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Collaborative Interlaboratory Studies for the Validation of ELISA Methods for the Detection of Allergenic Fining Agents Used in Wine According to the Criteria of OIV Resolution 427–2010 Modified by OIV–Comex 502–2012

Patrizia Restani · Francesca Uberti · Chiara Tarantino · Cinzia Ballabio · Francesca Gombac · Erica Bastiani · Laura Bolognini · Francesco Pavanello · Roberta Danzi

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Abstract The clarification or fining of wine removes undesired substances (mainly proteins, phenols, and tannins), which would roil the wine and cause bitterness and astringency. A common fining agent, egg white, can be directly added to wine through the inlet of a circulating pump, but more typically egg white comes as commercial preparation in powdered form (commercially named egg albumin). Skimmed milk or more frequently purified caseinates are used to remove bitterness and hardness of white wine and sherry. Both egg white and caseinates are fining agents with optimal enological properties, but their residues could represent a risk for subjects suffering from food allergy. The rules for allergen labeling were detailed in Directives 2003/89/EC, and Directive 2005/26/EC established a list of food ingredients provisionally excluded from labeling, that included wine fining agents. Extended till June 2012, wine labeling exemption can be now maintained only if (1) egg and milk derivatives are not used and cross-contamination is under control; and (2) wine clarified with such products is negative for the presence of residues using techniques with detection and quantification limits of 0.25 and 0.5 ppm, respectively. Analytical requirements were defined in the OIV resolution 427–2010 (OIV 2010) modified by OIV/COMEX 502–2012 (OIV 2012). On the basis of a previous experience, an interlaboratory collaborative trial was organized to validate a commercial ELISA kit designed to measure allergenic residues in red wine fined with egg white proteins. In the meantime, the performance of the commercial caseinate ELISA kit for white wine was rechecked according to the new limit of detection and limit of quantification values, recommended by OIV in 2012. The collaborative interlaboratory studies showed that both ELISA kits had good reproducibility, repeatability, and robustness in detecting residues of allergenic fining agents in wine, in good agreement with the requirements of the OIV resolution 427–2010 modified by OIV/COMEX 502–2012.

Keywords Fining agents · Egg white · Egg allergens · Milk allergens · Caseinates · ELISA

Introduction

A wide variety of agents has been used as wine fining agents: bovine blood, egg whites, milk caseinates, isinglass, horse gelatins, seaweed, clay, and others. Modern enology uses both organic and inorganic fining agents. Albumen/egg white proteins are common fining agents for red wine; their colloidal nature and a positively charged surface attracts negatively charged tannins, which can produce undesired astringency. Egg white proteins have optimal fining properties, but any residues left in wine could represent a risk for subjects with an allergy to eggs,

P. Restani (✉) · F. Uberti · C. Ballabio
Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, via Balzaretti 9, 20133 Milan, Italy
e-mail: patrizia.restani@unimi.it

L. Bolognini · F. Pavanello · R. Danzi
Unione Italiana Vini, via San Vittore al Teatro 3, 20123 Milan, Italy

C. Tarantino · F. Gombac · E. Bastiani
Euroclone SpA, via Figino 20/22, 20016 Pero, Milan, Italy

which can have dangerous clinical consequences. Though quite rare in adults, it does occur (Asero et al. 2009a, b).

The aim of Directive 2000/13/EC (Directive 2000/13/EC of the European Parliament and of the Council) was to harmonize the laws of EU Member States relating to labeling, presentation, and advertising of foodstuffs; the rules for allergen labeling were detailed in Directives 2003/89/EC (Directive 2003/89/EC of the European Parliament and of the Council) and 2006/142/EC (Commission Directive 2006/142/EC). Annex IIIa to Directive 2003/89/EC established a list of food ingredients (later including lupines and mollusks) likely to cause adverse reactions in susceptible individuals.

Directive 2005/26/EC (Commission Directive 2005/26/EC) established a list of food ingredients provisionally excluded from labeling until 25 November 2007 (extended with the Commission Directive, 2007/68/EC (Commission Directive 2007/68/EC) to July 2012). The exemption included agents used in wine production in order to allow clinical and experimental evaluation of the risk of adverse reactions in allergic subjects after consumption of treated wine. The Commission Implementing Regulation (EU) no. 579/2012 (Commission Implementing Regulation (EU) no. 579/2012) amended the previous regulations, establishing that its entry into force (as regards labeling procedures concerning milk and egg derivatives) was postponed to wine completely or partially made from grapes harvested in 2012 or later and labeled after 30 June 2012.

According to the new regulation, wine labeling exemption can be maintained only if (1) egg and milk derivatives are not used and cross-contamination is under control; and (2) wine clarified with such products is negative for the presence of residues using techniques with detection and quantification limits of 0.25 and 0.5 ppm, respectively. Analytical requirements were defined in the OIV resolution 427–2010 (OIV 2010) modified by OIV/COMEX 502–2012 (OIV 2012).

The aim of this study was the validation of a commercial ELISA kit designed to detect allergenic residues in wine fined with egg white proteins. To this purpose, an interlaboratory collaborative trial was organized according to the OIV Compendium of International Methods of Analysis MA-AS1-09:R2000 (Compendium of International Methods of Analysis of Wines and Musts - OIV 2013a), and the results were evaluated taking into consideration the analytical criteria listed by OIV resolution 427–2010 modified by 502–2012 (OIV 2010, 2012).

In the meantime, the performance of the commercial caseinate ELISA kit for white wine, previously validated (Restani et al. 2012), was rechecked according to the new limit of detection (LOD) and limit of quantification (LOQ) values, recommended by OIV in 2012 (OIV 2012).

Materials and Methods

ELISA Test

Commercial "sandwich" ELISA kit for detection of egg white in wine (Euroclone SpA) was used. The antibody, developed in rabbit versus commercial enological ovalbumin (egg white), was checked for its specificity. The immunoreactivity evaluated by immunoblotting technique showed that the antibody was capable to recognize all proteins present in the electrophoretic pattern of different fining agents (Restani et al. 2009).

Samples Included in the Study

Twelve blind red wine samples were prepared at the Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, and sent to the involved laboratories. Three different types of red wine were used:

- Samples 1–4: Quality wine (DOC-Geographical Origin Controlled) "RUPICOLO Castel del Monte"
- Samples 5–8: Quality wine (DOC) "Castelli Romani Rosso"
- Samples 9–12: Table wine "FRIULI"

The physicochemical characteristics of these types of wine are listed in Table 1. The wines were chosen to verify the performance of the test, when applied to samples having different alcoholic strenght, dry extract, and pH.

Twelve samples were prepared spiking wine with enological egg albumin to obtain a final concentration ranging from 0 to 7 ppm (Table 2):

Samples were thoroughly mixed to ensure a good dispersion of the fining agent and stabilized with gelatine at very low concentration (<0.001 %) to avoid any precipitation. Samples were identified by a serial number (blind samples) and sent to each interlaboratory test participant. Samples were stored and shipped at 4 °C.

Interlaboratory Trial Organization

The 11 laboratories included in this trial and listed below were experienced in ELISA analysis. A lab-code number was assigned to each laboratory.

- Laboratorio Chimico Camera Commercio Torino, I-10127 Torino
- Università di Genova; Di.C.T.F.A. - Facoltà di Farmacia, I-16147-Genova
- Università degli Studi di Milano, Facoltà di Farmacia, Unit of Food Chemistry, I- 20133 Milano
- Dal Cin Gildo SpA, I-20049 Concorezzo (MB)
- IASMA - Istituto Agrario San Michele all'Adige, I-38010 S. Michele all'Adige (TN)

Table 1 Physicochemical characteristics of red wines included in the interlaboratory trial

Characteristic	Rupicolo	Castelli Romani	Friuli
Alcoholic strength by volume (mL/100 mL)	12.95	14.17	9.27
Total alcoholic strength (mL/100 mL)	13.12	14.23	9.32
Reducing sugars (g/L)	2.9	1.0	0.8
Density	0.99427	0.99392	0.99833
pH	3.32	3.48	3.76
Total dry extract (g/L)	28.9	30.3	26.3
Reduced extract (g/L)	27.0	30.3	26.3
Total acidity (g/L)	5.5	5.7	5.2
Volatile acidity - SO_2 (g/L)	0.41	0.40	0.28
Ash (g/L)	2.34	3.11	3.41

Measured according to the Compendium of International Methods of Analysis of Wines and Musts - OIV (2013). Available at: http://www.oiv.int/oiv/info/enmethodesinternationalesvin

- ISPA- CNR, I-70126 Bari
- Università degli Studi di Milano, Facoltà di Farmacia – Unit of Toxicology, I- 20133 Milano
- ISS-Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare, I-00161 Roma
- Unione Italiana Vini soc. Coop., I-53100 - Siena
- Unione Italiana Vini soc. Coop., I- 37135 – Verona
- Veneto Agricoltura - Istituto per la Qualità e le Tecnologie Agroalimentari, I-36016 Thiene (VI)

Analysis

The parameters used for validating the ELISA method are described below.

Repeatability limit (r) is the value below which the absolute difference between two single test results, obtained using the same method on identical test material, under repeatability conditions (same sample, same operator, same apparatus, same laboratory, and a short interval of time) may be expected to lie within a specified probability (typically 95 %) and hence $r = 2.8 \times S_r$.

S_r is the standard deviation, calculated from results generated under repeatability conditions.

The *relative standard deviation* (RSD_r) is calculated from results generated under repeatability conditions, according to the equation: $S_r/\overline{x}$, where $\overline{x}$ is the mean of results over all laboratories and samples.

Reproducibility limit (R) is the value below which the absolute difference between two single test results, obtained under reproducibility conditions (on identical material, obtained by operators in different laboratories, using the standardized test method) may be expected to lie within a certain probability (typically 95 %); $R = 2.8 * S_R$. S_R is the standard deviation, calculated from results under reproducibility conditions.

The *relative standard deviation* (RSD_R) is calculated from results generated under reproducibility conditions according to the equation:

$$S_R/\overline{x} \times 100.$$

HORRAT (Ho_R) *value* is calculated by the ratio (AOAC 2012a):

$$Ho_R = \mathrm{RSD}_R/\mathrm{PRSD}_R$$

where:

PRSD_R Reproducibility relative standard deviation calculated from the Horwitz formula:

$$\mathrm{PRSD}_R = 2C^{-0.15}$$

Table 2 Blind samples included in the interlaboratory trial

Sample ID	Wine	Added egg white product (ppm)
1	Rupicolo Castel del Monte DOC	7
2	Rupicolo Castel del Monte DOC	3.5
3	Rupicolo Castel del Monte DOC	0
4	Rupicolo Castel del Monte DOC	1.2
5	Castelli Romani Rosso DOC	6.5
6	Castelli Romani Rosso DOC	3.5
7	Castelli Romani Rosso DOC	0
8	Castelli Romani Rosso DOC	1.5
9	Table wine "FRIULI"	4.5
10	Table wine "FRIULI"	1.5
11	Table wine "FRIULI"	7
12	Table wine "FRIULI"	0

where C is the concentration found or added, expressed as a mass fraction.

Trueness (T) is the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. It was calculated as follow (OIV 2010):

$$\left|\bar{x}-m\right| < 1,96*\sqrt{S_R(\text{lab})^2-S_r(\text{lab})^2\text{x}(1-1/n)}$$

where

- m is the certified value of the wine reference material and $\bar{x}$ is the average of n measurements of compound content in this wine, within the same laboratory.
- Sr (lab) are standard deviations, calculated from results within the same laboratory under repeatability conditions.
- SR (lab) are standard deviations, calculated from results within different laboratories under reproducibility conditions.

Recovery is the fraction or percentage of the analyte that is analytical determined when the test sample is conducted through the entire method (AOAC 2012b).

General Principles

The test was performed under the following conditions:

1. The protocol was clear and precise.
2. The samples used in the trials were taken from homogeneous batches of material.
3. The levels of the analyte to be determined covered the hypothetical concentrations of residues.
4. All laboratories taking part have a good experience in the technique employed.
5. For each participant, all analyses were performed within the same laboratory by the same analyst.
6. The method was well described and had to be followed as strictly as possible. Any change from the method described had to be documented.
7. The experimental values were determined under strictly identical conditions, which were described in the protocol given to all participants.
8. The experimental values were determined independently of each other and immediately one after the other.
9. The results were expressed by all laboratories in the same units (milligrams per liter), to the same number of decimal figures.

The ELISA kit was sent to each laboratory with the blind samples and the detailed protocol.

The protocol described in detail:

1. Samples and kit storage
2. Protocol for the ELISA reader check
3. Sample preparation
4. ELISA test protocol
5. Interpretation of results (a specific excel file was prepared and sent by e-mail)

Specific critical points were highlighted in the protocol in order to avoid any possible mistake during the analyses.

The *protocol of ELISA test* is summarized below:

1. All blinded samples (1–12), negative and positive controls must be diluted (1:10, *v*/*v*) with the buffer provided with the kit.
2. Egg white standards are supplied ready to use and must be directly loaded in the microplate to prepare a standard curve ranging from 0.061 to 2 ppm.
3. The blinded samples (1–12), the negative and positive controls, and different standard solutions must be loaded in duplicate into the microplate and incubated for 20 min at room temperature. All samples must be prepared twice and analyzed as two different samples (1a and 1b; 2a and 2b, etc.) (Table 3).
4. After a washing step, the specific antibody HRP conjugate must be added and allowed producing the suitable binding with an incubation of 20 min at room temperature.
5. The last incubation (25 min, at room temperature) with the chromogen substrate must be done after an additional washing step. The stop solution must be added and absorbances read at 450 nm with a calibrated ELISA reader.
6. Raw data (OD) must be reported in the excel file sent to all laboratories, which produced an automatic quantification.
7. All elaborated sheets must be sent to the interlaboratory trial coordinator (Università degli Studi di Milano), which collaborated with Unione Italiana Vini for the statistical elaboration.

Statistical Analysis

Statistical analysis was performed according to the norm UNI ISO 5725–2:2004 and to the OIV "Compendium of international methods of analysis" MA-AS1-10 (Compendium of International Methods of Analysis of Wines and Musts - OIV 2013b). In particular,

- Outlier data were assessed by Cochran test in order to compare variances among laboratories,
- Grubbs test was performed in order to evaluate variances inside a laboratory,
- Statistical analysis was performed to calculate repeatability and reproducibility data and all parameters required by the resolution OIV 427–2010/rev 2012 (OIV 2010, 2012).

Table 3 Loading distribution of samples into ELISA microplate (protocol supplied to all participants)

	1	2	3	4	5	6	7	8	9
A	Blank	Blank	Neg CRL	Neg CTL	7a	7	3b	3b	11b
B	STD 0	STD 0	Pos CRL	Pos CTL	8a	8a	4b	4b	11b
C	STD 0.0625	STD 0.0625	1a	1a	9a	9a	5b	5b	12b
D	STD 0.125	STD 0.125	2a	2a	10a	10a	6b	6b	12b
E	STD 0.25	STD 0.25	3a	3a	11a	11a	7b	7b	
F	STD 0.5	STD 0.5	4a	4a	12a	12a	8b	8b	
G	STD 1	STD 1	5a	5a	1b	1b	9b	9b	
H	STD 2	STD 2	6a	6a	2b	2b	10b	10b	

Results

Interlaboratory Trial to Validate the Egg White Protein ELISA Kit

Reproducibility, Repeatability, Horrat, Recovery, and Trueness

The assessment of the reproducibility and repeatability with Horrat and trueness values is summarized in Table 4.

All samples had Horrat value below or equal to 2, according to the OIV Resolution 427–2010 (OIV 2010) modified by OIV/COMEX 502–2012 (OIV 2012). Samples 3, 7, and 12 are not included in Table 4, since they were below the calculated LOQ (see "Detection and Quantification Limits"). For all samples, the trueness condition was satisfied. The mean recovery value 93.48 % obtained from the collaborative study is in accordance with the OIV resolution mentioned above.

Detection and Quantification Limits

To determine the detection limit (LOD) and the quantification limit (LOQ), the protocol described in the OIV-MA-AS1-10 (Compendium of International Methods of Analysis of Wines and Musts - OIV 2013b) was applied. The limits were calculated by the procedure "Determination on blank" using the data from all laboratories (Table 5).

Results from interlaboratory collaborative study showed that the commercial egg white ELISA kit for red wine is in agreement with the resolution OIV–Comex 502–2012 (OIV 2012).

Reevaluation of the Caseinate ELISA Kit

The validation of egg white protein ELISA kit allowed the authors to recheck the compliance of the caseinate kit included in the former interlaboratory trial (Restani et al. 2012). This was necessary, after the update of OIV 427–2010 resolution in 2012, where new and lower LOD and LOQ were approved.

Table 4 Reproducibility, repeatability, Horrat, trueness, and recovery obtained by the interlaboratory study on red wines

Sample	Spiked values (mg/mL)	Mean (mg/mL)	Total data	Valid data	*R*	RSD(*R*)	PRSD(*R*)	HoR	*r*	*Sr*	Trueness[a]	REC (%)
1	7.0	6.919	11	10	3.943	1.3942	20.1491	<**2**	2.450	0.8662	0.081<2.455	98.84
2	3.5	3.558	11	10	2.641	0.9337	26.2401	**2**	1.944	0.6874	0.058<1.562	101.66
4	1.2	1.041	11	10	0.902	0.3188	30.6277	<**2**	0.711	0.2513	0.159<0.519	86.75
5	6.5	5.959	11	10	4.329	1.5306	25.6870	**2**	2.327	0.8229	0.541<2.775	91.67
6	3.5	3.474	11	10	2.906	1.0275	29.5786	**2**	1.448	0.5120	0.026<1.885	99.26
8	1.5	1.286	11	10	1.198	0.4235	32.9279	**2**	1.002	0.3544	0.214<0.669	85.73
9	4.5	4.240	11	10	3.728	1.3180	31.0877	**2**	1.873	0.6621	0.260<2.415	94.22
10	1.5	1.368	11	10	1.195	0.4226	30.8852	**2**	0.892	0.3153	0.132<0.704	91.20
11	7.0	6.437	11	10	4.463	1.5778	24.5135	**2**	3.009	1.0639	0.563<2.718	91.96
											Mean	93.48%

Total data number of test results used for calculation, *Valid data* number of test results used for calculation after elimination of outliers, *R* Reproducibility limit, *RSD*(*R*) Reproducibility relative standard deviation, *PRSD*(*R*) Reproducibility relative standard deviation, *HoR* Horrat value for reproducibility, *r* repeatability limit, *Sr* estimate standard deviation of repeatability, *REC* (%) recovery (percentage)

[a] See "Materials and Methods" for the equation used to calculate Trueness

Table 5 LOD and LOQ (mg/mL) of egg white protein ELISA kit

Mean	0.0130
SD	0.0145
LOD=mean+3SD	0.0564
LOQ=mean+10SD	0.1578

Mean Mean of all blank values from calibration curves (10 labs), *SD* Standard deviation, *LOD= m+ 3SD* Limit of detection, *LOQ= m+ 10SD* Limit of quantification

Detection and Quantification Limits

The LOD and the LOQ reported in the article by Restani et al. (2012) were calculated using data coming from all laboratories involved in the study (10 laboratories), and the results were in accordance with OIV- MA-AS1 MA-AS1-10 (Compendium of International Methods of Analysis of Wines and Musts - OIV 2013b) and the OIV resolution 427–2010 (OIV 2010). In March 2012, after that the article was accepted (30 June 2011), the OIV–Comex 502–2012 (OIV 2012) amended the previous LOD and LOQ values, lowering them to 0.25 and 0.5 ppm, respectively.

To revalidate the caseinate ELISA kit, the LOD and LOQ were recalculated, eliminating the outliers according to the Cochran test, in order to compare variances among laboratories. One laboratory was outlier and the relative data were eliminated. The new LOD and LOQ values (Table 6) were in agreement with the new OIV resolution, since LOD was≤0.25 ppm (0.1517 ppm) and LOQ≤0.5 ppm (0.3813 ppm).

Recovery

Recovery was calculated from spiked samples and the average, calculated on 10 laboratories, was 91.05 % in accordance with the OIV resolution (OIV 2010).

Trueness

In order to calculate the trueness, the repeatability was evaluated for samples 5 and 10 that are the duplicate of samples 4 and 9, respectively. Data are reported in Table 7, showing that the caseinate ELISA kit is in line with the OIV 427–2010 (OIV 2010) resolution modified by OIV–Comex 502–2012 (OIV 2012).

Table 6 LOD and LOQ (mg/mL) of caseinate ELISA kit

Mean	0.0533
SD	0.0328
LOD=mean+3SD	0.1517
LOQ=mean+10SD	0.3813

Mean Mean of all blank values from calibration curve (10 labs), *SD* Standard deviation, *LOD= m+ 3SD* Limit of Detection, *LOQ= m+ 10SD* Limit of Quantification

Table 7 Trueness of caseinate ELISA kit values

Sample	Spiked values (mg/mL)	Mean (mg/mL)	Total valid data	RSD (*R*)	PRSD (*R*)	Trueness
4 and 5	1.6	1.5672	10	0.4246	0.0293	0.0328<0.831
9 and 10	7	6.7035	10	0.9650	0.0361	0.2965<1.891

Samples refer to the previous paper by Restani et al. (2012)

Final Validation of Caseinate and Egg White Protein ELISA Kits

To verify the performance of the validated ELISA tests at LOD and LOQ concentrations, three samples of white and two samples of red wine were spiked and measured according to the kit instructions. Results are reported in Table 8. The ELISA method showed optimal recovery at both LOD and LOQ spiking levels.

Conclusions

The ELISA test is one of the most usual analytical methods applied for the detection of allergens in food. The specificity and sensitivity of immunological procedures is well known. Moreover, for the limited equipment required, ELISA test can be applied in most laboratories involved in quality control.

Table 8 Final validation of caseinate and egg white protein ELISA kits

ELISA Kit	Sample	Spiking level (ppm)	Measured concentration (ppm)	Recovery (%)
Caseinate	White wine	0.25	0.24	96
	Pinot bianco	0.50	0.47	94
Caseinate	White wine	0.25	0.30	120
	Bianco di Sicilia	0.50	0.47	94
Caseinate	White wine	0.25	0.22	88
	Garganega	0.50	0.43	86
Egg white proteins	Red wine Rubicolo Castel del Monte	0.25 0.50	0.27 0.47	108 94
Egg white proteins	Red wine Castelli Romani	0.25 0.50	0.25 0.47	100 94

The critical point in ELISA methods is the effect of any food composition (matrix) on allergen determination. This is the reason why a specific protocol must be developed for any food/beverage analyzed. This paper illustrates the data obtained applying an ELISA test to the detection of allergenic residues in wine clarified with milk caseinates and egg white proteins.

The assay is extremely handy (reagents are mostly ready to use), internally monitored by negative and positive "wine" controls; finally, it is relatively quick (1 h to analyze roughly 80 samples).

The collaborative interlaboratory studies showed that the ELISA kits used in this study had good reproducibility, repeatability, and robustness in detecting residues of allergenic fining agents in wine till the LOD level, according to the OIV resolution 427–2010 (OIV 2010) modified by OIV/COMEX 502–2012 (OIV 2012) requirements.

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Conflict of Interest Patrizia Restani declares that she has no conflict of interest. Francesca Uberti declares that she has no conflict of interest. Chiara Tarantino declares that she has no conflict of interest. Cinzia Ballabio declares that she has no conflict of interest. Francesca Gombac declares that she has no conflict of interest. Erica Bastiani declares that she has no conflict of interest. Laura Bolognini declares that she has no conflict of interest. Francesco Pavanello declares that he has no conflict of interest. Roberta Danzi declares that she has no conflict of interest. This article does not contain any studies with human or animal subjects.

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