



Absence of allergenic residues in experimental and commercial wines fined with caseinates

Patrizia Restani^{a,*}, Francesca Uberti^a, Roberta Danzi^b, Cinzia Ballabio^a, Francesco Pavanello^b, Chiara Tarantino^c

^a Dept. Pharmacological Sciences, Università degli Studi di Milano, Via Balzaretti 9, 20133 Milan, Italy

^b Unione Italiana Vini, Via San Vittore al Teatro 3, 20123 Milan, Italy

^c Euroclone S.p.A., Via Figino 20/22, 20016 Pero, Milan, Italy

ARTICLE INFO

Article history:

Received 12 November 2010

Received in revised form 30 September 2011

Accepted 11 March 2012

Available online 21 March 2012

Keywords:

Food allergy
Fining agent
Caseinates
Milk allergens

ABSTRACT

The possible presence of allergenic residues in wines treated with one of the potassium caseinates used as fining agents has been investigated.

Samples of experimental (16) and commercial (63) wines that had been fined with caseinates with or without bentonites were studied. The leading physicochemical characteristics of each wine sample were determined to assess their possible role in promoting or hindering the elimination of allergenic residues.

The study used a specifically developed ELISA test as well as an immunoblotting technique in which membranes were incubated with specific anti-caseinate antibody, both tests having high sensitivity for allergenic residues.

No detectable allergenic residues were found in any of the samples by either immunochemical test, irrespective of the physicochemical characteristics of the wine, the type and dosage of refining agent and the enological process used.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Allergic reactions to foods are an increasing problem in the world, although their actual prevalence is still unknown. Milk allergy is frequent in childhood but quite rare in adults (Asero *et al.*, 2009a), in whom fruits and vegetables (Asero *et al.*, 2009b) or peanut and tree nuts (Metcalfe, 1997), are frequently the cause of anaphylactic or other clinical reactions.

Potassium caseinate is a water-soluble fining agent mainly used for white wines. It flocculates when added to wine because of the acidic pH, binding the compounds responsible for turbidity and enabling them to be removed mechanically. Caseinates have useful enological properties, but any residues left in the wines so clarified could constitute a risk for the rare individuals suffering from milk allergy in adulthood.

EC Directive 2000/13/EC (EC, 2000) incorporated laws of EU Member States relating to the labelling, presentation and advertising of foodstuffs. The rules for allergen labelling were detailed in Directives 2003/89/EC (EC, 2003) and 2006/142/EC (EC, 2006). Annex IIIa to Directive 2003/89/EC listed food ingredients (and, later, lupines and molluscs), likely to cause adverse reactions in susceptible individuals.

EC Directive 2005/26/EC listed food ingredients that were provisionally excluded from the labelling requirement (EC, 2005); but inclusion of wine fining agents in this list was postponed until the end of May 2009 (EC, 2007) and again to the end of 2010 to allow further studies (EC, 2009a). This paper records the results from an investigation into the presence of caseinate residues in several commercial wines that might pose a significant risk for adults suffering from milk allergy.

Some previous studies measured the presence of caseinate residues in wines (both experimentally made and commercially available), with mixed results (Liffrani *et al.*, 2009; Rolland, Apostolou, de Leon, Stockley, & O'Hehir, 2008; Weber, Hans, & Paschke, 2009; Weber, Steinhart, & Paschke, 2007). The present study, using two specially designed immunological tests, showed a large number of caseinate-treated wine samples to be free from detectable allergenic residues.

2. Materials and methods

2.1. Milk proteins

Highly purified cow's milk proteins (Sigma Chemical, St. Louis, MO, USA) were used in SDS-PAGE and immunoblotting as reference standards.

* Corresponding author. Tel.: +39 02 50318371; fax: +39 02 50318284.

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

2.2. Wine samples

2.2.1. Experimental wines

The following experimental wine samples were prepared: untreated wine (negative control), samples fined with 20 and 50 g/h L of commercial caseinate (Protein-100, purity 100%; Dal Cin, Sesto San Giovanni, MI, Italy), both these dosages with the addition of 0, 30, 60 or 90 g/h L of bentonite.

Aliquots of 2 L of wine were firstly added with the primary fining agent (caseinate) as described above and left at room temperature for 24 h. After addition of the secondary fining agent (bentonite), samples were maintained at room temperature for further 24 h.

All wines were microfiltered through membranes with pore size 3 µm. Detailed data on the 16 experimental wine samples are listed in Table 1. A further group of 14 samples not treated with caseinate were included as negative controls.

2.2.2. Commercial wines

Italian producers were asked to send commercial wines that had been fined with caseinates; only wine samples having known enological practices were included in the study. Detailed data on the 59 white, 2 rosé and 2 red wine samples are listed in Table 2. All commercial wines were filtered through membranes having pore size ≤ 1 µm. The commercial caseinates used in wine fining were: alpha caseinate (purity 96%; Pall Corporation, Italy), caseinato di potassio (purity 88–94%; Acca, Italy), Caseo Cell (containing potassium caseinate, montmorillonites and alpha cellulose; Pall Corporation, Italy), Clarasi DC (containing 65% caseinate, and bentonite; Dal Cin, Italy), Clarito Spray Dry (highly purified; Vason Group, Italy), Clarito SP (containing 12% caseinate, activate bentonite and potassium bicarbonate; Vason Group, Italy), Kaseomax (pure potassium caseinate; Tecnomax, Italy), Micron 96 (purity ≥ 96%; AEB, Italy), Micron XL (purity ≥ 90%; AEB, Italy), potassium caseinate S (purity 89.5%; DMW International, The Netherlands), Protoclar (purity > 90%; Esseco, Italy) and Vinpur Special (milk casein; Erbsloh Geisenheim, Germany).

2.3. Physicochemical characteristics of wines

2.3.1. Alcoholic strength by volume

The alcoholic strength by volume is the number of litres of ethanol contained in 100 L of wine, both volumes being measured at 20 °C. The symbol is “% vol.”.

The method involves distilling wine volume by volume; the volumetric weight of the distillate is measured by electronic densitometry using a frequency oscillator (OIV-TALVOL, 2009).

2.3.2. Total alcoholic strength by volume

Total alcoholic strength is a calculation of the potential alcohol concentration if all remaining sugars were to be fermented; it is calculated by adding potential alcoholic strength to alcoholic strength by volume (EC, 2009b).

Potential alcoholic strength by volume is 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 is calculated by multiplying the concentration of reducing sugars (g/L) by 0.06.

2.3.3. Reducing sugars

To prepare samples with a sugar content ranging between 0.5 and 5 g/L, dry wine (sugars < 5 g/L) was diluted 1:2 (v/v) with water; sweet wine was diluted suitably to reach the values reported above. Red wine was clarified with solutions of neutral lead acetate and calcium carbonate (Merck KGaA, Darmstadt, Germany). A specific quantity of an alkaline solution of copper salts is heated and the copper ions are titrated by the clarified/diluted wine, in the presence of methylene blue as indicator (UIV Internal Method, 2009).

2.3.4. Total acidity

Wine total acidity was determined by acid-base potentiometric titration, using 0.1 N NaOH, to pH 7, with an automatic titrator (OIV-ACITOT, 2009).

2.3.5. Volatile acidity

Carbon dioxide was first removed from the wine sample. Volatile acids were 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 titration by standard iodine solution (OIV-ACIVOL, 2009).

2.3.6. pH

pH was measured by potentiometry using a calibrated pH meter (OIV-pH, 2009).

Table 1

Physico-chemical characteristics and allergenic residues of experimental white wines.

Sample	Oenological treatment	ASV	TAS	RS	Specific gravity	pH	TDE	RE	TA	VA	Ash (g/L)	TPC	ELISA	IMM
C1G	–	11.87	11.95	1.4	0.99321	3.30	22.6	22.2	6.3	0.34	1.93	224	Neg	Neg
C2G	50 g/h L caseinate	11.70	11.76	1.0	0.99330	3.35	22.4	22.4	6.2	0.33	2.12	168	Neg	Neg
C3G	50 g/h L caseinate + 30 g/h L bentonite	11.62	11.69	1.2	0.99342	3.35	22.3	22.1	6.1	0.33	2.06	174	Neg	Neg
C4G	50 g/h L caseinate + 60 g/h L bentonite	11.48	11.54	1.0	0.99338	3.35	21.9	21.9	6.0	0.33	2.07	163	Neg	Neg
C5G	50 g/h L caseinate + 90 g/h L bentonite	11.41	11.48	1.1	0.99355	3.36	22.0	21.9	6.0	0.32	2.06	172	Neg	Neg
C6G	20 g/h L caseinate + 30 g/h L bentonite	11.61	11.68	1.1	0.99359	3.34	22.4	22.3	6.1	0.34	2.03	183	Neg	Neg
C7G	20 g/h L caseinate + 60 g/h L bentonite	11.43	11.49	1.0	0.99355	3.35	22.2	22.2	6.0	0.34	2.07	179	Neg	Neg
C8G	20 g/h L caseinate + 90 g/h L bentonite	11.44	11.52	1.3	0.99351	3.35	22.3	22.0	6.0	0.33	2.01	175	Neg	Neg
C1S	–	13.46	13.52	1.0	0.99289	3.46	27.2	27.2	6.4	0.33	2.73	295	Neg	Neg
C2S	50 g/h L caseinate	13.19	13.25	1.0	0.99314	3.47	27.2	27.3	6.4	0.31	2.67	266	Neg	Neg
C3S	50 g/h L caseinate + 30 g/h L bentonite	13.14	13.20	1.0	0.99314	3.46	27.0	27.0	6.3	0.32	2.70	281	Neg	Neg
C4S	50 g/h L caseinate + 60 g/h L bentonite	12.91	12.97	1.0	0.99336	3.45	26.9	26.9	6.2	0.31	2.73	254	Neg	Neg
C5S	50 g/h L caseinate + 90 g/h L bentonite	12.91	12.98	1.1	0.99318	3.46	26.7	26.6	6.2	0.31	2.62	252	Neg	Neg
C6S	20 g/h L caseinate + 30 g/h L bentonite	13.12	13.19	1.1	0.99325	3.45	27.2	27.1	6.3	0.32	2.63	250	Neg	Neg
C7S	20 g/h L caseinate + 60 g/h L bentonite	12.92	12.98	1.0	0.99337	3.45	26.9	26.9	6.3	0.32	2.67	248	Neg	Neg
C8S	20 g/h L caseinate + 90 g/h L bentonite	13.06	13.12	1.0	0.99314	3.45	26.6	26.6	6.2	0.31	2.69	246	Neg	Neg

G = Garganega; S = sicily white wine; Neg = negative; ASV = alcoholic strength by volume (mL/100 mL); TAS = total alcoholic strength (mL/100 mL); RE = reduced extract; RS = reducing sugars (g/L); TDE = total dry extract (g/L); TA = total acidity (g/L); TPC = total phenolic compounds; VA = volatile acidity (g/L); IMM = immunoblotting.

Table 2

Physico-chemical characteristics and allergenic residues of commercial wines – part I–IV.

N	Wine name	CAS (g/h L)	BEN	ASV	TAS	RS	Specific gravity	pH	TDE	RE	TA	VA	Ash (g/L)	TPC	ELISA	IMM
1	Bianco Toscano – Gruppo Italiano Vini	No	Yes	12.28	12.38	1.6	0.99171	3.32	20.8	20.2	5.6	0.32	1.97	154	Neg	Neg
2	Vernaccia di San Gimignano DOCG	VS 7.5	Yes	12.86	13.07	3.5	0.99058	3.19	20.3	17.8	5.1	0.24	1.09	150	Neg	Neg
3	Bianco di Toscana IGT 2008	VS 7.5	Yes	12.22	12.48	4.3	0.99224	3.24	22.3	19.0	5.5	0.28	1.59	206	Neg	Neg
4	Basilicata IGT 2008	VS 17.5	Yes	13.06	13.63	9.5	0.99560	3.27	33.4	24.9	5.9	0.27	1.96	232	Neg	Neg
5	Bianco Toscana IGT 2008	AC + CC 20 + 20	Yes	11.78	11.98	3.4	0.99178	3.14	20.0	17.6	5.9	0.33	1.22	167	Neg	Neg
6	Chardonnay Umbria IGT 2008	M96 + CL 30 + 25	Yes	12.71	12.85	2.3	0.99203	3.34	22.8	21.5	5.7	0.49	1.76	247	Neg	Neg
7	Chardonnay Umbria IGT 2007	M96 + CL 30 + 25	Yes	12.91	13.06	2.5	0.99146	3.27	22.3	20.8	6.1	0.45	1.55	182	Neg	Neg
8	Orvieto Classico DOC 2008 “Campogrande”	M96 30	Yes	11.62	11.96	5.7	0.99249	3.11	21.7	17.0	5.6	0.24	1.03	165	Neg	Neg
9	Bianco Toscana IGT 2008 “Villa Antinori”	AC + CC + CL 20 + 20 + 15	Yes	11.79	11.94	2.5	0.99137	3.14	18.8	17.3	5.7	0.26	1.13	172	Neg	Neg
10	Moscato d’Asti DOCG Vigneti Castelli del Poggio	CP 23	No	4.88	11.88	116.6	1.04709	3.24	139.4	23.8	5.8	0.35	1.77	207	Neg	Neg
11	Gavi DOCG 2008 Podere Saulino Novi Ligure	CP 10	Yes	12.40	12.59	3.1	0.99068	3.06	18.8	16.7	5.7	0.25	1.45	85	Neg	Neg
12	Trebbiano di Modena IGT Frizzante secco	CP 30	Yes	10.24	10.94	11.7	0.99771	2.41	26.4	15.7	8.4	0.43	2.06	10	Neg	Neg
13	Riesling Oltrepo’ Pavese DOC Torrevilla 2009	PTC 30	Yes	11.97	12.46	8.1	0.99465	3.17	27.4	20.3	6.2	0.24	1.70	242	Neg	Neg
14	Solbri 2008 Piemonte DOC Cortese	PC 40	Yes	11.89	12.20	5.1	0.99220	3.18	20.5	16.4	5.5	0.21	1.20	215	Neg	Neg
15	Vino Bianco Greco di Tufo DOCG 2005	M96 20	Yes	12.45	12.62	2.8	0.99102	3.23	20.0	18.2	5.8	0.31	1.35	269	Neg	Neg
16	Vino da Tavola Bianco – Vin del Sannio	AC 15	Yes	11.43	11.49	1.0	0.99160	3.31	18.1	18.1	4.4	0.26	1.47	226	Neg	Neg
17	Falanghina del Beneventano IGT Penguè	AC 15	Yes	12.40	12.46	1.0	0.99028	3.42	18.4	18.4	4.6	0.25	1.79	161	Neg	Neg
18	Sannio Falanghina DOC 2008	AC 15	Yes	12.61	12.67	1.0	0.99090	3.43	19.9	19.9	5.4	0.28	1.97	222	Neg	Neg
19	Sannio Greco DOC 2008	AC 18	Yes	13.28	13.34	1.0	0.99097	3.36	22.3	22.3	5.8	0.24	2.00	207	Neg	Neg
20	Coda di Volpe Sannio DOC 2008	M96 20	Yes	11.91	11.99	1.3	0.99123	3.27	18.7	18.4	5.7	0.20	1.76	394	Neg	Neg
21	Falanghina Sannio DOC 2008	M96 20	Yes	13.03	13.29	4.3	0.99105	3.14	21.8	18.5	5.7	0.20	1.05	381	Neg	Neg
22	Greco di Tufo DOCG 2008	CSD 10	Yes	13.24	13.30	1.0	0.99094	3.23	21.7	21.7	7.3	0.36	1.53	331	Neg	Neg
23	Fiano di Avellino DOCG 2008	CSD 10	Yes	12.97	13.03	1.0	0.99119	3.31	139.8	20.8	6.2	0.26	1.44	260	Neg	Neg
24	Moscato d’Asti DOCG 2009	PCS 20	Yes	4.56	11.09	108.8	1.04869	2.15	23.2	32.0	6.8	0.20	1.71	275	Neg	Neg
25	Pinot Grigio delle Venezie IGT 2009	CSD 30	Yes	11.50	11.80	5.0	0.99332	3.30	24.3	19.2	5.6	0.20	1.63	196	Neg	Neg
26	Oltrepo’ Pavese DOC Bianco 2008	PTC 10	Yes	11.61	12.10	8.1	0.99370	3.04	11.61	17.2	6.3	0.23	1.30	251	Neg	Neg
27	Chardonnay Friuli Aquileia DOC 2008	AC 15	Yes	13.22	13.30	1.3	0.99087	3.32	13.22	21.6	5.9	0.22	1.69	329	Neg	Neg
28	Chardonnay Friuli Aquileia DOC 2008	AC 25	Yes	13.18	13.27	1.5	0.99083	3.35	13.18	21.0	5.8	0.21	1.82	287	Neg	Neg
29	Sauvignon Friuli Aquileia DOC 2008	AC 15	Yes	12.80	12.91	1.9	0.99180	3.36	12.80	21.7	6.4	0.22	1.91	270	Neg	Neg
30	Oltrepo’ Pavese DOC Malvasia	PTC 10	Yes	11.06	11.71	10.9	0.99618	2.79	11.06	18.4	5.8	0.20	1.38	177	Neg	Neg
31	Vino Bianco Malvasia dell’Emilia IGT Frizzante	MXL 35	Yes	3.58	9.62	100.6	1.04689	1.84	130.9	31.3	7.3	0.20	2.16	315	Neg	Neg
32	Falanghina IGT Campania	CDC 5	Yes	12.86	13.16	5.00	0.99314	3.19	24.7	20.7	6.3	0.27	1.48	472	Neg	Neg
33	White wine San Marzano	PCS 40	Yes	12.37	12.68	5.20	0.99400	3.50	27.9	23.7	6.2	0.36	2.12	282	Neg	Neg
34	White wine Rufiano Feline 2008	PTC 35	Yes	13.91	14.18	4.5	0.99037	3.11	23.0	19.5	6.0	0.21	1.14	252	Neg	Neg
35	White wine Frascati Superiore 2008	PTC 25	Yes	12.91	13.01	1.6	0.99055	3.20	19.9	19.3	5.8	0.32	1.37	194	Neg	Neg
36	White wine Frascati Superiore 2008	PTC 25	Yes	13.04	13.13	1.5	0.99041	3.23	19.7	19.2	5.6	0.34	1.32	216	Neg	Neg
37	Lugana 2008	MXL 4	Yes	12.81	13.04	3.8	0.99157	3.24	22.6	19.8	5.5	0.2	1.35	218	Neg	Neg
38	Custoza 2008	MXL 5	Yes	12.37	12.65	4.7	0.99215	3.18	21.9	18.2	5.3	0.21	1.21	142	Neg	Neg
39	Chardonnay Villa Mura	MXL 2	Yes	12.23	12.39	2.7	0.99219	3.24	20.8	19.1	5.4	0.23	1.41	220	Neg	Neg
40	Pinot Grigio Trulli 2008	MXL 2	Yes	12.40	12.65	4.2	0.99245	3.17	22.8	19.6	5.5	0.24	1.40	190	Neg	Neg
41	Custoza 2009	MXL 2	Yes	12.21	12.46	4.2	0.99253	3.23	22.7	19.5	5.4	0.20	1.42	202	Neg	Neg
42	Chardonnay Feudo Sartanna 2009	MXL 3	Yes	13.34	13.50	2.7	0.99253	3.37	26.0	24.3	5.3	0.29	1.68	264	Neg	Neg
43	Pinot Grigio Villa Mura	MXL 3	Yes	12.21	12.44	3.9	0.99245	3.30	21.8	18.9	5.2	0.27	1.55	208	Neg	Neg
44	Pinot Grigio Grave 2009	MXL 3	Yes	12.78	13.03	4.1	0.99221	3.34	23.1	20.0	5.4	0.27	1.72	167	Neg	Neg
45	Pinot Grigio Trulli 2008	MXL 4	Yes	12.40	12.63	3.8	0.99283	3.30	23.9	21.1	6.0	0.31	1.93	198	Neg	Neg
46	Pinot Grigio Galico 2008	MXL 4	Yes	12.21	12.44	3.8	0.99271	3.27	22.7	19.9	5.8	0.25	1.60	213	Neg	Neg

Table 2 (continued)

N	Wine name	CAS (g/h L)	BEN	ASV	TAS	RS	Specific gravity	pH	TDE	RE	TA	VA	Ash (g/L)	TPC	ELISA	IMM
47	Pinot Grigio Arcole 2008	MXL 5	Yes	12.77	13.03	4.3	0.99297	3.39	25.4	22.1	5.7	0.26	1.95	204	Neg	Neg
48	Chardonnay Feudo Arancio 2007	CSD 6.6	No	13.57	13.79	3.6	0.99173	3.41	25.6	23.0	5.8	0.35	1.83	255	Neg	Neg
49	Chardonnay Vigneto delle Dolomiti 2007	CSP 5.5	No	13.12	13.34	3.7	0.99197	3.45	25.0	22.3	5.9	0.38	1.92	268	Neg	Neg
50	White wine Feudo Arancio 2007	CSD 6.6	No	12.89	13.08	3.1	0.99216	3.38	24.4	22.3	5.9	0.32	1.74	296	Neg	Neg
51	Pinot Grigio Mezzacorona 2007	CSD 5.7	No	12.51	12.70	3.2	0.99199	3.42	22.9	20.7	5.8	0.31	1.73	233	Neg	Neg
52	Rotary Talento Brut Arte Italiana	CSD 7.2	No	13.38	13.83	7.5	0.99290	3.41	27.9	21.4	6.3	0.30	1.77	226	Neg	Neg
53	Falanghina Vigne Sannite	CSP 15	Yes	11.91	12.16	4.1	0.99342	3.59	24.9	21.8	5.0	0.35	2.62	281	Neg	Neg
54	Fiano Vigne Sannite	CSP 20	Yes	10.85	11.14	4.8	0.99409	3.48	23.3	19.5	4.7	0.34	2.31	274	Neg	Neg
55	Falanghina Vigne Sannite	CSP 20	Yes	12.49	12.57	1.4	0.99171	3.46	22.3	21.9	5.6	0.31	2.63	300	Neg	Neg
56	Coda di Volpe Vigne Sannite	CSP 20	Yes	12.23	12.34	1.8	0.99183	3.32	22.2	21.4	6.2	0.33	2.13	356	Neg	Neg
57	Greco Vigne Sannite	CSP 20	Yes	12.58	12.66	1.4	0.99171	3.31	22.8	22.4	6.5	0.32	2.21	342	Neg	Neg
58	White Lambrusco Belgioioso	MXL 20	Yes	10.84	11.7	14.3	0.99908	3.05	35.6	22.3	7.3	0.20	1.55	154	Neg	Neg
59	Trebbiano Lamapopoli 2008	KSM 30	Yes	11.3	11.41	1.9	0.99258	3.37	20.8	19.9	5.0	0.30	2.30	223	Neg	Neg
60	Chardonnay Lama di Mucci 2009	KSM 40	Yes	13.01	13.34	5.6	0.99391	3.32	28.9	24.3	6.3	0.34	2.09	200	Neg	Neg
61	Bizantino Rosé Salento 2008	PTC 30	Yes	12.6	12.92	5.4	0.9931	3.37	25.8	21.4	5.3	0.25	2.21	356	Neg	Neg
62	Lambrusco Rosé Bosco d'Este	MXL 20	Yes	10.61	11.49	14.6	0.99999	3.22	36.8	23.2	7.0	0.21	1.97	420	Neg	Neg
63	Lambrusco Bosco d'Este	MXL 30	Yes	10.49	11.42	15.5	1.00197	3.05	41.4	26.9	7.5	0.26	2.22	1429	Neg	Neg
64	Lambrusco Nero di Corte	MXL 30	Yes	10.45	11.19	12.4	1.00197	3.08	41.4	30.0	6.5	0.2	2.20	3317	Neg	Neg

CAS = caseinates; BEN = bentonites.

Caseinates: AC = alpha caseinate; CP = caseinato di potassio; CC = Caseo Cell; CDC = Clarasi DC; CSD = Clarito Spray Dry; CSP = Clarito SP; KSM = Kaseomax; M96 = Micron 96; MXL = Micron XL; PCS = potassium caseinate S; PTC = Protoclar; VS = Vinpur Special.

Physicochemical parameters and analyses: ASV = alcoholic strength by volume (mL/100 mL); TAS = total alcoholic strength (mL/100 mL); RS = reducing sugars (g/L); TDE = total dry extract (g/L); RE = reduced extract; TA = total acidity (g/L); VA = volatile acidity (g/L); TPC = total phenolic compounds; ELISA = enzyme-linked immuno sorbent assay; IMM = immunoblotting.

2.3.7. Ash

The wine extract was ignited at 500–550 °C until complete combustion (oxidation) of organic material had been achieved. The amount of ash was obtained by weighing the residue after sample combustion using a balance having a sensitivity of 0.1 mg (OIV-CENDRE, 2009).

2.3.8. Total dry extract and reduced extract

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

2.3.9. Total phenolic compounds

Total phenolic compounds were measured using a modified version of the Folin–Ciocalteu Method (OIV-INFOL, 2009). All wine phenolic compounds were oxidized by Folin–Ciocalteu reagent. After dilution, wine samples were mixed with Folin–Ciocalteu reagent and sodium carbonate (Merck KGaA, Darmstadt, Germany); absorbance was measured by spectrophotometry at 760 nm.

2.4. SDS–PAGE

2.4.1. Gradient running gel

The composition of the gradient running gel was: 9–19% acrylamide; 0.08–0.17% bis-acrylamide; 0.36 M Tris–HCl buffer pH 8.8; 35% glycerol; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% N,N,N',N'-tetramethylethylenediamine (TEMED).

2.4.2. Stacking gel

The composition of the stacking gel was: 3.5% acrylamide; 0.09% bis-acrylamide; 0.125 M Tris–HCl buffer pH 6.8; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% TEMED.

2.4.3. Running buffer

The composition of the running buffer was: 25 mM Tris, 0.19 M glycine and 0.1% SDS (w/v), pH 8.8. For further details, see Ballabio et al. (2007). All reagents were from BioRad (Segrate, MI, Italy).

2.4.4. Sample buffers

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

The composition of the 4× sample buffer was: 0.50 M Tris–HCl buffer pH 6.8, 45.0% glycerol, 4% SDS and 10% β-mercaptoethanol.

2.4.5. Standard preparation

Standard caseinates were solubilized at a concentration of 2 mg/mL in water and then mixed 1:1 (v/v) with 2× sample buffer.

2.4.6. Sample preparation

Wine samples were mixed 3:1 (v/v) with 4× sample buffer and 25 µL were loaded onto the gel.

2.4.7. Gromova staining

After electrophoresis, gels were stained according to Gromova and Celis (2006). Protein detection depends on the binding of silver ions to the aminoacid side chains, primary the sulfhydryl and carboxyl groups of proteins, followed by reduction to free metallic silver. The protein bands are visualized as spots where the reduction occurs and, as a result, the image of protein distribution within the gel is based on the difference in oxidation–reduction potential between the gel area occupied by proteins and the free adjacent sites.

2.5. Immunoblotting

After SDS–PAGE, proteins were transferred to PVDF membrane (Millipore, Vimodrone, MI, Italy) 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 (in 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% Triton-X) to prevent nonspecific adsorption of the immunological reagents. The membrane was then immersed in 10 mL of 0.25% gelatin solution containing 10 μ L of rabbit anti-caseinate IgG polyclonal antibodies. The antibody was developed for this research by using total casein fraction (Sigma–Aldrich, Milan, Italy) for immunization; the same antibody was used both in immunoblotting and ELISA test (Restani, Ballabio, Mottini, & Tarantino, 2009).

Antigen–IgG complexes were detected using 10 μ L of goat anti-rabbit IgG antibodies labelled with alkaline phosphatase (Sigma–Aldrich). The developing solution contained 15% bromochloroindolyl phosphate (BCIP) and 30% nitro blue tetrazolium (NBT) (Sigma–Aldrich) in alkaline phosphatase buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Tests were performed at least three times.

2.6. ELISA test

An ELISA kit (Casein Wine Kit, Euroclone S.p.A., Pero Milano), specifically modified for the quantification of caseinates in wine, was used (Restani et al., 2009). It is a sandwich ELISA where the microplate is first coated with the specific anti-casein antibody also used in immunoblotting; after incubation with the wine sample a secondary anti-casein antibody conjugated with horse radish peroxidase (HRP) is added to form a 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-LIMDET, 2009) was used. The limits were calculated according to the procedure “Determination on blank” using the data from 10 laboratories participating in a collaborative interlaboratory study. For quantification, standard solutions contained increasing amounts of a fining caseinate (Pro-tean 100, Dal Cin, Sesto San Giovanni, MI, Italy) were used. Wine samples were diluted 1:5 (v/v) with the buffer supplied with the kit. Tests were performed at least three times.

3. Results

This study included 16 experimental and 63 commercial wines fined with the aid of caseinates with or without bentonites; all samples were microfiltered. Untreated wines were examined in parallel as negative controls.

3.1. Physicochemical characteristics

The physicochemical properties of wine samples were examined in order to identify (in case residues were detected) parameters most likely to inhibit or promote the elimination of allergens during the fining process. Although the selection was performed only among Italian wines, the wide variety of samples considered makes the results significant also for the international market. Few rosé and red wines were examined since in Italy caseinates are rarely used in these products. Data are reported in Table 1 (experimental wines) and Table 2 (commercial wines).

3.2. SDS–PAGE and Gromova staining

After the optimization of SDS–PAGE method, its detection limit was determined by loading decreasing quantities of enological caseinates. LOD was 8.6 ng of caseinate, corresponding to 0.43 ppm in the wine sample due to the sample dilution (data not shown).

The method was then applied to wine samples. To assess the usefulness of this technique to quantify allergenic residues, some caseinate-free white wine samples were also analyzed. As an

example, Fig. 1 shows the electrophoretic separation of some commercial wines and an untreated sample.

Several bands are visible in the gel, even when wines were not fined with caseinates. Unfortunately, the proteins present in wine (Dizy & Bisson, 1999) have electrophoretic properties similar to those of caseinates. The wine samples were always assessed by SDS–PAGE, but the results were not considered specific enough for caseinate detection and are not described in this paper in detail.

3.3. Specificity of anti-caseinate antibody

The quality of antibody is critical in immunochemical determinations; for this reason the antibody specifically developed for this research was characterized by its binding capacity for the main proteins contained in fining agents (α - and β -caseins) and their possible proteolytic fragments (γ -caseins).

Fig. 2 shows the electrophoresis (left) and immunoblotting (right) of an enological caseinate alongside the purified caseins from cow's milk. In the fining agent, α - and β -caseins are clearly visible and identifiable.

As Fig. 2 (right) shows, the specificity of the antibody used both in ELISA test and in immunoblotting is capable of detecting each casein fraction, although it was developed mainly against α - and β -caseins. The antibody also recognized the proteolytic fragments of β -casein (γ -caseins), which guarantees the detection of casein in commercial wines even when there has been proteolysis during the maturation.

The specificity of antibody was also assayed with most fining agents used in experimental and commercial wines. All tested fining agents were specifically recognized by the antibody (data not shown); the differences observed depended on the percentage of caseinate present in each product as described in Section 2.

3.4. Immunoblotting

3.4.1. Detection limit of immunoblotting

Through the loading of decreasing quantities, it was possible to determine the lowest detectable amount of enological caseinates; this value was below 1 ppm in the wine sample. This limit could be reduced by concentrating the wine sample, but it was considered low enough to confirm data from the ELISA test.

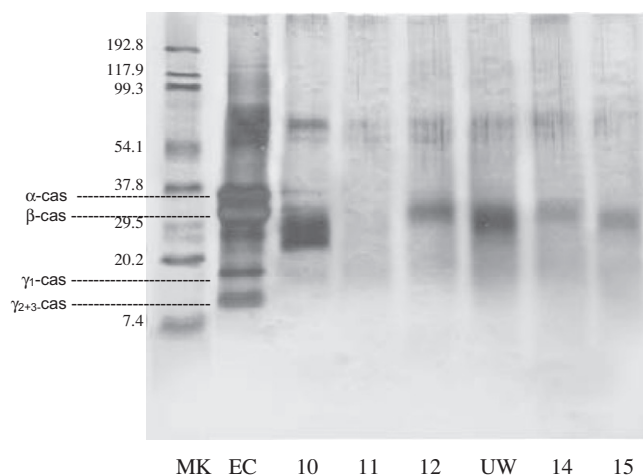


Fig. 1. SDS–PAGE of commercial wine samples fined with caseinates and an untreated experimental wine. EC = potassium caseinate (fining agent); 10 = Moscato d'Asti; 11 = Trebbiano di Modena; 12 = untreated wine; 14 = Solbri DOC; 15 = Greco di Tufo DOCG; MK = molecular weight standard solution.

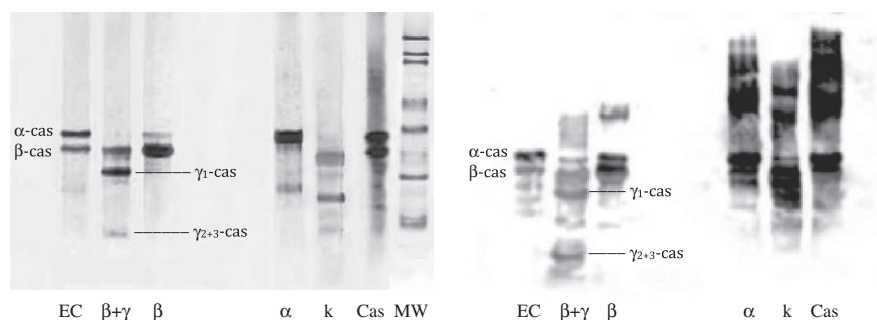


Fig. 2. SDS–PAGE (left) and immunoblotting (right) of a fining agent containing caseinate and purified casein fractions from cow's milk. EC = enologic caseinate; $\beta + \gamma = \beta + \gamma$ casein; $\beta = \beta$ casein; $\alpha = \alpha$ -casein; $\kappa = \kappa$ casein; Cas = standard total casein; MK = molecular weight standard solution.

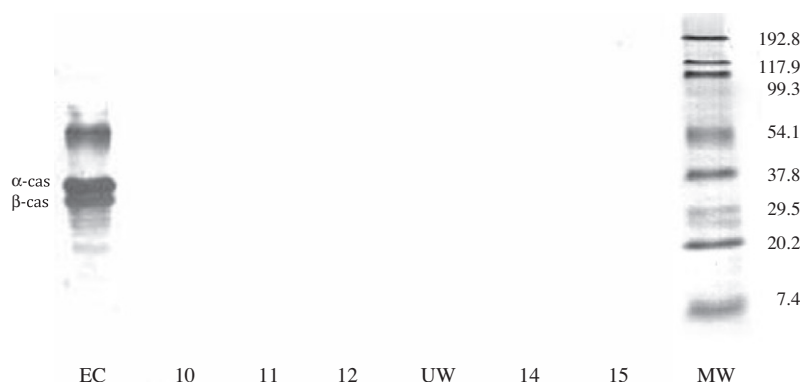


Fig. 3. Immunoblotting of commercial wine samples fined with caseinates and an untreated experimental wine. EC = potassium caseinate (fining agent); 10 = Moscato d'Asti; 11 = Gavi DOCG; 12 = Trebbiano di Modena; UT = untreated wine; 14 = Solbri DOC; 15 = Greco di Tufo DOCG; MK = molecular weight standard solution.

3.4.2. Immunoblotting of wine samples

An example of immunoblotting performed on wine samples is shown in Fig. 3. Samples shown in this figure are the same loaded onto SDS–PAGE illustrated in Fig. 1. All experimental and commercial wines tested in this project were analyzed by immunoblotting and were free from residues of caseinates as reported in Tables 1 and 2, respectively. As expected, all untreated samples were free from caseinate.

3.5. ELISA

The ELISA sandwich test specifically developed for this project is now commercially available. According to the collaborative inter-laboratory study, this method showed a reliable limit of detection equivalent to 0.28 ppm and a limit of quantification of 0.76 ppm, and quality parameters (reproducibility, repeatability and robustness) in line with the criteria established by the OIV in the Compendium of International Methods of Analysis (OIV-ETCOL, 2009).

All the samples proved to be free from detectable allergenic residues, irrespective of the physicochemical characteristics of the wine, type and dosage of fining agent used and enological practices applied to experimental and commercial wines as reported in Tables 1 and 2, respectively. As expected, all untreated samples were free from caseinates (data not shown).

4. Discussion and conclusions

The prevalence of food allergy in adults is lower than in children and clinical data show wide heterogeneity, possibly because of differences in study design and methodologies. Moreover, there is

a significant discrepancy between perception of food allergy and the results of the double-blind placebo-controlled food challenges (actual prevalence) both in adults and in young subjects (Roehr et al., 2004; Young, Stoneham, Petrukevitch, Barton, & Rona, 1994). According to the study by Asero et al. (2009a, 2009b) performed in 17 Italian allergologic centres, the percentage of subjects suffering for food and milk allergy among adults are respectively 2% and 0.062%; moreover, in the case of milk allergy in adulthood the frequency of anaphylaxis is very rare. Similar prevalences were reported by other authors (Osterballe, Hansen, Mortz, Høst, & Bindslev-Jensen, 2005; Sicherer & Sampson, 2006).

As to the risk associated to wines fined by caseinates, we can unequivocally state that because the number of adults suffering for milk allergy is low and because of the undetectable levels of residues we found, clinical reactions to fined wine will be highly improbable. Kirschner, Belloni, Kugler, Ring, and Brockow (2009) did show that some patients gave a skin-prick test positive to wines fined with caseinates, but the subjects actually tolerated the same product assayed by double-blind, placebo-controlled food challenge.

Our investigation showed residues of less than 0.28 ppm in all the wines examined. This result was independent of the physicochemical characteristics of the wines, despite the wide range of values for each parameter included; the type and dosage of fining agent that had been used (20–50 g/h L of caseinate for experimental and 2–40 g/h L for commercial wines) and the enological practices applied (use or not of bentonite in both experimental and commercial wines) apart from microfiltration.

We nevertheless searched for further data that might indicate the need to protect the most sensitive subjects. Some authors have

determined milk protein thresholds: Taylor et al. (2002) summarized the work of different teams and concluded that 0.33% of children allergic to milk could react to as little as 0.6 mg of milk proteins (see comments in Moneret-Vautrin & Kanny, 2004), while Bindslev-Jensen, Briggs, and Osterballe (2002) defined threshold values that would protect 99 of 100 allergic individuals as 8.6 mg of proteins for milk, 3.4 mg for egg, 1.2 mg for peanut, and 2.2 mg for soybean, and Bellioni-Busino et al. (1999) and Fiocchi et al. (2008) found LOAELs of 1 and 0.1 mL of whole milk in children (approximately 36.2 and 3.6 mg of proteins, respectively).

In adults, Pastorello et al. (1989) found a LOAEL of 0.5 g of freeze-dried milk (approximately 187 mg of proteins). In agreement with the different values reported, the threshold of milk capable of eliciting an objective clinical reaction ranged between 0.6 and 180 mg of protein. On the basis of these findings, a limit of detection (LOD) of 0.5 ppm and a limit of quantification (LOQ) of 1 ppm was considered amply sufficient for methods to be capable of estimating caseinate residues in fined wines, since in the very worst case (a child sensitive to 0.6 mg of milk protein) the subject would have to drink 1.2 L of wine in a short time to suffer a clinical reaction; the adult thresholds seemed to be above 10 mg. The ELISA test is therefore amply sufficient for the routine assessment of caseinate residues in wine.

Although the ELISA methods published previously (Rolland, Apostolou, de Leon, Stockley, & O'Hehir, 2008; Weber et al., 2007, 2009) are scientifically sound, our method was developed specifically for wine samples, in commercial form in order to standardize its performances and to make it available for producers and laboratories involved in food control. The use of mass spectrometry, though highly sensitive and specific (Monaci, Norgaard, & van Hengel, 2010), or animal tests (Lifrani et al., 2009) cannot be considered suitable methods for routine control.

Our results provide strong evidence of the safety of wines fined with caseinates, and are particularly important in view of the current assessment by the EU of the possibility of exempting caseinates from wine labelling requirements.

Acknowledgements

This study was funded by the Italian Ministero dell'Agricoltura e delle Politiche Agricole e Forestali (MIPAAF) – VINALL.

References

- Asero, R., Antoniceili, L., Arena, A., Bommarito, L., Caruso, B., Colombo, G., et al. (2009b). Cases of food-induced anaphylaxis in Italian adults: A multi centre study. *International Archives of Allergy and Immunology*, 150, 271–277.
- Asero, R., Antoniceili, L., Arena, A., Bommarito, L., Caruso, B., Crivellaro, M., et al. (2009a). EpidemAAITO: Feature of food allergy in Italian adults attending allergy clinics: A multi-centre study. *Clinical and Experimental Allergy*, 39, 547–555.
- Ballabio, C., Bertino, E., Coscia, A., Fabris, C., Fuggetta, D., Molino, S., et al. (2007). Immunoglobulin-A profile in breast milk from mothers delivering full-term and pre-term infants. *International Journal of Immunopathology and Pharmacology*, 20, 119–128.
- Bellioni-Busino, B., Paganelli, R., Lucenti, P., Giampietro, P. G., Perborn, H., & Busino, L. (1999). Allergenicity of goat's milk in children with cow's milk allergy. *Journal of Allergy and Clinical Immunology*, 103, 1191–1194.
- Bindslev-Jensen, C., Briggs, D., & Osterballe, M. (2002). Can we determine a threshold level for allergenic foods by statistical analysis of published data in the literature? *Allergy*, 57, 741–746.
- Dizy, M., & Bisson, L. F. (1999). White wine protein analysis by capillary electrophoresis. *American Journal of Enology and Viticulture*, 50, 120–127.
- EC (2000). Directive 2000/13/EC of the European Parliament and of the Council of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs. *Official Journal of the European Union* L 109/29, 6 May 2000.
- EC (2003). Directive 2003/89/EC of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. *Official Journal of the European Union* L 308/15, 25 November 2003.
- EC (2005). Directive 2005/26/EC of 21 March 2005 establishing a list of food ingredients or substances provisionally excluded from Annex IIIa of the Directive 2000/13/EC of the European Parliament and of the Council. *Official Journal of the European Union* L 75/33, 23 March 2005.
- EC (2006). Directive 2006/142/EC of 22 December 2006 amending Annex IIIa of Directive 2000/13/EC of the European Parliament and of the Council listing the ingredients which must under all circumstances appear on the labelling of foodstuffs. *Official Journal of the European Union* L 368/110, 23 December 2006.
- EC (2007). Directive 2007/68/EC of 27 November 2007 amending Annex IIIa of the Directive 2000/13/EC of the European Parliament and of the Council as regards certain food ingredients. *Official Journal of the European Union* L 310/11, 28 November 2007.
- EC (2009a). Commission Regulation (EC) No 607/2009 of 14 July 2009 laying down certain detailed rules for the implementation of Council Regulation (EC) No 479/2008 as regards protected designations of origin and geographical indications, traditional terms, labelling and presentation of certain wine sector products. *Official Journal of the European Union* L 193/60, 24 July 2009.
- EC (2009b). Total alcoholic strength by volume Reg. 491/09/EC All I + All III GU L154 17/06/2009.
- Fiocchi, A., Terracciano, L., Bouygue, G. R., Veglia, F., Sarraud, T., Martelli, A., et al. (2008). Incremental prognostic factors associated with cow's milk allergy outcomes in infant and child referrals: The Milan Cow's Milk Allergy Cohort study. *Annals of Allergy Clinical Immunology*, 101, 166–173.
- Gromova, I., & Celis, J. E. (2006). Protein detection in gels by silver staining: A procedure compatible with mass-spectrometry. In J. E. Celis, N. Carter, T. Hunter, K. Simons, J. V. Small, & D. Shotton (Eds.). *Cell biology: A laboratory handbook* (Vol. 4, pp. 219–225). Academic Press.
- Kirschner, S., Belloni, B., Kugler, C., Ring, J., & Brockow, K. (2009). Allergenicity of wine containing processing aids: A double-blind, placebo-controlled food challenge. *Journal of Investigational Allergology and Clinical Immunology*, 19, 210–217.
- Lifrani, A., Dos Santos, J., Dubarry, M., Rautureau, M., Blachier, F., & Tomé, D. (2009). Development of animal models and sandwich-ELISA tests to detect the allergenicity and antigenicity of fining agent residues in wines. *Journal of Agriculture and Food Chemistry*, 57, 525–534.
- Metcalfe, D. D. (1997). Food allergy in adults. In D. D. Metcalfe, H. A. Sampson, & R. A. Simon (Eds.), *Food allergy: Adverse reactions to foods and food additives* (pp. 183–192). Cambridge, Mass: Blackwell Science.
- Monaci, L., Norgaard, J. V., & van Hengel, J. (2010). Feasibility of a capillary LC/ESI-Q-TOF MS method for the detection of milk allergens in an incurred model food matrix. *Analytical Methods*, 2010(2), 967–972.
- Moneret-Vautrin, D. A., & Kanny, G. (2004). Update on threshold doses of food allergens: Implications for patients and the food industry. *Current Opinion in Allergy and Clinical Immunology*, 4, 215–219.
- OIV-ACITOT (2009). OIV MA-E-AS313-01-ACITOT. *Compendium of International methods of wine and must analysis*. http://news.reseau-concept.net/pls/news/p_entree?i_sid=1775932271663370238258&i_type_edition_id=20473&i_section_id=20486&i_lang=33#20489.
- OIV-ACIVOL (2009). OIV MA-E-AS313-02-ACIVOL. *Compendium of International methods of wine and must analysis*. http://news.reseau-concept.net/pls/news/p_entree?i_sid=1775932271663370238258&i_type_edition_id=20473&i_section_id=20486&i_lang=33#20489.
- OIV-CENDRE (2009). OIV MA-E-AS2-04-CENDRE. *Compendium of International methods of wine and must analysis*. http://news.reseau-concept.net/pls/news/p_entree?i_sid=1775932271663370238258&i_type_edition_id=20473&i_section_id=20486&i_lang=33#20489.
- OIV-ETCOL (2009). OIV MA-EAS1-07-ETCOL. *Compendium of International methods of wine and must analysis*. http://news.reseau-concept.net/pls/news/p_entree?i_sid=1775932271663370238258&i_type_edition_id=20473&i_section_id=20486&i_lang=33#20489.
- OIV-EXTSEC (2009). OIV MA-E-AS2-03-EXTSEC. *Compendium of International methods of wine and must analysis*. http://news.reseau-concept.net/pls/news/p_entree?i_sid=1775932271663370238258&i_type_edition_id=20473&i_section_id=20486&i_lang=33#20489.
- OIV-INFOL (2009). OIV MA-E-AS2-10-INFOL. *Compendium of International methods of wine and must analysis*. http://news.reseau-concept.net/pls/news/p_entree?i_sid=1775932271663370238258&i_type_edition_id=20473&i_section_id=20486&i_lang=33#20489.
- OIV-LIMDET (2009). OIV E-AS1-10-LIMDET. *Compendium of International methods of wine and must analysis*. http://news.reseau-concept.net/pls/news/p_entree?i_sid=1775932271663370238258&i_type_edition_id=20473&i_section_id=20486&i_lang=33#20489.
- OIV-pH (2009). OIV MA-E-AS313-15-PH. *Compendium of International methods of wine and must analysis*. http://news.reseau-concept.net/pls/news/p_entree?i_sid=1775932271663370238258&i_type_edition_id=20473&i_section_id=20486&i_lang=33#20489.
- OIV-TALVOL (2009). OIV MA-E-AS312-01-TALVOL. *Compendium of International methods of wine and must analysis*. http://news.reseau-concept.net/pls/news/p_entree?i_sid=1775932271663370238258&i_type_edition_id=20473&i_section_id=20486&i_lang=33#20489.
- Osterballe, M., Hansen, T. K., Mortz, C. G., Høst, A., & Bindslev-Jensen, C. (2005). The prevalence of food hypersensitivity in an unselected population of children and adults. *Pediatric Allergy and Immunology*, 16, 567–573.
- Pastorello, E. A., Stocchi, L., Pravettoni, V., Bigi, A., Schilke, M. L., Incorvaia, C., et al. (1989). Role of the elimination diet in adults with food allergy. *Journal of Allergy and Clinical Immunology*, 84, 475–483.

- Restani, P., Ballabio, C., Mottini, M., & Tarantino, C. (2009). Development of ELISA methods to detect the presence of milk and egg proteins in wine after refining. *Le Bulletin de l'OIV – Revue Technique Internationale*, 82, 386–394.
- Roehr, C. C., Edenharter, G., Reimann, S., Ehlers, I., Worm, M., Zuberbier, T., et al. (2004). Food allergy and non-allergic food hypersensitivity in children and adolescent. *Clinical and Experimental Allergy*, 34, 1534–1541.
- Rolland, J. M., Apostolou, E., de Leon, M. P., Stockley, C. S., & O'Hehir, R. E. (2008). Specific and sensitive enzyme-linked immunosorbent assays for analysis of residual allergenic food proteins in commercial bottle wine fined with egg white, milk and non-grape-derived tannins. *Journal of Agricultural and Food Chemistry*, 56, 349–354.
- Sicherer, S. H., & Sampson, H. A. (2006). Food allergy. *Journal of Allergy and Clinical Immunology*, 117(2 Suppl. Mini-Primer), S470–S475.
- Taylor, S. L., Hefle, S. L., Bindslev-Jensen, C., Bock, S. A., Burks, A. W., Jr., Christie, L., et al. (2002). Factors affecting the determination of threshold doses for allergenic foods: How much is too much. *Journal of Allergy and Clinical Immunology*, 109, 24–30.
- UIV Internal Method (2009). Based on the OIV MA-AS311-01-SUCRED. *Compendium of International methods of wine and must analysis*. http://news.reseau-concept.net/pls/news/p_entree?i_sid=1775932271663370238258&i_type_edition_id=20473&i_section_id=20486&i_lang=33#20489.
- Weber, P., Hans, S., & Paschke, A. (2009). Determination of the bovine food allergen casein in white wines by quantitative indirect ELISA, SDS-PAGE, Western blot and immunostaining. *Journal of Agricultural and Food Chemistry*, 57, 8399–8405.
- Weber, P., Steinhart, H., & Paschke, A. (2007). Investigation of the allergenic potential of wines fined with various proteinogenic fining agents by ELISA. *Journal of Agricultural and Food Chemistry*, 55, 3127–3133.
- Young, J., Stoneham, M. D., Petrukevitch, A., Barton, J., & Rona, R. (1994). A population study of food intolerance. *Lancet*, 343, 1127–1130.