;+2; y_{12}^{+2})[a,b]
		PINFQTAADQAR (*m/z* 1331.7;+1; y_{12}^{+1})
	VASMASEK (*m/z* 411.9;+2)	VASMASEK (*m/z* 402.8;+2; water loss)[a]
		SMASEK (*m/z* 652.2;+1; y_6^{+1})

[a] Most intense MS/MS transition.
[b] *m/z* transition monitored for the calculation of the validation parameters.