JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Immunochemical and Mass Spectrometry Detection of Residual Proteins in Gluten Fined Red Wine

Barbara Simonato,[†] Federica Mainente,[†] Serena Tolin,[‡] and Gabriella Pasini*[,‡]

[†]Dipartimento di Biotecnologie, Università di Verona, Strada Le Grazie 15, 37134, Verona, Italy

[‡]Dipartimento di Biotecnologie Agrarie, Università di Padova, Viale dell'Università 16, 35020 Legnaro (PD), Italy

Supporting Information

ABSTRACT: Recently, wheat gluten has been proposed as technological adjuvant in order to clarify wines. However, the possibility that residual gluten proteins remain in treated wines cannot be excluded, representing a hazard for wheat allergic or celiac disease patients. In this work, commercial wheat glutens, in both partially hydrolyzed (GBS-P51) and nonhydrolyzed (Gluvital 21000) forms, were used as fining agents in red wine at different concentrations. Beside immunoenzymatic analyses using anti-gliadin, anti-prolamin antibodies and pooled sera of wheat allergic patients, a method based on liquid chromatography coupled to mass spectrometry has been proposed to detect residues of gluten proteins. Residual gluten proteins were detected by anti-prolamin antibodies, anti-gliadin antibodies and sera-IgE only in the wine treated with GBS-P51 at concentration 50, 150, and 300 g/hL, respectively, whereas no residual proteins were detected by these systems in the wine treated with Gluvital 21000. In contrast liquid chromatography−mass spectrometry analyses allowed the detection of proteins in red wines fined down to 1 g/hL of Gluvital 21000 and GBS-P51. Our results indicate that MS methods are superior to immunochemical methods in detecting gluten proteins in wines and that adverse reactions against gluten treated wines cannot be excluded.

KEYWORDS: wheat gluten, fining, wine, mass spectrometry

INTRODUCTION

Wheat gluten is a flour fraction, mainly constituted of water-insoluble proteins (gliadins and glutenins that represent about 80% of total wheat proteins), containing also lipids (ca. 3.5−6.8%), minerals (ca. 0.5−0.9%) and carbohydrates (7.0 − 16.0%), the latter mainly consisting of starch and lesser amounts of nonstarch polysaccharides.[1]

Wheat gluten is widely used in the food industry as an ingredient in processed foods, and it could be utilized as a dough strengthener, formulation aid, nutrient supplement, stabilizer and thickener and texturing agent.[1] Recently, gluten has also been proposed as wine fining agent and its use was authorized by Food Standards Australia New Zealand (FSANZ)[2] and EC REGULATION No. 2165 (2005).[3] Fining wine is the process of removing undesirable substances such as phenolic compounds in order to prevent colloidal precipitation phenomena and to reduce bitterness and astringency.[4] In red wines this is commonly done by adding proteins such as gelatin, isinglass, albumin and casein.[5] These protein−phenol complexes are then removed by decanting, centrifugation or filtration.

Previous studies showed that commercial wheat gluten, especially when in the form of a hydrolyzed preparation, allows very efficient clarification of the wine,[6,7] and it has also been demonstrated that wheat gluten selectively precipitates condensed tannins from red wine and from a model one.[8]

Nevertheless, it is well-known that the gluten proteins are involved in both food allergy and celiac disease (CD) that occur in sensitive individuals after consumption of wheat products.[9] It has been demonstrated that several gluten proteins, such as α/β-, γ-, ω-gliadins, are implicated in wheat allergy.[10] Gliadins, particularly the α-gliadins, are also responsible for celiac disease, producing the most severe effects.[11] The gluten proteins are soluble in alcoholic and acidic solution,[12] and their solubility could increase after hydrolysis due to their conversion in low molecular mass protein derivatives. On the basis of these considerations, the possibility that gluten proteins remain in the wine after treatment cannot be excluded, representing a potential hazard for wheat allergic or CD patients.

As there are no safety threshold values for allergen contents in food, it is necessary to provide consumers with all the information on food composition on the label.[13] A list of allergenic foods to be declared in food labels has been drafted by legislation in USA and Europe. In particular the Directive 2006/142/EC of the European Commission extended the list to 14 foods, including cereal containing gluten.[14]

So, detection of residual gluten proteins in gluten treated wines has become an essential safety and legal issue.

In previous studies no gluten-associated immunochemical reaction was observed, by immunoblotting or dot-blot, in red and white wine treated with raw, hydrolyzed and deamidated gluten by using polyclonal specific antibodies or allergic patients' sera.[6,15,16] Also, gluten antibodies were not able to recognize their antigens by ELISA in a Muscat must treated with deamidated gluten.[17] These results seem to exclude a risk for subjects with celiac disease or gluten allergy.

Recently, mass spectrometry (MS) methods for the direct and absolute identification and quantification of food allergens have

Received: July 23, 2010
Accepted: February 13, 2011
Revised: February 10, 2011
Published: March 04, 2011

Table 1. Analytical Characteristics of the Merlot Wine before Fining

total acidity (g/L tartaric acid)	6.0
pH	3.34
alcohol content (% v/v)	12.1
anthocyanins[a] (mg/L)	498
proanthocyanidins[a] (mg/L)	2257

[a] Means of the three determinations. Standard deviation <0.5%.

been developed. MS allows the detection of allergenic proteins in trace amounts, thanks to its high sensitivity. Previously, Weber et al.[18] detected caseins in cookies through a method based on nano liquid chromatography−tandem mass spectrometry (LC−MS/MS), while Monaci and van Hengel[19] developed a method for the quantification of whey allergen traces in mixed-fruit juice by using liquid chromatography coupled to MS. Also the major allergens of peanut were detected by using LC−MS/MS in vanilla ice cream[20] and in dark chocolate.[21] Moreover, a method to detect allergenic milk proteins in fined white wines based on capillary liquid chromatography combined with electrospray ionization tandem mass spectrometry has also been proposed.[22] Recently, a method based on combinatorial peptide ligand libraries (CPLLs) coupled to MS has been applied for identifying traces of casein, used as fining agent, in white[23] and red wines.[24]

Nevertheless, the detection of residual fining agents seems to be particularly complicated in red wines, as proteins are difficult to analyze for the presence of a large quantity of interfering compounds, such as polyphenols and carbohydrates.[25]

The aim of the present study is to evaluate the presence of gluten residual proteins in a red wine after its clarification with two commercial gluten samples. Beside the classical immunochemical analyses, using a polyclonal antibody against a wide range of gluten proteins and sera of wheat allergic patients, a method based on liquid chromatography coupled to MS for the detection of residual gluten proteins in red wine has been developed.

MATERIALS AND METHODS

Materials. A commercial wheat vital gluten (Gluvital 21000, Cerestar, Italia), a partially hydrolyzed commercial wheat gluten (GBS-P51, Chamtor, Francia) and a wheat gluten prepared in our laboratory from a commercial wheat semolina (WG) were used.

The red wine (cv. Merlot) used for the fining experiments was produced during the 2008 vintage. Some analytical characteristics of the wine are reported in Table 1.

Wine Analyses. Alcohol, pH and titratable acidity were determined using the OIV methods.[26]

Anthocyanins were determined using the method of Riberau-Gayon and Stonestreet.[27] Proanthocyanidins were quantified according to Pompei et al.[28]

In-House Wheat Gluten Preparation. 100 g of durum wheat semolina was mixed with 40 mL of water to obtain dough. Gluten was prepared by washing the dough under tap water for 2−3 min in order to eliminate water-soluble proteins and starch. The gluten ball preparation was freeze-dried and grinded into a soft powder (WG).

Nitrogen Quantification and Protein Content Determination. Samples were mineralized according to the method of Hach et al.[29] and nitrogen was quantified by the AOAC method 33.056.[30] The protein content was calculated as $N \times 5.7$.

Evaluation of Wheat Gluten Solubility. The solubility of the different wheat gluten samples was evaluated in model wine. 50 mg of each sample was solubilized in 50 mL of model wine (12% (v/v) ethanol, 5% tartaric acid, pH 3.5). After 24 h in continuous stirring, the samples were centrifuged at 12000*g* for 30 min and the soluble nitrogen was measured according to the method of Hach et al.[29]

Statistical Analysis. Total protein and protein soluble in model wine were performed in triplicate. Statistical analysis was calculated by Student's *t* test.

Fining Experiments. Gluvital 21000 and GBS-P51 were used for fining experiments in an unfined Merlot red wine.

Each commercial gluten was suspended in water at a final concentration of 5% (w/v) and stirred for 12 h at room temperature before use. The suspensions were added to 500 mL of wine to final concentrations of 1, 5, 10, 15, 50, 150, 250, 300 g/hL. After settling for 48 h at 20 °C, wine samples were centrifuged at 1900*g* for 10 min.[31]

Protein Recovery from the Clarified Wine. Protein recovery from the clarified wine was made by the potassium dodecyl sulfate method (KDS method).[32] Briefly, 10% (w/v) sodium dodecyl sulfate (SDS) was added to samples (25 mL) to a 0.2% (w/v) final concentration. Samples were heated in boiling water for 10 min. 2 M KCl was then added to reach a final concentration of 400 mM, and samples were gently mixed for a further 45 min at 4 °C. KDS−protein pellets were recovered by centrifugation at 14000*g* for 15 min at 4 °C.

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to Laemmli[33] in a Mini Protean II cell (Bio-Rad, Milan, Italy) with a gel containing a total polyacrylamide concentration of 15%.

Ten milligrams of each gluten sample was solubilized in 1 mL of buffer A [0.3 M Tris-HCl, pH 7.4 containing 2% (w/v) SDS and 8% (w/v) glycerol]. After stirring for 1 h, samples were centrifuged at 10000*g* for 20 min. Fifteen microliter portions of the resulting protein solutions were loaded on the gels.

KDS−protein pellets were resuspendend in 500 μL of buffer A, and 10 μL of each sample was loaded onto the gels.

Electrophoresis was run at 50 mA constant current until the tracking dye bromophenol blue reached the bottom of the gel. Molecular weight standard proteins (Bio-Rad) were phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovoalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soy trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

Dot-Blot. KDS−protein pellets (from 25 mL of clarified wine) were resuspendend in 500 μL of buffer B (0.3 M Tris-HCl pH 6.0) and used for dot-blotting. 45 μL of resuspended KDS−protein pellets and 10 μL of a solution (1 mg/mL) of each commercial wheat gluten (as a control) were spotted on the nitrocellulose membrane as described by De Zorzi et al.[34]

Immunoenzymatic Analysis. Dot-blots were performed according to the method reported by Pasini et al.[35] using a polyclonal antibody against wheat prolamins (high molecular weight, low molecular weight and α/β- and γ-gliadins)[36] diluted 1:200 as a primary antibody. Anti-rabbit IgG horseradish peroxidase conjugate diluted 1:10000 (Sigma, A-9169) was secondary antibody.

Dot-blots were also developed using an anti-gliadin antibody (Sigma G-9144) diluted 1:3000.

IgE-binding proteins on dot-blots (using pooled sera) were detected by an anti-human IgE peroxidase−conjugate antibody (Sigma) diluted 1:6000. The negative control was a spot of bovine serum albumin (BSA). Peroxidase activity was visualized by chemioluminescence using the Super Signal detection kit (Pierce), following the manufacturer's instructions.

Pooled sera were collected by patients (4 women, 3 men, aged between 25 and 45 years) reporting gastrointestinal symptoms after ingestion of wheat-based foods, selected on the basis of positivity to RAST (radioallergosorbent test) and SPT (skin prick test).

Reverse-Phase LC−MS/MS Analyses. Wines clarified with 25, 15, 10, 5, and 1 g/hL of GBS-P51 and Gluvital 21000 were used for mass

spectrometry analysis. Proteins recovered from 50 mL of clarified wines by the KDS method were washed with acetone, in order to eliminate SDS, and centrifuged at 12000*g* for 10 min. The obtained pellets were dried and then dissolved in 50 mM NH_4HCO_3, 8 M urea, reduced with 10 mM dithiothreitol (DTT) (1 h, 37 °C, in the dark) and alkylated with 30 mM iodoacetamide (1 h, at room temperature, in the dark). One microgram of sequencing grade modified trypsin (Promega, Madison, WI, USA) was added after 1:10 dilution of the samples with 50 mM NH_4HCO_3 to reach a final concentration of 0.8 M urea, and digestion was carried out overnight at 37 °C. Samples were desalted with C18 cartridges (Strata C18-E, 50 mg/1 mL, Phenomenex). Extracted peptides were dissolved in 50 μL of 0.1% formic acid. Liquid chromatography−tandem mass spectrometry (LC−MS/MS) analyses were performed with a 6520 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) coupled to a chip-based chromatographic interface. A large capacity chip was used, 2 μL samples were injected into the enrichment column (C18, 9 mm, 160 nL volume) at a flow rate of 4 μL/min, and peptides were separated in the C18 nanocolumn (150 mm × 75 μm) at a flow rate of 0.5 μL/min. Water/formic acid 0.1% and acetonitrile/formic acid 0.1% were used as eluents A and B respectively. The chromatographic separation was achieved by using a gradient of eluent B from 3% to 50% in 50 min. Mass spectra were acquired in a data dependent mode: MS/MS spectra of the 3 most intense ions were acquired for each MS scan in the range of 350−2400 Da. Scan speed was set to 4 MS spectra/s and 3 MS/MS spectra/s. Capillary voltage was set to 1750 V and drying gas to 5 L/s. Raw data files were converted into Mascot Generic Format (MGF) files with MassHunter Qualitative Analysis Software version B.03.01 (Agilent Technologies) and searched using Mascot Search Engine (version 2.2.4 Matrix Science, London, U.K.) against the UniRef100 database (version 15.14, 9757328 sequences, 3429197968 residuals). Enzyme specificity was set to semitrypsin with one missed cleavage using a mass tolerance window of 10 ppm for the precursor ion and 0.05 Da for the fragment ions and carbamidomethylcysteine as fixed modification. Proteins with at least 2 peptides with individual significant score ($p < 0.05$) were considered as positively identified.

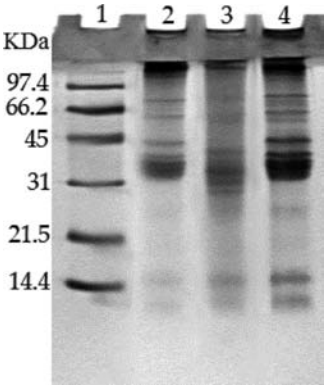

Figure 1. SDS−PAGE in unreducing conditions of wheat glutens used for wine fining, stained with Coomassie Brilliant Blue. Lane 1: MW markers. Lane 2: wheat gluten (WG). Lane 3: GBS-P51 (partially hydrolyzed). Lane 4: Gluvital 21000.

RESULTS

Wheat Gluten Analyses. *Total Protein Content.* Gluvital 21000 contained 89 (SD ±3.6), GBS-P51 84 (SD ±2.8) and WG 83 (SD ±4.3) mg of protein/100 mg of sample, and the remainder of constituents was mainly starch.

Electrophoresis. The protein patterns of the two commercial glutens were analyzed by electrophoresis in nonreducing conditions and compared to WG (Figure 1). As expected, the electrophoretic profile of Gluvital 21000, a gluten retaining its functionality (Figure 1, lane 4), is very similar to WG (Figure 1, lane 2) showing the classical protein components of the wheat flour. These latter comprised α/β- and γ-gliadins (31−45 kDa); ω-gliadins (60−80 kDa) and glutenin polymers remaining blocked at the top of the gel, due to their high molecular mass.[37] Protein bands corresponding to members of the protein

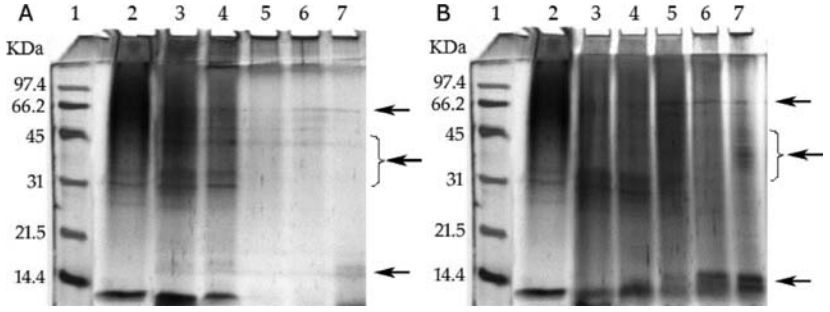

Figure 2. SDS−PAGE of red wine proteins recovered through the KDS method after clarification with wheat glutens (A, GBS-P51; B, Gluvital 21000) at different concentrations, stained with silver. Lane 1: MW markers. Lane 2: unclarified wine. Lane 3−7: wine + 10, 15, 50, 150, 300 g/hL of wheat glutens. The arrows indicate residual gluten proteins with M_r 14 kDa (α-amylase inhibitors), 31−45 kDa (gliadins) and 60 kDa (ω-gliadin).

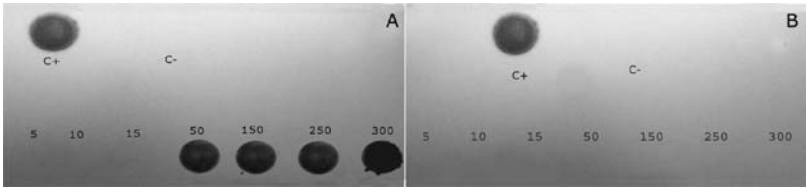

Figure 3. Dot-blot of wine fined with wheat glutens (A, GBS-P51; B, Gluvital 21000) at different concentration (5−300 g/hL), performed with antiprolamin antibodies. C+ = positive control (GBS-P51 or Gluvital 21000); C− = negative control (untreated wine).

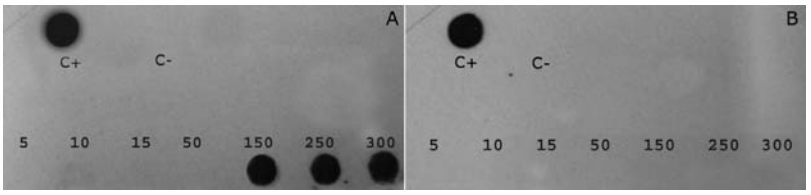

Figure 4. Dot-blot of wine fined with wheat glutens (A, GBS-P51; B, Gluvital 21000) at different concentration (5−300 g/hL), performed with anti-gliadin antibodies. C+ = positive control (GBS-P51 or Gluvital 21000); C− = negative control (untreated wine).

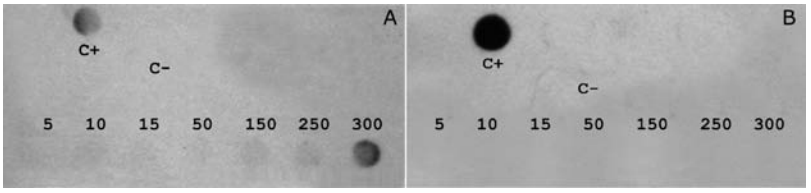

Figure 5. IgE-dot blot of wine fined with wheat glutens (A, GBS-P51; B, Gluvital 21000) at different concentration (5−300 g/hL), performed with pool wheat allergic sera. C+ = positive control (GBS-P51 or Gluvital 21000); C− = negative control (untreated wine).

Table 2. Proteins Identified in Red Wines Fined with 5 g/hL of Gluvital 21000 or GBS-P51[a]

(A) Gluvital 21000		
accession number	identified protein	species
B2Y2Q1	**LMW glutenin subunit**	*Triticum aestivum* (wheat)
Q0GNF9	LMW glutenin	*Triticum aestivum* (wheat)
B2Y2R4	LMW glutenin subunit	*Triticum aestivum* (wheat)
Q6QGV8	LMW glutenin pGM107	*Triticum aestivum* (wheat)
Q1WA40	**alpha-gliadin Gli2-LM2-12**	*Triticum aestivum* (wheat)
P17314	**alpha-amylase/trypsin inhibitor CM3**	*Triticum aestivum* (wheat)
D0ES81	gamma-gliadin	*Triticum aestivum* (wheat)
A5JSA7	**alpha-gliadin**	*Triticum aestivum* (wheat)
B6UKP6	gamma-gliadin	*Triticum aestivum* (wheat)
Q4U195	**dimeric alpha-amylase inhibitor**	*Triticum aestivum* (wheat)
D2KFG9	gliadin/avenin-like seed protein	*Triticum aestivum* (wheat)
B5B0D5	**major allergen CM16**	*Triticum aestivum* (wheat)
Q2A784	putative avenin-like a	*Triticum aestivum* (wheat)
A4ZIX1	monomeric alpha-amylase inhibitor	*Triticum aestivum* (wheat)
B8XU29	alpha-gliadin	*Triticum monococcum*
Q6UJY7	HMW-glutenin By subunit	*Triticum turgidum*
A9YSK5	HMW glutenin subunit	*Triticum aestivum* (wheat)
Q41540	CM 17 protein	*Triticum aestivum* (wheat)
Q9M4G6	Putative thaumatin-like protein	*Vitis vinifera* (grape)
Q7XAU7	thaumatin-like protein	*Vitis vinifera* (grape)
D1IBD6	cysteine-type endopeptidase	*Vitis vinifera* (grape)
D1IP80	chitinase-like	*Vitis vinifera* (grape)
A5AYU8	ATP synthase subunit beta	*Vitis vinifera* (grape)
D1HU00	hydrolase/glycosidase activity	*Vitis vinifera* (grape)
B6VJY3	ATP synthase subunit alpha	*Vitis vinifera* (grape)
A5BAX1	putative uncharacterized protein	*Vitis vinifera* (grape)
D1HDI5	cysteine-type endopeptidase	*Vitis vinifera* (grape)
D1HTT2	enolase family	*Vitis vinifera* (grape)
Q7XAU6	class IV chitinase	*Vitis vinifera* (grape)
A5BUW9	putative uncharacterized protein	*Vitis vinifera* (grape)
A5AKD8	peptidyl-prolyl cis−trans isomerase	*Vitis vinifera* (grape)
Q09VU3	phospholipase D alpha	*Vitis vinifera* (grape)
D1IRB3	ATPase (P-type) family	*Vitis vinifera* (grape)

Table 2. Continued

(A) Gluvital 21000		
accession number	identified protein	species
B3LP15	protein YGP1	*Saccharomyces cerevisiae*
B5VJM6	YGR282Cp-like protein	*Saccharomyces cerevisiae*
Q04E64	60 kDa chaperonin	*Oenococcus oeni*
(B) GBS-P51		
accession number	identified protein	species
Q0GNF9	LMW glutenin	*Triticum aestivum* (wheat)
B2Y2Q1	**LMW glutenin subunit**	*Triticum aestivum* (wheat)
Q6QGV8	LMW glutenin pGM107	*Triticum aestivum* (wheat)
B5ANT6	LMW glutenin	*Triticum aestivum* (wheat)
Q1ZZT4	LMW glutenin subunit	*Triticum aestivum* (wheat)
B2Y2S1	**LMW glutenin subunit**	*Triticum aestivum* (wheat)
Q571Q5	putative LMW-glutenin subunit	*Triticum aestivum* (wheat)
A7LHB3	**alpha-gliadin**	*Triticum aestivum* (wheat)
C0SUC3	HMW glutenin subunit x5	*Triticum aestivum* (wheat)
Q41593	**serpin**	*Triticum aestivum* (wheat)
B6UKL5	gamma-gliadin	*Triticum turgidum*
B6UKM7	gamma-gliadin	*Triticum aestivum* (wheat)
B9VRI0	gamma-gliadin	*Triticum aestivum* (wheat)
A7LHA4	alpha-gliadin	*Lophopyrum elongatum*
P17314	**alpha-amylase/trypsin inhibitor CM3**	*Triticum aestivum* (wheat)
D1MJA1	HMW glutenin subunit Ax-dp	*Triticum turgidum*
A1EHE7	gamma-gliadin	*Triticum aestivum* (wheat)
Q1WA39	**alpha-gliadin**	*Triticum aestivum* (wheat)
Q0Q5D8	Y-type HMW glutenin	*Triticum aestivum* (wheat)
B2CGM6	triticin	*Triticum aestivum* (wheat)
Q1WA40	**alpha-gliadin Gli2-LM2−12**	*Triticum aestivum* (wheat)
A4ZIX1	Monomeric alpha-amylase inhibitor	*Triticum aestivum* (wheat)
Q6UJY5	HMW-glutenin Bx subunit	*Triticum turgidum*
Q41528	**alpha-gliadin**	*Triticum aestivum* (wheat)
B7U6L4	globulin 3	*Triticum aestivum* (wheat)
B5B0D5	**major allergen CM16**	*Triticum aestivum* (wheat)
Q2A784	putative avenin-like a	*Triticum aestivum* (wheat)
Q41540	CM 17 protein	*Triticum aestivum* (wheat)
Q9M4G6	putative thaumatin-like protein	*Vitis vinifera* (grape)
D1HU00	hydrolase/glycosidase activity	*Vitis vinifera* (grape)
Q7XAU7	thaumatin-like protein	*Vitis vinifera* (grape)
D1IBD6	cysteine-type endopeptidase	*Vitis vinifera* (grape)
A5AKD8	peptidyl-prolyl cis−trans isomerase	*Vitis vinifera* (grape)
A5AYU8	ATP synthase subunit beta	*Vitis vinifera* (grape)
Q04E64	60 kDa chaperonin	*Oenococcus oeni*

[a] Potential allergens are in bold.

family of the α-amylase/trypsin inhibitors (M_r 14−16 kDa) were also evident.

Since WG and Gluvital 21000 were very similar (Figure 1, lanes 2 and 4), only the latter was considered for the following studies.

In GBS-P51 (Figure 1, lane 3), the partially hydrolyzed wheat gluten, protein bands belonging to gliadins (31−45 kDa) were less evident obviously due to the enzymatic breakdown that gives rise to the accumulation of bands with molecular masses in the range of 31−14 kDa. ω-Gliadins (60−80 kDa) and α-amylase inhibitors (M_r 14−16 kDa),[37] were also evident in GBS-P51 (Figure 1, lane 3), indicating their resistance to the enzymatic treatment used to produce this latter gluten preparation.[35]

Protein Solubility. As gluten proteins are partially soluble in 70% alcohol/water mixture (especially gliadins) and partially soluble in acid solution (especially glutenins),[12] we have evaluated the solubility in model wine. The protein solubility in model wine was 19.9 (SD ±1.39) and 49.2 (SD ±3.2) mg/100 mg of Gluvital 21000 and GBS-51, respectively. As GBS-P51 presented higher solubility than Gluvital 21000, this is probably due to the smaller molecular mass of the proteins deriving from hydrolysis process.

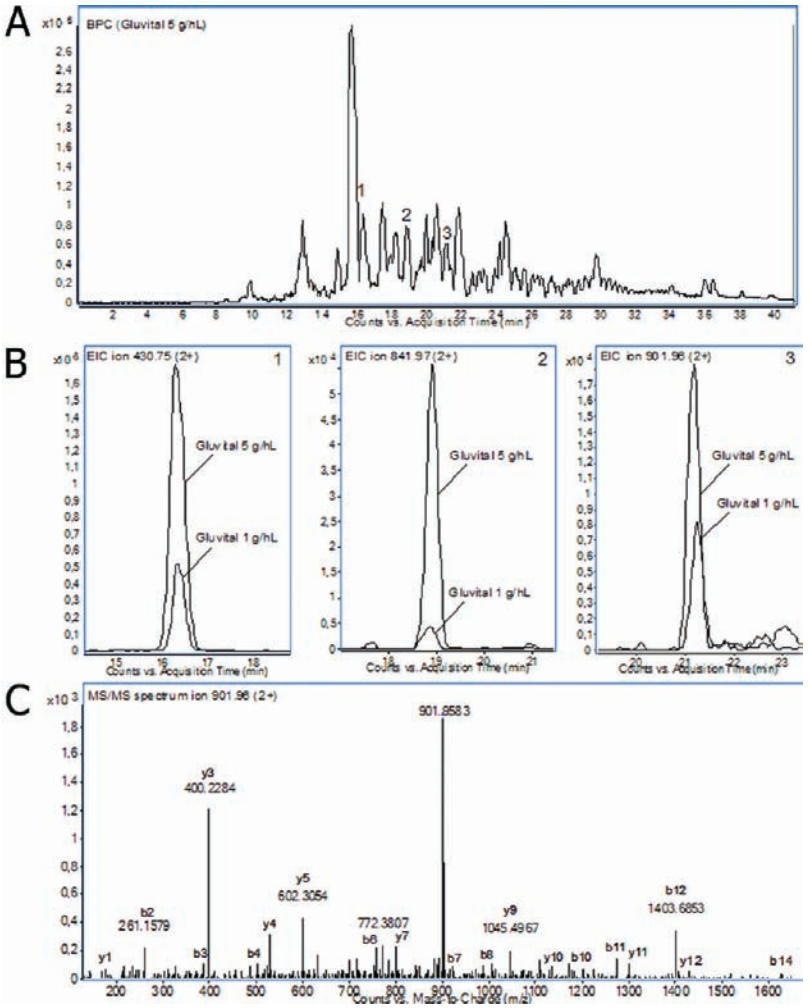

Figure 6. A: Base peak chromatogram (BPC) of a tryptic digest of red wine fined with 5 g/hL Gluvital. B: Extracted ion chromatogram (EIC) of 3 peptide ions arising from gliadin and glutenin, detected in sample fined with 1 or 5 g/hL Gluvital, as indicated. 1: Peptide VNVPLYR (ion 430.75, 2+), low molecular weight glutenin subunit (UniProt accession number B2Y2Q1). 2: Peptide IIQPQQPAQYEVIR (ion 841.97, 2+), gamma gliadin (UniProt accession number B6UKL5). 3: Peptide LFLQQQCSPVAMPQR (ion 901.96, 2+), low molecular weight glutenin subunit (UniProt accession number B2LS34). C: MS/MS spectrum of peptide ion 901.96 (2+) (LFLQQQCSPVAMPQR) detected in red wine fined with 1 g/hL Gluvital. The main matching b and y product ions are indicated in the spectrum and in Table 3.

Electrophoretic and Dot-Blotting Analyses of Wine Treated with Wheat Glutens. SDS−PAGE analyses of the proteins (with MW > 10 kDa) recovered through the KDS method from the wines treated with wheat glutens at different concentrations (10, 15, 50, 150, 300 g/hL) are reported in Figure 2. The control (untreated wine) (Figure 2A,B, lanes 2) showed the classical profile of the main endogenous wine proteins, including chitinase.[38] In general, adding wheat glutens at increasing concentrations resulted in electrophoretic patterns of increasing quality (Figure 2A,B). This can be due to removal of tannins by the gluten proteins. Bands corresponding to gluten proteins were detectable in the wine samples treated at high concentration of fining agents, especially Gluvital 21000. In particular, bands with molecular masses around 14 kDa (α-amylase inhibitors) (arrows in Figure 2) and 31−45 kDa (α/β- and γ-gliadins) (arrows in Figure 2) and a band with electrophoretic mobility corresponding to that of ω-gliadins (around 60 kDa) were revealed by silver staining (arrows in Figure 2).

In order to detect the possible presence of fragments of wheat gluten proteins with a small molecular mass too low to be retained in the electrophoretic gel, dot-blotting experiments using two antibodies, one specific for the wheat prolamins (high molecular weight, low molecular weight and α/β- and γ-gliadins) and the other for gliadins, were performed. Moreover dot-blots were also assayed using pooled sera of wheat allergic patients.

Residual wheat proteins were immunodetected by anti-prolamin antibodies only in the wine treated with the hydrolyzed gluten GBS-P51 (Figure 3A) from the 50 g/hL concentration upward; the anti-prolamin antibodies did not react with the wine treated with Gluvital 21000 (Figure 3B).

The commercial anti-gliadin antibody was able to bind residual proteins in the wine treated with GBS-P51 from the 150 g/hL concentration upward (Figure 4A). Also in this case no residual wheat proteins were detectable in the wine treated with Gluvital 21000 (Figure 4B).

IgE-dot-blots (Figure 5) showed IgE-binding proteins only in the wine treated with GBS-P51 (Figure 5A) at the concentration of 300 g/hL, whereas no IgE-binding was detectable in wine clarified with Gluvital 21000 (Figure 5B).

Reverse-Phase LC−MS/MS Analyses. Liquid chromatography−mass spectrometry (LC−MS/MS) analysis of the wines treated with decreasing concentrations (25, 15, 10, 5, and 1 g/hL) of glutens (Gluvital 21000 and GBS-P51) led to the identification of many wheat proteins. Grape proteins belonging to thaumatin-like proteins and chitinases were also identified as well as a few yeast proteins (*Saccharomyces cerevisiae*) and a protein from *Oenococcus oeni*. However the majority of identified proteins were from wheat. In particular, it was possible to clearly identify the presence of low and high molecular weight glutenin subunits, as well as components belonging to α/β- and γ-gliadins. Table 2, sections A and B, lists all the proteins that were identified in red wines fined with 5 g/hL of Gluvital 21000 and GBS-P51, respectively. The complete information on the identifications is provided in Table V (see Supporting Information), reporting the list of peptides identified by MS/MS analysis per each protein. The same kinds of proteins but, as expected, with a lower coverage were also identified in wines fined with Gluvital 21000 and GBS-P51 at a concentration of 1 g/hL.

The base peak chromatogram (BPC) of the tryptic digest of red wine fined with 5 g/hL of Gluvital 21000 is reported in Figure 6A. The profiles of three representative ions arising from gliadin and glutenin peptides were extracted from the chromatograms of samples fined with 1 and 5 g/hL Gluvital 21000 (Figure 6B). Figure 6C shows the MS/MS spectrum of a peptide from glutenin identified in the sample fined with 1 g/hL of Gluvital 21000. The spectrum is of very good quality, as shown also by the almost complete sequence of y and b ion matches reported in Table 3, indicating that the identification of gluten proteins could be unambiguously achieved also at such a low concentration of fining agent. In parallel, the BPC of the tryptic digest of red wine fined with 5 g/hL of GBS-P51 is reported in Figure 7A. Similar results were obtained for this sample, and three ion profiles arising from glutenin and gliadin peptides are reported in Figure 7B. The MS/MS spectrum of a peptide from gliadin identified in the sample fined with 1 g/hL of GBS-P51 is shown in Figure 7C, together with the list of b and y ions matched (Table 4). Also in the case of GBS-P51, gluten proteins could be clearly detected at this low dosage of fining agent.

Table 3. Fragment Ions of Precursor 901.96 (2+) (Peptide LFLQQQCSPVAMPQR, Low Molecular Weight Glutenin Subunit, Accession Number B2LS34)[a]

no.	b	seq	y	no.
1	114.09	L		15
2	**261.16**	F	1689.82	14
3	**374.24**	L	1542.75	13
4	**502.30**	Q	**1429.67**	12
5	630.36	Q	**1301.61**	11
6	758.42	Q	**1173.55**	10
7	918.45	C	**1045.49**	9
8	1005.48	S	885.46	8
9	1102.53	P	**798.43**	7
10	**1201.60**	V	701.38	6
11	**1272.64**	A	**602.31**	5
12	**1403.68**	M	**531.27**	4
13	1500.73	P	**400.23**	3
14	**1628.79**	Q	303.18	2
15		R	**175.12**	1

[a] b and y ions matched by Mascot are indicated in bold, and the amino acid sequence to which they correspond is indicated.

DISCUSSION

The use of plant proteins, including wheat gluten, as wine fining agents has been permitted by the Food Standards of Australia and New Zealand (2004)[2] and by EC REGULATION No. 2165 (2005).[3] Nevertheless, it is well-known that gluten proteins are the causal agents in pathologies such as celiac disease (CD) and food allergy.[9,37] While Codex Alimentarius establishes a limit for gluten quantity in gluten-free foods[39] intended for celiac patients, a safe limit cannot be established for food allergies.[9]

Previously,[6,15] it has been reported that wines fined with wheat glutens could be safe for patients affected by wheat allergy and CD, although only the presence of gliadins[15] and deamidated gluten proteins[6] was investigated. In our study, vital gluten containing approximately equal amounts of gliadins and glutenins has been considered; so the use of antibodies against the total prolamins seems to be mandatory. As it has been reported that several gluten proteins (α/β-, γ-, ω-gliadins and low molecular weight subunits) can be responsible for wheat allergy[10,37] while α-gliadin elicits CD,[9] the role of all the native proteins in such pathologies has to be investigated.

In the present paper, the research of residual wheat proteins was performed after their recovery from treated wine by using the KDS method. It has been demonstrated that this method is more efficient, not only for the white[32] but also for the red wines (personal data not shown) than protein precipitation by trichloroacetic acid (TCA) method, which has been previously used.[15] Dot-blotting experiments carried out with both the antibodies and also with wheat allergic patient sera showed residual proteins only in the wine treated with partially hydrolyzed gluten (GBS-P51). As a matter of fact, the anti-prolamin antibodies and the commercial anti-gliadin antibodies recognized residual proteins only for doses of 50 and 150 g/hL of gluten upward, respectively. This indicates that the type of antibody used has a major effect on the possibility to detect residual allergens, raising some doubts on the analyses by immunochemical methods.

A further step to evaluate the potential allergenicity can be the use of wheat allergic patients' sera whose IgE-binding was evident at 300 g/hL treated wine. It is interesting to note that the wine treated with the vital gluten (Gluvital 21000) which contains native proteins did not show any binding with both the antibodies (anti-prolamins and anti-gliadins) and sera-IgE. This result is obviously due to the low solubility of gluten proteins in wine. In contrast, hydrolyzed gluten proteins (GBS-P51) could remain in the clarified wines to a higher extent with respect to the native ones. Moreover, the possible alteration of the protein epitope structures after their absorption on the membrane surface in dot-blotting experiments might also compromise the results of the immunochemical reaction.[40] The partially hydrolyzed gluten (GBS-P51), having a good solubility in hydro-alcoholic solution, could remain in higher quantity in wine, and its epitopes could also be better exposed when spotted on solid matrices.

In any case, residual wheat proteins were detectable by the immunological methods only in wines treated with concentrations of gluten (>50 g/hL) higher than those usually employed in wine fining treatments. This could be ascribed to the sensitivity of the immunological techniques,[41] and indicates that these methods could be inappropriate for detection of allergens present in trace amounts.

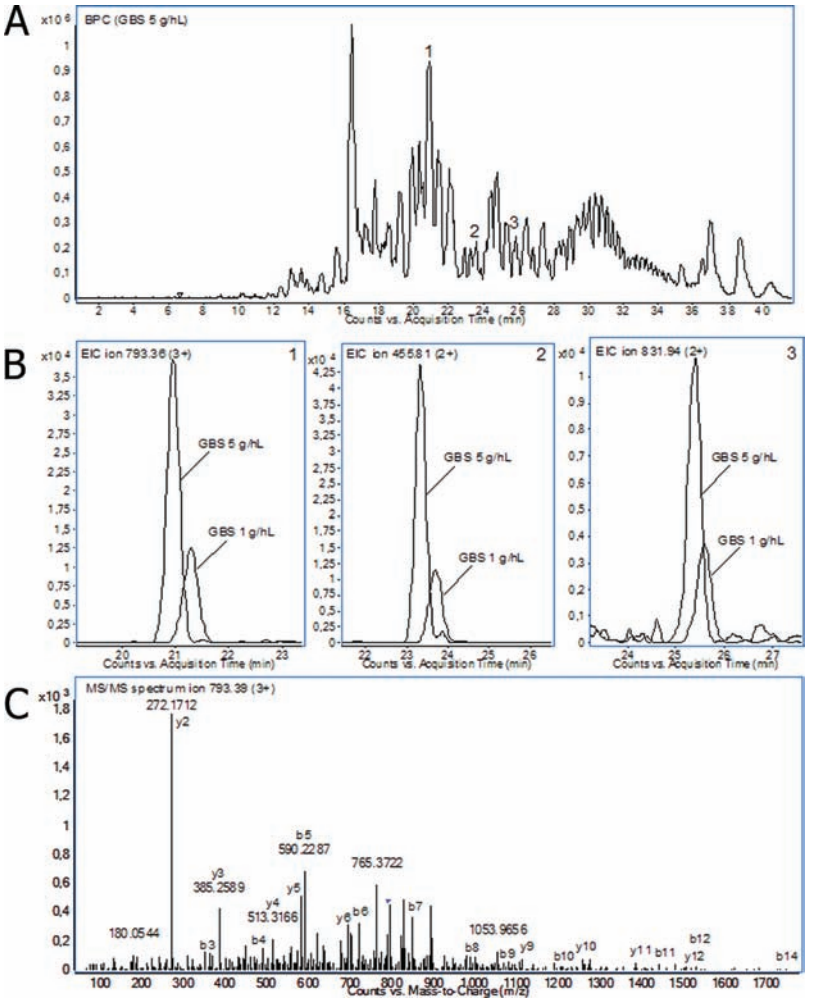

Figure 7. A: Base peak chromatogram (BPC) of a tryptic digest of red wine fined with 5 g/hL GBS. B: Extracted ion chromatogram (EIC) of 3 peptide ions arising from gliadin and glutenin, detected in sample fined with 1 or 5 g/hL GBS, as indicated. 1: Peptide SDCQVMQQQCCQQLAQIPR (ion 793.36, 3+), gamma gliadin (UniProt accession number B6UKL5). 2: Peptide LVSIILPR (ion 455.81, 2+), gamma gliadin (UniProt accession number B6UKL5). 3: Peptide ILPTMCSVNVPLYR (ion 831.9421, 2+), low molecular weight glutenin pGM107 (UniProt accession number Q6QGV8). C: MS/MS spectrum of peptide ion 793.36 (3+) (SDCQVMQQQCCQQLAQIPR) detected in red wine fined with 1 g/hL GBS. The main matching b and y product ions are indicated in the spectrum and in Table 4.

Recently, mass spectrometry (MS) methods for the direct and absolute identification and quantification of food allergens have been developed. Thanks to its high sensitivity MS allows the detection of allergenic proteins in trace, and makes the identification independent of the structure of the allergens[40,41] which can in contrast affect their detection by immunochemical methods. Here we have shown that KDS protein precipitation combined with the LC−MS/MS approach is a powerful method for the detection of residual proteins in red wines fined with glutens. The detection of proteins can be difficult in red wines, because of the presence of a large quantity of interfering compounds, such as polyphenols and carbohydrates.[25]

In this study protein samples have been recovered through the KDS method,[32,42] which is based on protein complexation with dodecyl sulfate followed by their precipitation as potassium salts. The KDS-recovered proteins can be used not only for quantitative and electrophoretic analyses[42] but, after washing with acetone in order to eliminate SDS, also for the MS analyses.

To the best of our knowledge, a study has been published regarding the analysis of red wine proteins[43] by a combination of SDS−PAGE and mass spectrometry.

Recently, a method based on combinatorial peptide ligand libraries (CPLLs) coupled to SDS−PAGE and MS has been applied for identifying traces of casein, used as fining agent, in red wines.[24] This method allows the amplification of rare protein signals that normally avoid detection even by MS. The preparation of wine samples provides the use of a different range of wine volumes (from 200 up to 750 mL), a partial removing of phenolic substances by incubation overnight with PVPP, then the absorption (about 3 h), the subsequent desorption of the captured proteins from the ProteoMiner beads, their separation on SDS−PAGE, in-gel digestion, and MS analysis.

In our paper we have also demonstrated a gel-free approach suitable for MS analysis of the proteins present in red wines. Moreover our method allows an easy and rapid sample preparation starting from a lesser quantity of wine and avoiding the removal of phenolic compounds step. Our data clearly showed that many wheat gluten proteins with well established allergenic properties (listed in Table 2, sections A and B) can remain in wines fined with gluten. In conclusion, residual gluten proteins can be clearly detected in red wines treated with as low as 1 g/hL

Table 4. Fragment Ions of Precursor 793.36 (3+) (Peptide SDCQVMQQQCCQQLAQIPR, Gamma Gliadin, Accession Number B6UKL5)[a]

no.	b	seq	y	no.
1	88.04	S		19
2	203.07	D	2291.01	18
3	**363.10**	C	2175.99	17
4	**491.16**	Q	2015.96	16
5	**590.22**	V	1887.90	15
6	**721.26**	M	1788.83	14
7	**849.32**	Q	1657.79	13
8	**977.38**	Q	**1529.73**	12
9	**1105.44**	Q	**1401.67**	11
10	**1265.47**	C	**1273.61**	10
11	**1425.50**	C	1113.58	9
12	**1553.56**	Q	953.55	8
13	1681.63	Q	825.49	7
14	**1794.70**	L	**697.44**	6
15	1865.74	A	**584.35**	5
16	1993.80	Q	**513.31**	4
17	2106.88	I	**385.26**	3
18	2203.94	P	**272.17**	2
19		R	175.12	1

[a] b and y ions matched by Mascot are indicated in bold, and the amino acid sequence to which they correspond is indicated.

of gluten (Table 2, Figures 6 and 7, Table V in the Supporting Information), a dose lower than those usually used for wine fining.

Therefore, it can be concluded that wine samples resulting negative for the presence of gluten proteins by the commonly employed immunochemical methods can potentially trigger an adverse reaction in gluten-sensitized people.

■ ASSOCIATED CONTENT

Supporting Information. Complete information on the identifications is provided in Table V, reporting the list of peptides identified by MS/MS analysis in wine fined with 5 and 1 g/hL Gluvital 21000 and GBS-P51. The observed and theoretical molecular weights of peptides are reported, together with the percent of sequence coverage, the difference between observed and calculated molecular weight (in ppm), the Mascot score and *p* value, the ranking of the identification, and the amino acid sequences of the identified peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +390498272919. E-mail: gabriella.pasini@unipd.it.

■ ACKNOWLEDGMENT

We thank dott. Mara Vegro for technical assistance.

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