



Analytical Methods

Immunochemical investigation of allergenic residues in experimental and commercially-available wines fined with egg white proteins



Francesca Uberti^a, Roberta Danzi^b, Creina Stockley^c, Elena Peñas^a, Cinzia Ballabio^a, Chiara Di Lorenzo^a, Chiara Tarantino^d, Patrizia Restani^{a,*}

^a Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, via Balzaretti 9, 20133 Milano, Italy

^b Unione Italiana Vini, via San Vittore al Teatro 3, 20123 Milano, Italy

^c The Australian Wine Research Institute, PO Box 197, Glen Osmond, South Australia 5064, Australia

^d Euroclone SpA, via Figino 20/22, 20016 Pero, MI, Italy

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ABSTRACT

Proteinaceous egg whites are widely used as a fining agent during the production of red wines. Residues of egg white in the final wine could present a risk for individuals allergic to eggs. This study investigated the presence of allergenic residues in both red and white wines fined with egg whites. Experimental and commercially available wines fined with egg whites, with or without subsequent bentonite fining, were studied. Unfined wines were used as negative controls. The physicochemical characteristics of each wine were determined to assess their possible role in enhancing or hindering the elimination of allergenic residues from wine. The amount of egg white protein residues was investigated both by a specifically developed/validated ELISA test and by immunoblotting. Both immunochemical tests used the same anti-total egg white protein antibody and were highly sensitive to the allergen. No egg white protein was detected in the wines studied in either immunochemical test, irrespective of the physicochemical characteristics of the wine, the type and dosage of the fining agent and the oenological process used. The risk of adverse reactions in egg-allergic individuals should therefore be considered negligible, but the exemption from labelling should be allowed only when the absence of residues is confirmed by analytical controls.

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1. Introduction

Fining is one of the least expensive operations in wine production, but it has a major impact on wine quality. The aims of the fining process are to soften or reduce the astringency and/or bitterness of the wine; clarify and remove proteins capable of haze formation; and/or stabilise and reduce the colour by the adsorption and precipitation of polymeric phenolic compounds and tannins (Yokosuka & Singleton, 1995). Several proteinaceous fining agents are currently used, including gelatine, milk proteins, egg proteins, isinglass and, more recently, proteins derived from plants such as wheat and white lupin (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2000). Water-soluble egg white (albumin or albumen) is the most commonly used fining agent for red wines. Its positively charged surface binds with negatively charged compounds such as tannins. The insolubility of the resulting aggregates allows

them to be eliminated by racking and/or filtration prior to bottling or further maturation. A second fining agent may be used, such as the inorganic fining agent bentonite, which adsorbs proteins and helps to remove residual proteins from the wine (D'Amato, Kravchuk, Bachi, & Righetti, 2010).

Egg white contains several allergenic proteins such as ovalbumin, ovomucoid, ovotransferrin and lysozyme (Mine & Zhang, 2002).

If fining agents are used and removed according to a good manufacturing practice, it can be assumed that these proteins are not present in the final wine product. Good manufacturing practice for fining is essentially defined as using the smallest amount of fining agent needed to achieve the desired result when followed by racking and pre-bottling filtration processes. To date, however, there is limited evidence that commercially available wines are free from residues of proteinaceous fining agents. Studies that have evaluated wines for residual protein have produced conflicting results, perhaps reflecting different analytical methodologies as well as differences in manufacturing practice (Liffrani et al., 2009; Restani et al., 2012; Rolland, Apostolou, de Leon, Stockley, & O'Hehir, 2008; Weber, Steinhart, & Paschke, 2009). Although allergy

* Corresponding author. Address: Dept. Pharmacological Sciences, Università degli Studi di Milano, via Balzaretti 9, 20133 Milano, Italy. Tel.: +39 0250318350–8371; fax: +39 0250318284.

E-mail address: patrizia.restani@unimi.it (P. Restani).

to egg proteins is rare in adults (Asero et al., 2009; Clark et al., 2010; EFSA, 2007; Sampson, 2004), the presence of allergenic proteins in wine could cause an adverse reaction in sensitised individuals. As a consequence, to protect these consumers it is important to demonstrate the absence of egg protein residues in commercial treated wines.

European Union Directive 2003/89/EC (2003), last amended by Directive 2007/68/EC (2007), contains a list of allergenic substances (Annex III), including egg and egg derivatives, which must be declared on the label of foodstuffs. EC Directive 2005/26/EC (2005) listed food ingredients that were provisionally excluded from the labelling requirement; inclusion of wine fining agents in this list was postponed until June 2012 to allow for further studies (Commission of the European Communities, 2007; Commission of the European Communities, 2009a). The inclusion of a statement such as “contains egg proteins” on the wine label can cause uncertainty amongst consumers (allergic or not) and, thereby, damage perception of the product. In order to maintain wine labelling exemption, it is crucial to provide evidences for ensuring that wines are free from egg protein residues.

Taking into consideration the different problems described above, the European Commission published the implementing regulation No. 579/2012 (European Commission, 2012) stating that “it is therefore necessary to establish detailed rules for labelling these beverages, including a mention of the substances referred to in Annex IIIa to Directive 2000/13/EC and used when making the beverages, if their presence can be detected in the final product....”. This decision was based on scientific data from different studies, including those presented in this paper, where the presence of allergenic residues was evaluated in 14 experimental and 85 international, commercially-available wines (7 untreated and 78 wines fined with egg white). The allergenic residues were measured by a newly developed/validated ELISA test with enhanced limits of detection and quantification, and also by immunoblotting; both tests used antibodies specifically developed against the egg white fining agent.

2. Materials and methods

2.1. Wine samples

Experimental and commercially-available wines fined with egg proteins were included.

Experimental wines included red wines fined with 3 or 10 g/hL of egg white, both concentrations with the subsequent addition of 0, 10, 20 or 30 g/hL of bentonite. All wine samples were microfiltered through a 3- μ m membrane pore size. The detailed characteristics of these wines are listed in Table 1. A further group of 12 wines that had not been treated with egg albumin were included as negative controls (data not shown).

Commercially available bottled wines were supplied by several wine producers from five countries. Among them, 48 red wines and one white wine were supplied by Italian winemakers; seven of these samples were untreated wines and were used as negative controls. Other wines were from France (20 samples), Australia (12 samples), New Zealand (two samples) and Spain (two samples). Only wines where the oenological practices were documented were included. Table 2 lists the 85 commercially available wines and shows their physicochemical characteristics. The fining agents used were as follows: Albapur (Tecnofood, Italy); Albovo (Oliver Ogar, Italy); Albuclar (Vason Group, Italy); Albumin Dry (Enolife srl, Italy); Egg albumin (Dal Cin SpA, Sesto San Giovanni, Italy); Albumin powder (Laffort Oenologie, France); Albumin powder (Lamothe Abiet, France); Blancoll (Esseco srl, Italy); Oviclair (La Littorale, France); Ovoclar (Pall Corporation, Italy); Ovoclaryl (Laffort, France); and Ovocol L (Martin Vialatte Oenology, France).

2.2. Physicochemical characteristics of wines

The physicochemical characteristics that were evaluated in wine samples are listed below:

2.2.1. Alcoholic strength by volume (% vol.)

This is defined as the number of litres of ethanol contained in 100 litres of wine, measured at 20 °C, measured by distilling the wine volume by volume. The distillate was measured by electronic densitometry using a frequency oscillator (OIV., 2009).

2.2.2. Total alcoholic strength by volume

This is the potential alcohol concentration if all remaining sugars were to be fermented, calculated by adding potential alcoholic strength to the alcoholic strength by volume (Commission of the European Communities., 2009b). Potential alcoholic strength is defined as the number of volumes of pure alcohol at 20 °C produced by total fermentation of the sugars contained in 100 volumes of the product at that temperature and was calculated by multiplying the concentration of reducing sugars (g/L) by 0.06.

2.2.3. Reducing sugars

To prepare samples with a sugar content ranging between 0.5 and 5 g/L, dry wine (sugar concentration <5 g/L) was diluted 1:2 (v:v) with water; sweet wine was diluted to achieve the same concentration. Red wine was clarified with solutions of neutral lead acetate and calcium carbonate (Merck KGaA, Darmstadt, Germany). An alkaline solution of copper salts was heated and the copper titrated against the clarified/diluted wine in the presence of methylene blue as indicator (OIV internal method).

2.2.4. Total acidity

Wine total acidity was determined by acid–base potentiometric titration, using 0.1 mol/L NaOH, to pH 7 with an automatic titrator (OIV, 2009).

2.2.5. Volatile acidity

To determinate the volatile acidity of wines, carbon dioxide was first removed from the wine sample. Volatile acids were then separated from wine by steam distillation and titrated using NaOH. The acidity of free and combined sulphur dioxide distilled under these conditions was subtracted from the acidity of the distillate, after filtration by standard iodine solution (OIV, 2009).

2.2.6. pH

The pH value of wine samples was determined by potentiometry using a calibrated pH-metre (OIV, 2009).

2.2.7. Ash content

Ash amount was measured by ignition of wine extract at 500–550 °C until complete combustion (oxidation) of organic material had been achieved. The residue was weighed using a balance sensitive to 0.1 mg (OIV, 2009).

2.2.8. Total dry extract and reduced extract

The total dry wine extract was calculated indirectly from the specific gravity of the alcohol-free wine after measuring the specific gravity (20 °C) of the wine and the water-alcohol mixture obtained by distillation of wine. The reduced extract was calculated as the difference between the total dry extract and the reducing sugars in excess of 1 g/L (OIV, 2009).

2.2.9. Total phenolic compounds

The total phenolic compounds were analysed using the Folin–Ciocalteu Method, with some modifications (OIV, 2009). Wine samples were diluted and then mixed with the Folin–Ciocalteu

Table 1

Physicochemical characteristics and allergenic residues of experimental red wines.

Sample	Oenological treatment	ASV (mL/ 100 mL)	TAS (mL/ 100 mL)	RS (g/L)	Specific gravity (g/L)	pH	TDE (g/L)	RE (g/L)	TA (g/L)	VA-SO ₂ (g/L)	Ash (g/L)	TPC (g/L)	TAC (g/L)	ELISA	Imm
1A	–	16.99	17.12	2.2	0.99220	3.51	35.8	34.6	5.5	0.55	2.82	2.19	0.18	Neg	Neg
2A	10 g/hL egg white	16.67	16.86	3.1	0.99325	3.53	37.4	35.3	5.6	0.60	2.90	2.43	0.13	Neg	Neg
3A	10 g/hL egg white + 10 g/hL bentonite	17.01	17.17	2.7	0.99274	3.54	36.9	35.2	5.6	0.59	2.93	2.24	0.13	Neg	Neg
4A	10 g/hL egg white + 20 g/hL bentonite	17.18	17.33	2.5	0.99242	3.53	36.6	35.1	5.6	0.61	2.83	2.53	0.12	Neg	Neg
5A	10 g/hL egg white + 30 g/hL bentonite	16.83	17.02	3.1	0.99303	3.53	36.9	34.8	5.6	0.60	2.90	2.57	0.12	Neg	Neg
6A	3 g/hL egg white + 10 g/hL bentonite	17.01	17.15	2.4	0.99276	3.51	37.0	35.6	5.6	0.61	2.90	2.64	0.12	Neg	Neg
7A	3 g/hL egg white + 20 g/hL bentonite	16.99	17.15	2.6	0.99278	3.52	36.8	35.2	5.6	0.60	2.90	2.40	0.13	Neg	Neg
8A	3 g/hL egg white + 30 g/hL bentonite	16.95	17.12	2.9	0.99281	3.53	36.8	34.9	5.5	0.61	2.92	2.45	0.12	Neg	Neg
1V	–	13.03	13.12	1.5	0.99408	3.34	29.0	28.5	6.0	0.38	2.79	1.67	0.27	Neg	Neg
2V	10 g/hL egg white	12.98	13.09	1.9	0.99413	3.32	29.1	28.2	6.4	0.69	2.80	1.81	0.12	Neg	Neg
3V	10 g/hL egg white + 10 g/hL bentonite	13.10	13.21	1.9	0.99385	3.31	28.6	27.7	6.3	0.71	2.71	1.82	0.12	Neg	Neg
4V	10 g/hL egg white + 20 g/hL bentonite	12.49	12.60	1.9	0.99472	3.32	29.0	28.1	6.1	0.55	2.76	1.83	0.13	Neg	Neg
5V	10 g/hL egg white + 30 g/hL bentonite	12.63	12.73	1.6	0.99447	3.34	28.7	28.1	6.0	0.47	2.71	1.90	0.13	Neg	Neg
6V	3 g/hL egg white + 10 g/hL bentonite	13.16	13.25	1.5	0.99375	3.32	28.5	28.0	5.9	0.45	2.76	1.74	0.10	Neg	Neg
7V	3 g/hL egg white + 20 g/hL bentonite	13.15	13.22	1.2	0.99387	3.32	28.8	28.6	6.0	0.45	2.73	1.83	0.12	Neg	Neg
8V	3 g/hL egg white + 30 g/hL bentonite	12.87	12.97	1.6	0.99422	3.33	28.9	28.3	6.0	0.50	2.75	1.83	0.13	Neg	Neg

A: Amarone wine; V: Valpolicella wine; ASV: Alcoholic Strength by Volume; TAS: Total Alcoholic Strength; RS: Reducing Sugars; TDE, Total Dry Extract; RE, Reduced Extract; TA: Total Acidity; VA-SO₂: Volatile Acidity SO₂; TPC: Total Phenolic Compounds; TAC: Total Anthocyanins; Imm: Immunoblotting; Neg: negative.

Table 2

Physicochemical characteristics and allergenic residues of commercial wines.

N°	Wine name	Origin	Fining agent	F. agent concentration (g/hL)	Ben	ASV (mL/ 100 mL)	TAS (mL/ 100 mL)	RS (g/L)	Specific gravity (g/L)	pH	TDE (g/L)	RE (g/L)	TA (g/L)	VA (g/L)	Ash (g/L)	TPC (g/L)	TAC (g/L)	ELISA	Imm
1	Chianti classico Riserva Fontale DOCG	Italy	No albumin	–	NO	14.05	14.11	1.0	0.99257	3.16	29.3	29.3	6.2	0.33	2.12	1.73	0.23	Neg	Neg
2	Chianti classico Riserva – Vigneti La Selvanella DOCG 2006	Italy	No albumin	–	NO	13.39	13.45	1.0	0.99282	3.20	27.2	27.2	5.7	0.25	2.11	1.63	0.31	Neg	Neg
3	Merlot delle Maestrelle (Santa Cristina) IGT2008	Italy	No albumin	–	NO	13.14	13.33	3.2	0.99460	3.43	30.1	27.9	5.4	0.37	2.61	1.69	0.42	Neg	Neg
4	Il Bruciato – Bolgheri DOC 2007	Italy	No albumin	–	NO	14.32	14.40	1.3	0.99284	3.37	29.7	29.4	5.6	0.46	2.51	1.94	0.26	Neg	Neg
5	Badia a Passignano – Chianti Classico DOCG 2007	Italy	No albumin	–	NO	14.23	14.29	1.0	0.99385	3.43	31.7	31.7	5.8	0.52	3.01	2.19	0.37	Neg	Neg
6	Il Bruciato – Bolgheri DOC 2008	Italy	No albumin	–	NO	14.37	14.43	1.0	0.99311	3.42	30.1	30.1	5.6	0.47	2.78	2.06	0.25	Neg	Neg
7	Badia a Passignano – Chianti Classico DOCG 2006	Italy	No albumin	–	NO	13.57	13.65	1.4	0.99273	3.38	27.1	26.7	5.7	0.49	2.39	2.00	0.16	Neg	Neg
8	Rosso Toscano “i coltri” IGT 2007	Italy	Egg white	4.5	YES	13.31	13.42	1.8	0.99340	3.37	28.4	27.6	5.2	0.38	2.72	1.64	0.28	Neg	Neg

(continued on next page)

Table 2 (continued)

N°	Wine name	Origin	Fining agent	F. agent concentration (g/hL)	Ben	ASV (mL/100 mL)	TAS (mL/100 mL)	RS (g/L)	Specific gravity (g/L)	pH	TDE (g/L)	RE (g/L)	TA (g/L)	VA (g/L)	Ash (g/L)	TPC (g/L)	TAC (g/L)	ELISA	Imm
9	Chianti DOCG 2008	Italy	Egg white	4	YES	12.69	12.81	2.0	0.99407	3.39	28.5	27.5	5.3	0.4	2.47	1.88	0.42	Neg	Neg
10	Barbera d'Asti – Vigneti Castello del Poggio DOC 2006	Italy	Egg white	10	NO	13.10	13.54	7.4	0.99686	3.25	34.9	28.5	6.3	0.42	2.55	1.71	0.16	Neg	Neg
11	Lambrusco Grasparossa di Castelvetro – Passione vino secco frizzante DOC	Italy	Ovoclar	10	YES	10.24	11.03	13.1	1.00057	2.11	33.6	21.5	7.1	0.20	3.84	1.16	0.22	Neg	Neg
12	Taurasi DOCG 2000	Italy	Albuclar	10	NO	14.16	14.28	2.0	0.99383	3.32	32.2	31.2	7.1	0.57	2.28	2.66	0.19	Neg	Neg
13	Chianti Classico 2006	Italy	Ovoclar	7	NO	13.39	13.48	1.5	0.99261	3.30	26.9	26.4	6.0	0.42	2.22	2.33	0.12	Neg	Neg
14	Alturio – Refosco dal Ped. Rosso DOC 2005	Italy	Blancoll	6	YES	13.60	13.75	2.5	0.99362	3.40	30.1	28.6	6.0	0.39	2.76	2.54	0.46	Neg	Neg
15	Sangiovese maremma toscana IGT 2008	Italy	Ovoclar	8	YES	13.55	13.69	2.4	0.99382	3.43	29.6	28.2	5.8	0.44	2.53	1.85	0.29	Neg	Neg
16	Oltrepò Pavese – Pinot nero Poggio Pelato DOC 2005	Italy	Blancoll	20	YES	13.49	13.57	1.3	0.99160	3.55	25.4	25.1	5.2	0.64	2.68	3.01	0.066	Neg	Neg
17	Oltrepò pavese – Bonarda vivace DOC 2008	Italy	Blancoll	6	YES	11.84	12.55	11.9	0.99783	2.97	34.1	23.2	6.7	0.24	2.13	1.95	0.36	Neg	Neg
18	Montepulciano d'Abruzzo DOC 2008	Italy	Blancoll	10	YES	12.42	12.83	6.8	0.99659	3.43	34.3	28.5	5.9	0.43	2.65	2.00	0.32	Neg	Neg
19	Negroamaro Cabernet Sauvignon IGT 2007	Italy	Albapur	4	YES	13.43	13.97	9.0	0.99921	3.74	43.8	35.8	5.7	0.56	4.03	2.76	0.35	Neg	Neg
20	Salice salentino – Masseria Trajone DOC 2005	Italy	Albapur	4	YES	13.14	13.75	10.2	0.99990	3.65	44.3	35.1	5.9	0.52	3.83	2.78	0.28	Neg	Neg
21	Primitivo di Manduria – Epicuro DOC 2007	Italy	Albapur	4	YES	14.53	15.05	8.7	0.99875	3.85	46.0	38.3	5.6	0.59	4.22	3.00	0.34	Neg	Neg
22	Aglianico IGT 2007	Italy	Albuclar	3	NO	12.69	13.13	7.4	0.99719	3.38	36.1	29.7	5.7	0.58	3.21	3.24	0.36	Neg	Neg
23	Vino Rosso Primitivo IGT 2008	Italy	Albumin dry	10	YES	13.68	14.27	9.8	0.99962	3.61	45.4	36.6	5.5	0.59	3.46	2.89	0.55	Neg	Neg
24	Primitivo di Manduria – Felline DOC 2006	Italy	Albumin dry	20	YES	14.77	15.12	5.8	0.99705	3.47	41.2	36.4	6.6	0.47	3.59	2.94	0.54	Neg	Neg
25	Negroamaro del Salento – Pietraluna Torreguaceto IGT 2009	Italy	Albumin dry	20	YES	13.70	14.06	6.0	0.99792	3.33	40.3	35.3	7.3	0.42	3.28	2.88	0.60	Neg	Neg
26	Vino Rosso del Salento – Alberello IGT 2007	Italy	Albumin dry	20	YES	13.32	13.64	5.4	0.99740	3.40	37.9	33.5	6.3	0.42	3.36	2.69	0.56	Neg	Neg
27	Primitivo di Manduria – Archidamo Peruini DOC 2007	Italy	Albumin dry	20	YES	14.70	15.02	5.4	0.99699	3.42	41.3	36.9	6.8	0.38	3.41	3.10	0.52	Neg	Neg
28	Valpolicella classico DOC 2007	Italy	Ovoclar	6	YES	13.33	13.60	4.5	0.99544	3.34	32.8	29.3	6.0	0.40	2.68	1.78	0.24	Neg	Neg
29	Merlot Colli Berici DOC 2008	Italy	Ovoclar	4	YES	12.50	12.77	4.5	0.99552	3.39	31.1	27.6	5.6	0.33	2.86	1.65	0.36	Neg	Neg
30	Montepulciano d'Abruzzo – Cerulli Spinozzi DOC 2008	Italy	Ovoclar	4	YES	13.23	13.51	4.7	0.99553	3.30	33.4	29.7	6.4	0.45	2.54	2.20	0.46	Neg	Neg
31	Bardolino classico DOC 2009	Italy	Ovoclar	3	YES	12.47	12.74	4.5	0.99572	3.32	30.9	27.4	5.6	0.30	2.64	1.53	0.47	Neg	Neg
32	Nero d'Avola Syrah – Feudo Sartanna IGT 2009	Italy	Ovoclar	3	YES	14.06	14.26	3.4	0.99501	3.42	33.3	30.9	5.7	0.45	2.83	2.06	0.42	Neg	Neg
33	Bardolino classico DOC 2008	Italy	Ovoclar	4	YES	12.32	12.6	4.6	0.99576	3.25	30.9	27.3	5.5	0.30	2.66	1.63	0.44	Neg	Neg
34	Regolo rosso veronese IGT 2006	Italy	Ovoclar	3	YES	13.84	14.26	7.0	0.99669	3.33	37.7	31.7	6.2	0.45	3.01	2.26	0.32	Neg	Neg
35	Bardolino – Murari DOC 2008	Italy	Ovoclar	3	YES	12.2	12.49	4.9	0.99596	3.29	31.2	27.3	5.6	0.32	2.68	1.45	0.40	Neg	Neg
36	Valpolicella classico superiore – Vigneti di Montegradella DOC 2006	Italy	Ovoclar	5	YES	13.59	13.95	6.0	0.99645	3.34	36.1	31.1	6.3	0.44	3.18	2.14	0.28	Neg	Neg

Table 2 (continued)

N°	Wine name	Origin	Fining agent	F. agent concentration (g/hL)	Ben	ASV (mL/100 mL)	TAS (mL/100 mL)	RS (g/L)	Specific gravity (g/L)	pH	TDE (g/L)	RE (g/L)	TA (g/L)	VA (g/L)	Ash (g/L)	TPC (g/L)	TAC (g/L)	ELISA	Imm
37	Valpolicella superiore Ripasso – Vigneti di Valdimizzo DOC 2007	Italy	Ovoclar	8	YES	13.71	14.13	7.0	0.99701	3.35	37.4	31.4	6.1	0.42	3.23	2.21	0.32	Neg	Neg
38	Amarone della Valpolicella DOC 2005	Italy	Ovoclar	8	YES	15.25	15.73	8.0	0.99657	3.39	41.2	34.2	6.2	0.50	3.29	2.43	0.22	Neg	Neg
39	Aglianico – Vigne Sannite IGT 2008	Italy	Albuclar	5	YES	13.18	13.29	1.8	0.99388	3.38	29.8	29.0	6.1	0.59	2.45	2.62	0.16	Neg	Neg
40	Refosco dal Peduncolo Rosso DOC 2007	Italy	Potassium Caseinate – Egg White – Lysozyme	20 + 20	NO	14.16	14.31	2.5	0.99526	3.46	35.7	34.2	6.1	0.59	3.62	2.40	0.33	Neg	Neg
41	Merlot – Daunia Teanum Alta IGT 2008	Italy	Egg white	30	YES	14.98	15.06	1.3	0.99615	3.54	38	37.7	5.4	0.40	3.32	3.17	0.39	Neg	Neg
42	Cabernet sauvignon Teanum Alta IGT 2008	Italy	Egg white	50	YES	13.54	13.68	2.3	0.99786	3.41	38.3	37.0	5.4	0.38	3.20	3.47	0.49	Neg	Neg
43	Aglianico IGT 2008	Italy	Egg white	100	YES	14.3	14.4	1.7	0.99602	3.35	36.3	35.6	5.6	0.38	2.89	3.34	0.34	Neg	Neg
44	Lareith Sudtiroil Lagrein	Italy	Blancoll	4	YES	13.27	13.39	2.0	0.99499	3.47	30.6	29.6	5.3	0.51	3.23	2.96	0.64	Neg	Neg
45	Grobnerhof – Santa Maddalena Sudtiroil DOC 2008	Italy	Vinpur Blancoll	1 2	YES	13.31	13.45	2.4	0.99430	3.46	28.4	27.0	4.7	0.43	2.93	1.85	0.37	Neg	Neg
46	Leuchtenburg Sudtiroil – Kalterersee Lago di Caldaro DOC 2009	Italy	Vinpur Biancoll	1 3	YES	13.12	13.26	2.4	0.99328	3.44	26.0	24.6	4.5	0.43	2.60	1.91	0.29	Neg	Neg
47	Primitivo di Manduria DOC 2008	Italy	Albovo Oliver Ogar	6	NO	14.41	14.5	1.5	0.99450	3.29	32.9	32.4	5.9	0.43	2.72	1.86	0.23	Neg	Neg
48	Taurasi – Vigna Quattro confini DOCG 2009	Italy	Egg white	1 g/ 5 hL	YES	13.69	13.75	1.0	0.99237	3.16	26.7	26.7	6.8	0.42	1.95	1.89	0.086	Neg	Neg
49	Greco di Tufo DOCG 2009	Italy	1% Egg white Oliver Star (caseinate)	1	YES	13.23	13.39	2.7	0.99102	3.20	21.4	19.7	6.1	0.27	1.44	0.26	–	Neg	Neg
50	Cuxac IGP 2009	France	Ovoclar	8	NO	14.03	14.09	1.0	0.99342	3.63	28.9	28.9	4.9	0.51	2.90	2.13	0.56	Neg	Neg
51	Cabernet Franc/ Mourvedre IGP 2009	France	Oviclair	10	NO	14.46	14.56	1.7	0.99382	3.58	31.3	30.6	5.1	0.53	2.76	2.20	0.44	Neg	Neg
52	Petit Verdot IGP 2007	France	Oviclair	12	NO	13.88	14.02	2.4	0.99398	3.48	30.4	29.0	5.3	0.51	2.58	1.93	0.27	Neg	Neg
53	Cabecac AOP 2007	France	Albumin poudre (Laffort)	6	NO	13.93	14.04	1.8	0.99343	3.52	28.7	27.9	5.2	0.43	2.34	2.15	0.29	Neg	Neg
54	Carignan – Sicard Ignan AOP 2009	France	Albumin poudre (Laffort)	10	NO	14.16	14.37	3.5	0.99591	3.55	35.1	32.6	5.5	0.45	3.01	2.36	0.59	Neg	Neg
55	La Cuvée Ghislain AOP 2004	France	Albumin poudre (Laffort)	5–6	NO	13.51	13.61	1.7	0.99264	3.48	26.9	26.2	4.8	0.55	2.57	2.27	0.18	Neg	Neg
56	Caraguilhes AOP 2009	France	Albumin poudre (Laffort)	10	NO	13.75	13.87	2.0	0.99379	3.39	29.9	28.9	6.0	0.44	3.03	3.00	0.22	Neg	Neg
57	Caraguilhes AOP 2007	France	Albumin poudre (Laffort)	10	NO	13.12	13.20	1.3	0.99444	3.55	28.8	28.5	5.1	0.46	2.55	1.72	0.28	Neg	Neg
58	Merlot – Cabernet – Domaine du Vieux Parc IGP 2008	France	Oviclair	10	NO	13.91	13.97	1.0	0.99360	3.57	29.1	29.1	5.5	0.53	2.83	2.18	0.39	Neg	Neg
59	Corbières AOP 2008	France	Oviclair	10	NO	13.67	13.81	2.4	0.99300	3.47	27.2	25.8	4.7	0.48	2.49	2.42	0.450	Neg	Neg
60	Chateau du Grand Caumont AOP 2008	France	Oviclair	10	NO	12.61	12.73	2.0	0.99334	3.43	25.5	24.5	4.9	0.44	2.29	1.78	0.35	Neg	Neg
61	Château du Grand Caumont AOP 2009	France	Oviclair	10	NO	12.76	12.86	1.6	0.99282	3.32	24.2	23.6	5.3	0.39	2.04	1.49	0.22	Neg	Neg
62	Listrac – Cuvé 98 AOC 2008	France	Egg white (40 days)	66	NO	13.17	13.24	1.2	0.99326	3.42	26.7	26.5	5.3	0.45	2.51	2.46	0.28	Neg	Neg
63	Moulis – Cuvé 56 cru bourgeois AOC 2008	France	Egg white (40 days)	66	NO	12.95	13.01	1.0	0.99334	3.42	26.0	26.0	5.2	0.42	2.36	2.28	0.26	Neg	Neg

(continued on next page)

Table 2 (continued)

N°	Wine name	Origin	Fining agent	F. agent concentration (g/hL)	Ben	ASV (mL/100 mL)	TAS (mL/100 mL)	RS (g/L)	Specific gravity (g/L)	pH	TDE (g/L)	RE (g/L)	TA (g/L)	VA (g/L)	Ash (g/L)	TPC (g/L)	TAC (g/L)	ELISA	Imm
64	Malleret-Cru Bourgeois AOC 2009	France	Egg white (3 months)	40	NO	12.73	12.84	1.9	0.99496	3.58	29.7	28.8	5.0	0.51	3.18	2.38	0.29	Neg	Neg
65	Corbière Cru Signé AOC 2009	France	Egg white	US	NO	13.73	13.82	1.5	0.99354	3.45	28.7	28.2	5.1	0.39	2.44	1.87	0.42	Neg	Neg
66	Bois du roi AOC 2007	France	Egg white	US	NO	14.61	14.74	2.2	0.99330	3.72	30.9	29.7	5.4	0.94	2.58	1.60	0.13	Neg	Neg
67	Montplaisir AOC 2007	France	Egg white	US	NO	14.2	14.39	3.1	0.99300	3.65	28.5	26.4	4.8	0.60	2.49	1.29	0.099	Neg	Neg
68	Echantillon 9B	France	Egg white	Maximum dose	NO	10.84	12.39	25.9	1.00504	3.42	49.5	24.6	5.4	0.76	2.72	1.58	0.17	Neg	Neg
69	Echantillon 8	France	Egg white	Maximum dose	NO	11.25	11.41	2.6	0.99507	3.44	25.8	24.2	5.0	0.45	2.74	1.48	0.19	Neg	Neg
70	Grenache 58.13%; Shiraz 38.74% 2009	Australia	Egg white	3	Yes (37.00)	14.29	14.42	2.1	0.99371	3.28	30.7	29.6	5.8	0.43	2.24	1.43	0.21	Neg	Neg
71	Cabernet-Sauvignon 2009	Australia	Egg white	4	Yes (1.20)	13.29	13.37	1.4	0.99525	3.05	31.2	30.8	6.4	0.38	1.91	2.17	0.35	Neg	Neg
72	Merlot 2009	Australia	Egg white	5.9	Yes (0.90)	13.31	13.37	1.0	0.99454	3.08	28.9	28.9	6.2	0.34	1.53	1.97	0.089	Neg	Neg
73	Shiraz 55.87%; Cabernet Sauvignon 29.90% 2008	Australia	Egg white	4	Yes (1.30)	13.39	13.52	2.1	0.99527	3.03	31.5	30.4	6.3	0.41	1.95	1.73	0.26	Neg	Neg
74	Shiraz 2008	Australia	Egg white	4	Yes (0.18)	13.84	13.97	2.2	0.99493	3.26	32.3	31.1	6.0	0.49	2.65	1.59	0.26	Neg	Neg
75	Pinot Noir 2010	Australia	Egg white	3.2	Yes (0.01)	12.95	13.03	1.4	0.99351	3.38	26.1	25.7	5.5	0.63	2.00	1.61	0.18	Neg	Neg
76	Cabernet Sauvignon 2008	Australia	Egg white	5.3	Yes (4.50)	14.23	14.30	1.1	0.99363	3.23	30.5	30.4	6.2	0.44	2.16	2.37	0.35	Neg	Neg
77	Cabernet Sauvignon 49.14%; Merlot 47.78% 2008	Australia	Egg white	4.1	Yes (0.72)	14.41	14.47	1.0	0.99396	3.21	31.4	31.4	6.5	0.43	2.25	2.19	0.31	Neg	Neg
78	Shiraz 2008	Australia	Egg white	1.7	Yes (8.50)	13.66	14.00	5.7	0.99677	3.03	36.7	32.0	6.4	0.41	2.05	1.88	0.36	Neg	Neg
79	Shiraz 2007	Australia	Egg white	3	Yes (2.20)	14.58	14.65	1.1	0.99440	3.23	33.6	33.5	6.4	0.51	3.00	1.84	0.24	Neg	Neg
80	Pinot Noir 2009	Australia	–	–	Yes (0.02)	13.26	13.37	1.8	0.99461	3.16	29.4	28.6	6.4	0.60	1.70	2.08	0.22	Neg	Neg
81	Cabernet Sauvignon 2008	Australia	Egg white	1	Yes (0.51)	14.15	14.21	1.0	0.99406	3.06	31.2	31.2	6.6	0.42	1.97	2.41	0.36	Neg	Neg
82	Pinot Noir 2009	New Zealand	CuSO ₄ ·5H ₂ O 1.12 mg/L, Egg white 2.5 mg/L, Laffort Gecoll 2.42 mg/L	0.60	NO	14.85	14.98	2.1	0.99212	3.47	28.4	27.3	5.4	0.64	2.14	1.40	0.15	Neg	Neg
83	Pinot Noir 2009	New Zealand	CuSO ₄ ·5H ₂ O 0.59 mg/L, Laffort Gecoll 0.2 mg/L	0.075	NO	14.60	14.71	1.9	0.99340	3.62	30.7	29.8	5.4	0.67	2.52	1.41	0.15	Neg	Neg
84	Vino Navarra Reserva 2004	Spain	Albumin (Lamothe Abiet)	5	NO	13.64	13.73	1.5	0.99446	3.13	30.3	29.8	6.2	0.41	1.66	2.30	0.13	Neg	Neg
85	Ysios Reserva 2005	Spain	Ovocol L (Martin vialatte)	46.6	NO	13.66	13.80	2.4	0.99389	3.27	29.3	27.9	5.3	0.47	2.21	1.97	0.22	Neg	Neg

Ben: Use of bentonite; ASV: Alcoholic Strength by Volume; TAS: Total Alcoholic Strength; RS: Reducing Sugars; TDE, Total Dry Extract; RE, Reduced Extract; TA: Total Acidity; VA: Volatile Acidity; TPC: Total Phenolic Compounds; TAC: Total Anthocyanins; Imm: Immunoblotting; Neg: negative.

reagent, which oxidises all the phenolic compounds, and sodium carbonate (Merck KgaA, Darmstadt, Germany). Absorbance was then measured at 760 nm.

2.2.10. Anthocyanins

Total anthocyanins were determined according to Di Stefano and Cravero (1989) with some modifications. Briefly, wine samples were diluted with an acidulated ethanol–water solution. The absorbance spectrum was then determined between 420 and 620 nm.

2.3. SDS–PAGE and immunoblotting

Wine samples were analysed by SDS–PAGE according to Ballabio et al. (2007), on a gel having the following characteristics:

2.3.1. Gradient running gel

9–19% acrylamide; 0.08–0.17% bis-acrylamide; 0.36 mol/L TRIS–HCl buffer pH 8.8; 35% glycerol; 0.1% SDS; 0.02% ammonium persulphate; and 0.15% N,N,N',N'-tetramethylethylenediamine (TEMED).

2.3.2. Stacking gel

3.5% acrylamide; 0.09% bis-acrylamide; 0.125 mol/L TRIS–HCl buffer pH 6.8; 0.1% SDS; 0.02% ammonium persulphate; and 0.15% (TEMED).

2.3.3. Running buffer

0.025 mol/L TRIS, 0.19 mol/L glycine and 0.1% SDS (w/v), pH 8.8.

2.3.4. Sample buffer

The composition of the 2× sample buffer was: 0.25 mol/L Tris–HCl buffer pH 6.8, 22.5% glycerol, 2% SDS and 5% β-mercaptoethanol.

Purified egg proteins and fining agents were diluted in water at the final concentration of 4 g/L and then mixed with 2× sample buffer (1:1, v/v). Aliquots of 0.001 L of each wine sample were dried under nitrogen at room temperature until a dry extract was obtained. Afterwards, 0.2 L of a solution containing water: 2× sample buffer (1:1, v/v) was added to the dry extract. Sample aliquots of 25 µL were loaded onto the gel. Prestained SDS–PAGE standard Broad Range (BioRad), containing proteins in the range 6.7–202.8 kDa, was used to control the electrophoretic run.

After the electrophoretic run, proteins were transferred onto a PVDF membrane (Millipore, Billerica, MA) by western blotting in a Trans-blot Electrophoretic Transfer Cell (Bio-Rad). The membranes were blocked with 1% gelatin and washed three times with 0.25% gelatin solution (150 mmol/L NaCl, 5 mmol/L TRIS, 0.05% Triton-X) to prevent non-specific adsorption of the immunological reagents. Afterwards, the membrane was immersed in 10 mL of 0.25% gelatin solution containing 10 µL of rabbit anti-total egg white protein IgG polyclonal antibodies. This antibody was specifically developed for this research using total egg proteins for immunisation. The antibody was characterised in order to ensure its capability to detect the different egg white allergens. Antigen–IgG complexes were detected by using 10 µL of goat anti-rabbit IgG antibodies labelled with peroxidase (Sigma Aldrich, Milan, Italy). The developing solution contained DAB (3,3'-diaminobenzidine) Plus substrate and DAB Plus Chromogen (Sigma Aldrich).

2.4. ELISA test

A sandwich ELISA kit (Euroclone SpA, Pero, Milano), specifically developed for the quantification of egg white proteins in wine (Restani et al., 2013), was used. This method was specifically developed in agreement with the OIV Resolution 427–2010, modified in 2012 (OIV, 2012).

Briefly, the microplate was first coated with the specific anti-egg white protein antibody also used for immunoblotting. After incubation with the wine sample, a secondary anti-egg white protein antibody conjugated with horseradish peroxidase (HRP) was added to form the sandwich. To determine the detection limit (LOD) and the quantification limit (LOQ), the protocol described in the OIV “Compendium of international methods of analysis” E-AS1–10-LIMDET (OIV, 2009) was used. The limits were calculated according to the procedure “Determination on blank” using the data from 11 laboratories participating to a collaborative inter-laboratory study (Restani et al., 2013). For quantification, standard solutions contained increasing amounts of egg proteins and spiked wine samples containing egg white in the range 0–7 ppm were used. All standards and wines were diluted 1:5 (v/v) with the buffer supplied with the kit. Results were analysed statistically according to UNI ISO 5725-2:2004 and the OIV “Compendium of international methods of analysis” MA-EAS1–07-ETCOL; the repeatability and reproducibility of the ELISA method were determined.

3. Results

The study examined 85 commercially-available bottled wines collected from five different countries and 14 experimental wines, fined by adding egg white proteins with or without subsequent bentonite fining. Untreated wines were evaluated in parallel as negative controls. All samples, with the exception of one, corresponded to red wines since egg white proteins are generally only used to fine red wines (Ribéreau-Gayon et al., 2000).

3.1. Physicochemical properties

Different physicochemical characteristics of the wines were recorded (Tables 1 and 2), with the aim of assessing, if allergenic residues were detected, any possible role in inhibiting or enhancing the elimination of allergens during the fining process.

3.2. SDS–PAGE and silver staining

SDS–PAGE was assayed for its sensitivity using silver staining (Gromova & Celis, 2006). For these purposes, decreasing quantities of oenological egg white proteins were loaded onto the gel, and the detection limit was calculated as 0.78 µg of oenological egg albumin.

The method was then applied to fined and unfined wine samples. Some protein bands were present in all untreated wine samples, but all were associated with grape proteins (data not shown). Due to these grape proteins, it is difficult to evaluate in the SDS–PAGE the presence of traces of egg white proteins. For this reason, SDS–PAGE was used for the separation step but not quantification of allergenic residues.

3.3. Specificity of anti-egg white protein antibody

Since the quality of antibody is critical in immunochemical determinations, several antibodies specifically developed for this project were characterised for their binding capacity with egg white proteins. Two antibodies were developed against total egg white proteins and two against ovomucoid, since ELISA plates used for the detection of egg white allergens in food are usually coated with anti-ovomucoid antibodies. Fig. 1 illustrates the specificity of the selected anti-total egg white protein antibody. It recognised all the albumen proteins (ovotransferrin, ovomucoid, ovalbumin and lysozyme). Anti-ovomucoid antibodies (data not shown) bound different egg white proteins but their affinities were lower and for this reason considered unsuitable for this project. The selected antibody was also used to coat the ELISA plates.

The binding capacity of the selected antibody was also verified against most of the fining agents containing egg white proteins currently available. The antibody recognised the egg white proteins in all fining agents evaluated; the differences in the bound affinity depended on the percentage of each egg white protein present in each product (results not shown).

3.4. Immunoblotting

The detection limit for egg white proteins in immunoblotting was determined as indicated in materials and methods section and was 1.5 ng, corresponding to 0.122 mg/L in the wine sample.

Once the limit of detection was calculated, the different wine samples were examined by immunoblotting. Fig. 2 illustrates, as an example, some results of the immunoblotting. No residues of egg white proteins were detected in any of the experimental or commercial wines analysed (see last column of Tables 1 and 2). As expected, no egg white protein was found in the unfined wines.

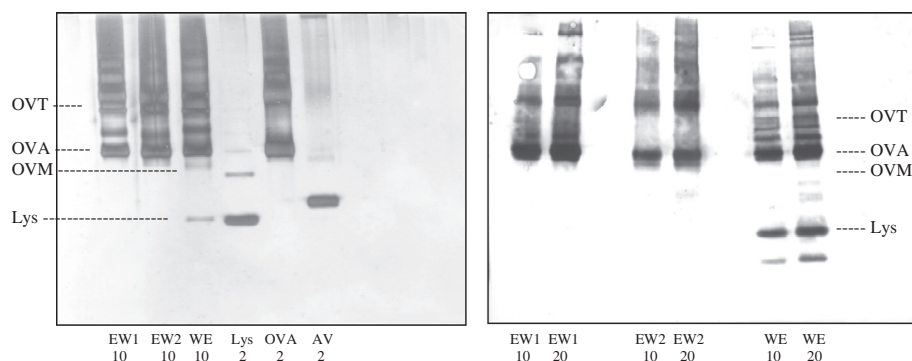


Fig. 1. SDS–PAGE (left) and Immunoblotting (right) of two fining agents containing egg white proteins and purified fractions from hen egg white. An antibody against total egg white proteins was used. Purified proteins and fining agents were prepared as described in Material and Methods and the volume loaded onto the gel (in μL) is reported under the sample identification. Legend: EW1 = Oenologic egg white 1; EW2 = Oenologic egg white 2; WE = Whole egg; Lys = Lysozyme; OVA = Ovalbumin; AV = Avidin; OVT = Ovotransferrin; OVM = Ovomucoid.

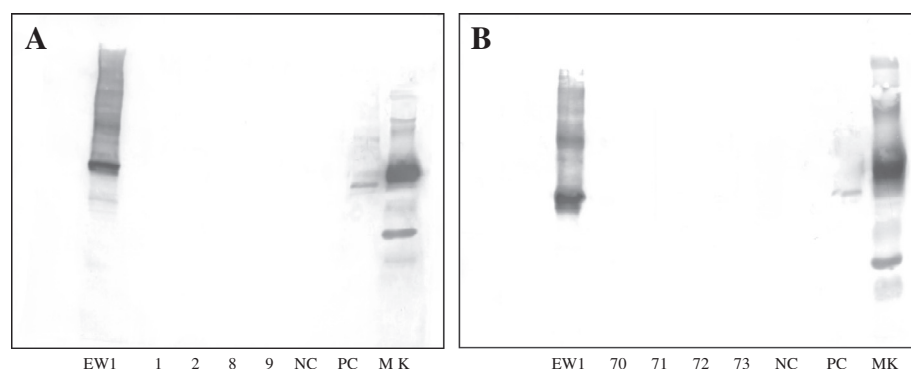


Fig. 2. Immunoblotting of different commercial red wine samples obtained by incubating the PVDF membrane with the anti-total egg white protein antibody. (A) Italian red wine samples; (B) Australian red wine samples. Legend: EW = egg white protein standard solution; 1–2 = Commercial Italian red wines fined with egg white proteins; 8–9 = untreated commercial Italian red wines; 70–73 = Commercial Australian red wines fined with egg white proteins; NC = negative control (untreated red wine); PC = positive control (wine spiked with 1 mg/L albumen); MK = Prestained SDS–PAGE standards.

3.5. ELISA test

An ELISA test was specifically developed for this investigation to detect traces of egg white proteins in wine, and was validated by a collaborative inter-laboratory study involving 11 laboratories (Restani et al., 2013). This method showed a reliable limit of detection of 0.056 mg/L in wine and a limit of quantification of 0.158 mg/L. The quality parameters of the method (reproducibility, repeatability and robustness) were in line with the criteria established by the Organisation Internationale de la Vigne et du Vin (OIV) in the Compendium of International Methods of Analysis (OIV, 2009). None of the wine samples analysed contained detectable amount of egg white proteins (see the penultimate column of Tables 1 and 2). As expected, all unfined wines were free of egg white proteins (data not shown).

4. Discussion

Egg allergy is one of the most common food allergies in infancy and childhood (Heine, Laske, & Hill, 2006). Although its prevalence in adults is considerably lower (Asero et al., 2009; Sampson, 2004), the presence of egg white proteins in fined wines should be avoided if the most sensitised subjects are to be protected. To our knowledge, no case of an allergic reaction after wine consumption due to the presence of residues of egg white proteins has been reported, despite the presence of unlabelled egg white-fined wines for sale in countries such as Canada, the EU, USA and those of South

East Asia, which do not require allergen labelling for wine or have recently implemented it. This could be due to the absence of egg white residues or to the lack of awareness about the oenological practice of fining with egg white proteins as a potential trigger of allergenic reactions.

In this study, no egg proteins were detected in any of the 78 commercially-available wines fined with egg white proteins analysed (detection limit of 0.0564 mg/L), irrespective of the physico-chemical characteristics of wines, despite the wide range of values for each parameter included. Specifically, this result was independent of:

- The type and dose of the agent used for fining: 0.075–100 g/hL of fining agent for experimental and 3–10 g/hL for commercially available wines; and
- The oenological practice: whether or not bentonite fining was subsequently used in both experimental and commercially available wines.

Our findings are consistent with those reported by Rolland, Apostolou, de Leon, Stockley, and O'Hehir (2008) who found no residue of ovalbumin in 40 commercially available Australian wines fined with egg white proteins. The same research group found no significant clinical response in a group of adults allergic to eggs tested by a double-blind, placebo-controlled challenge with wines fined with egg white protein (Rolland et al., 2006). It should be emphasised that although ELISA tests previously reported (Rolland, Apostolou, de Leon, Stockley, & O'Hehir, 2008; Weber,

Steinhart, & Paschke, 2009) did show good quality characteristics, our method was specifically developed for commercial wine samples, in order to standardise its performance and to make it available for wine producers and laboratories involved in quality control.

Only a few reports illustrate cases of allergy to wine, especially in Mediterranean countries, but these adverse reactions were associated with grape proteins (Borghesan, Basso, Chieco Bianchi, Favero, & Plebani, 2004; Kalogeromitros, Makris, Gregoriou, Katoulis, & Straurianeas, 2006), or intolerance to acetaldehyde, biogenic amines such as tyramine or sulphur dioxide (Alibrandi, Parodi, & Varadlo 1990; Littlewood, Gibb, Glover, Sandler, Davies, & Rose, 1988).

Identification of the safe threshold for the most sensitive individuals is critical. Bindeslev-Jensen, Briggs, and Osterballe (2002) defined a threshold value for egg of 8.6 mg that would protect 99% of egg-allergic individuals. Moneret-Vautrin and Kanny (2004) reported that 18% of egg-allergic individuals can react to a concentration equal to or lower than 65 mg, while the threshold for egg white capable of triggering an allergic reaction in 1% of sensitised people was between 1 and 2 mg. Similarly, Morriset et al. (2003) performed a double-blind, placebo-controlled food challenge with egg-allergic individuals and reported that the lowest adverse effect level (LOAEL) for crude egg was 2 mg.

Taking these values into account, the limit of detection of the methods used in this study (0.0564 mg/L) and the limit of quantification (0.1578 mg/L) should be more than sufficient to protect egg-allergic individuals. In fact, an egg-allergic individual drinking 1 L of wine at the limit of detection would consume less than 0.06 mg of egg white. Thus, the risk of an allergic reaction from consuming wine should be considered negligible even in the most sensitive subjects. On the other hand, to ensure the safety of all allergic consumers the exemption from labelling should be allowed only when the absence of residues is confirmed by analytical controls.

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