

Collaborative Tests of ELISA Methods for the Determination of Egg White Protein and Caseins Used as Fining Agents in Red and White Wines

M. Lacorn · R. Ristow · T. Weiss · U. Immer

Received: 28 January 2013 / Accepted: 9 May 2013 / Published online: 28 May 2013
© Springer Science+Business Media New York 2013

Abstract Methods are needed for detecting allergenic proteins in wine after fining processes, both for legal requirements and to estimate any risk for allergic individuals. Two of the critical allergens in this case are the protein fraction from egg white and caseins. Requirements for methods were set up both by the International Organisation of Vine and Wine (OIV) and the European Commission and included collaborative tests to determine repeatability, reproducibility, limit of detection (LOD), and recovery. Two collaborative tests with 18 participants from all over the world were conducted using commercial enzyme-linked immunosorbent assay kits to quantify egg white protein and casein. For each analyte, nine red and white wines (RW and WW, respectively) with different analyte concentrations were prepared and sent to the participants. Resulting sets of data were analyzed according to the AOAC by eliminating outliers and calculating the performance statistics for each assay. For wines with no or very low analyte concentrations, the values were extrapolated from the calibration curve and the resulting LODs are in accordance with the performance criteria set by the OIV method OIV-MA-AS315-23: R2012. For casein in WW and egg white protein in RW, all Horwitz ratio values for samples with concentrations above the required limit of quantification (0.5 mg/L) were below 2. While the recovery of egg white protein from RW is 90 % or more, the recovery of casein from WW is between 73 and

76 %, which is attributable to the instability/solubility of casein in wine.

Keywords Collaborative tests · Casein · Egg white protein · ELISA · Wine · Allergens

Introduction

Fining processes during winemaking are used to clarify must and wine or to remove substances which otherwise would influence the flavor of the wine in a negative way. Traditionally, proteins from cow's milk and hen's egg have been used for "fine-tuning" of the flavor of wine due to their capacity for selective tannin adsorption. Since cow's milk and hen's egg proteins are barely soluble in wine, they coprecipitate with tannins and other absorbed substances within hours and can be easily removed partly by decantation and sometimes completely by filtration (Lacorn et al. 2011). Whereas milk and its constituents are typically used for white wine (WW) fining, egg is usually applied for red wine (RW). In case of egg, the whole raw egg white as a liquid or a powder (containing the allergenic proteins ovalbumin, ovomucoid, ovotransferrin, and lysozyme) could be used, whereas the allergenic caseins and whey proteins (consisting of mainly β -lactoglobulin and α -lactalbumin) are used in case of milk (Paschke-Kratzin, personal communication). A special case is the addition of pure lysozyme (isolated from egg white) which is added to terminate bacterial growth that otherwise would result in undesirable aroma compounds, e.g., ethylphenol, or uncontrolled malolactic fermentation (Lopez et al. 2009; Gerbaux et al. 1997; Delfini et al. 2004).

In recent years, reasonable suspicion has emerged that residues of proteins used for fining processes can remain in

M. Lacorn (✉) · T. Weiss · U. Immer
R-Biopharm AG, An der neuen Bergstr. 17,
64297, Darmstadt, Germany
e-mail: m.lacorn@r-biopharm.de

R. Ristow
Albert-Schweitzer-Str. 6a,
67346, Speyer, Germany

wine after filtration (Lacorn et al. 2011). This could elicit an allergic reaction in sensitized consumers. Allergies to milk and egg proteins are rare (Kanny et al. 2001; Savage et al. 2007; Lam et al. 2008) but severe in adults as shown for milk proteins (Lam et al. 2008) and egg (Hu et al. 2007). Minimum eliciting doses for egg proteins are as low as 0.13 mg (Taylor et al. 2002) and for milk proteins are 0.3 mg (Lam et al. 2008).

Rolland et al. (2006) described a double-blind placebo-controlled wine challenge using fined and non-fined wines. Unfortunately, the number of patients was very low (five patients with allergy to egg and only one patient with allergy to milk) and the few specific reactions to fined wines were not significant compared to reactions on control wines (non-fined wines). This is not exceptional because Rolland et al. (2008) reported recently that values of casein and egg proteins in fined wines using noncommercial enzyme-linked immunosorbent assays (ELISA) were below the limit of detection (LOD) of 1 µg ovalbumin/L (corresponds to approximately 1.85 µg egg white proteins/L) or of 8 µg casein/L (corresponds to approximately 10 µg milk proteins/L).

Nevertheless, according to the EU Directive 2007/68/EC (European Commission Directive 2007), all wines labeled after May 31, 2009 must declare if allergens like egg and milk were used during production and are still detectable. An extension of the term until June 30, 2012 was decided by the Standing Committee on the Food Chain and Animal Health of the European Union. It is decided now that the wine with the beginning of the grape harvest of 2012 has to be tested for fining agents and that it has to be declared if residues are found.

In summer 2012, the International Organisation of Vine and Wine (OIV) set up requirements (mainly LOD, limit of quantification [LOQ], and recovery) for antibody-based detection methods in their resolutions OIV-MA-AS315-23: R2010 (2010) and OIV-COMEX 502-2012 (2012). In contrast to the EU regulation, which mention “egg” and “milk,” these resolutions specify the analytes. Unfortunately, they did not name egg white proteins but ovalbumin, which is misleading because commercial fining products contain a dry egg white powder and the regulation does not reflect the real measurand used during production. In the meantime, a revision of OIV-MA-AS315-23: R2012 (2012) was published in 2012 by the OIV. The criteria defined in this publication are valid for residues of “fining agents” which are in reference to caseins and egg white protein in this publication. Therefore, the confusion from the first version of this resolution was somewhat corrected. This is the first case that special minimum requirements for antibody-based methods and fractionated allergens (e.g., casein from milk; egg white protein and lysozyme from egg) were described by regulations. The LOD and LOQ should be 0.25 and 0.5 mg/kg, respectively. It was the aim of this study to conduct

international collaborative tests for ELISA-based methods that detect caseins and egg white proteins in RW and WW.

Materials and Methods

Samples and Sample Preparation

Caseins and egg white proteins are barely soluble in wine. They denature and precipitate after a short time even if they were partly solved after application as a fining agent. Therefore, special attention is necessary to produce samples for a collaborative study and to maintain their quality during transport and until measurement by the participants.

Wine samples that went through a whole fining process will never cover the concentration range that was needed for the collaborative tests. The values are unpredictable due to the facts mentioned previously. Therefore, the organizers decided to spike the wine samples, which is much more defined and under more control. As spiking materials, a highly purified casein (C5890; Sigma-Aldrich, St. Louis, MO, USA), a defined egg white powder (protein content, 78.3 %; Colman's, Norwich, UK), and two commercially available fining agents were used (AlbuVin and KalCasin; Erbslöh, Geisenheim, Germany). A casein stock solution was prepared by weighing 10 mg of casein and solving this amount with 10 ml of a PBS–Tween buffer containing 6 M urea. An egg white protein stock solution was prepared by weighing 10 mg of egg white protein and solving this amount with 10 ml of a 0.05-M Tris–HCl buffer, pH 8.5. The commercial products AlbuVin and KalCasin were treated like egg white protein or casein, respectively.

Defined volumes of the stock solution were pipetted under slow stirring into 250 ml of wine. The wines were stirred for 6 h and allowed to settle down overnight at room temperature. A portion of 3 ml was bottled the next day into 5-ml vials. The wine was controlled over a period of 3 days by ELISA; afterwards, homogeneity was tested 10 times per sample. This was done by taking 10 times 1-ml aliquots from the bulk sample and analyzing them by ELISA. The coefficients of variation were 15% or less and passed the fitness-for-purpose criteria which were derived from in-house validation data.

Due to the limited stability of the spike (data not shown), the organizers of this collaborative study decided to use assigned values for each sample which were derived from the data of homogeneity testing. Another measure to maintain the quality of the samples was the short time window between preparation and sending of the samples and the measurement of the samples by the participants (see the succeeding sections).

For casein, nine wines were prepared with assigned casein concentrations up to 10.5 and 7.2 mg/L for RW and

WW, respectively (see Tables 1 and 2). For egg white protein, nine wines were prepared with assigned egg concentrations up to 7.6 and 7.5 mg/L for RW and WW, respectively (see Tables 3 and 4). It should be noted that the results in Tables 3 and 4 are expressed as whole egg powder because the assay is calibrated to a whole egg powder from National Institute of Standards and Technology (NIST) (reference material [RM] 8445), recommended for ELISA calibration. Since casein is typically used for WW and egg proteins for RW, the main focus of the spiking was set accordingly.

For spiking of casein, a white Mosel wine (Germany) and a red Barbera wine (Italy) were used. For egg white protein, a white Cerra de la Cruz wine (Spain) and a red Bordeaux wine (France) were used. The organizers decided to keep the commercial wines used for spiking anonymous for the most part. The wines were controlled to be free of casein and free of egg before spiking.

Sample Presentation to Laboratories

All samples were sent in duplicate with a randomized number to the participants. Besides the number, they were labeled with the laboratory code (A to R) and a “C” for a sample to be measured in the casein assay or an “E” for a sample to be measured in the egg assay.

In order to check the preparation and transport of the samples, requirements, paper works, and open questions, a pre-collaborative study with four laboratories from Europe preceded the full collaborative study. After receiving encouraging results out of this pre-study, only minor changes of the study design were undertaken, and the full collaborative study went on as scheduled. Since wines spiked with casein or egg white proteins have a limited stability of the spiked proteins, the participants were advised to strictly follow the time schedule which set a maximum of 14 days between sample shipment and sample measurement.

Together with the samples, the participants obtained the letter from the organizer of the collaborative study containing the general outline of the study as well as general comments, a detailed analytical protocol, one data return sheet each for the casein and egg measurement, one CD with the RIDA®SOFT Win software, two kits RIDASCREEN®FAST Casein R4612, and two kits RIDASCREEN®FAST Ei/Egg R6402.

Participating Laboratories

As a prerequisite, all laboratories needed to be familiar with immunological tests. Eighteen laboratories designated from A to R participated. The following gives the alphabetic order:

1. ABC-Labor GmbH, Mülheim, Germany
2. Araldica Vini Piemontesi, Castel Boglione, Italy
3. Centro Nacional de Alimentacion (AESAN), Madrid, Spain
4. Chemisches und Veterinäruntersuchungsamt Karlsruhe, Karlsruhe, Germany
5. Ciati AC, Rio Negro, Argentina
6. Estacion Enologica de Haro, Haro-La Rioja, Spain
7. Eurofins Analytics France, Nantes, France
8. Institut Heidger, Kesten, Germany
9. ISVEA, Poggibonsi, Italy
10. Laboratorio Arbitral Agroalimentario, Madrid, Spain
11. LHL, Kassel, Germany
12. Landesuntersuchungsamt, Institut für Lebensmittelchemie, Trier, Germany
13. R-Biopharm AG, Darmstadt, Germany
14. SGS Institut Fresenius, Taunusstein, Germany
15. Staatliches Weinbauinstitut Freiburg, Freiburg, Germany
16. Staatl. Lehr- und Versuchsanstalt für Wein- und Obstbau, Weinsberg, Germany
17. Universität Hamburg, Institut für Lebensmittelchemie, Hamburg, Germany

Table 1 Performance statistics for the RIDASCREEN®FAST Casein R4612 in RW

Sample	RW-C0	RW-C1	RW-C2	RW-C3
Total number of accepted laboratories (<i>p</i>)	13	17	18	17
Total number of outlying laboratories	5	1	0	0
Total number of replicates (<i>n</i>)	26	33	36	34
Grand mean [mg/L]	−0.03	1.02	5.63	8.46
Repeatability standard deviation (<i>s_r</i>)	0.05	0.12	0.83	2.03
Reproducibility standard deviation (<i>s_R</i>)	0.08	0.20	1.40	3.01
Repeatability relative standard deviation (RSD _r %)		11.7	14.7	24.0
Reproducibility relative standard deviation (RSD _R %)		19.4	24.8	35.6
HORRAT value		1.20	2.00	3.10
Assigned value [mg/L]	0	1.1	6.8	10.5
Recovery [%]		93.2	82.8	80.6

Table 2 Performance statistics for the RIDASCREEN®FAST Casein R4612 in WW

Sample	WW-C0	WW-C1	WW-C2	WW-C3	WW-C4
Total number of accepted laboratories (<i>p</i>)	13	13	18	13	15
Total number of outlying laboratories	5	5	0	4	3
Total number of replicates (<i>n</i>)	26	26	35	26	29
Grand mean [mg/L]	−0.03	0.12	0.80	5.48	4.05
Repeatability standard deviation (<i>s_r</i>)	0.05	0.04	0.07	0.75	0.44
Reproducibility standard deviation (<i>s_R</i>)	0.09	0.07	0.11	1.00	0.68
Repeatability relative standard deviation (RSD _r %)		35.0	9.1	13.6	10.8
Reproducibility relative standard deviation (RSD _R %)		53.8	13.7	18.3	16.7
HORRAT value		2.46	0.85	1.50	1.30
Assigned value [mg/L]	0	0.37	1.1	7.2	5.3
Recovery [%]		32.4	72.7	76.1	76.4

18. Zentrallabor Witowski GbR, Alzey, Germany

ELISA Kits: Assay Protocol and Calculation Software

R-Biopharm AG provided the sandwich ELISA kits for the quantification of egg (RIDASCREEN®FAST Ei/Egg R6402) and caseins (RIDASCREEN®FAST Casein R4612) in wine for the study.

One milliliter of each wine was mixed with 19 ml extraction buffer (pre-warmed to 60 °C) and incubated at 60 °C for 10 min. After cooling down to room temperature, the extracts were centrifuged (10 min, 2,500×*g*) or filtered. One hundred microliters of each extract supernatant or calibrator was pipetted in duplicates into wells of the antibody-coated microtiter plate and incubated for 10 min at room temperature. After this time, the liquid was poured out of the wells and the microtiter plate was tapped upside down vigorously on an absorbent paper to ensure complete removal of liquid from the wells. Each well was filled with 250 μl washing buffer, emptied again, and tapped on absorbent paper. This

procedure was repeated twice, followed by pipetting 100 μl of the finally diluted conjugate solution. The plate was incubated for 10 min at room temperature and washed again as described before. One hundred microliters of substrate/chromogen solution was added to each well and incubated for 10 min at room temperature in the dark followed by 100 μl of stop solution to each well. The absorbance (optical density [OD]) was read at 450 nm against air. All OD values had to be recorded in a ready-to-use Excel form sheet.

Together with the test kits, a software (RIDA®SOFT Win Z9999) was provided to construct calibration curves (cubic spline curve fitting) and to calculate concentrations from the measured ODs of calibrators.

A cubic spline is a spline function constructed of piecewise third-order polynomials which pass through a set of *m* control points. The second derivative of each polynomial is commonly set to zero at the end points, since this provides a boundary condition that completes the system of *m*−2 equations. This produces a so-called natural cubic spline and leads to a simple tridiagonal system which can be solved

Table 3 Performance statistics for the RIDASCREEN®FAST Ei/Egg R6402 in RW

Sample	RW-E0	RW-E1	RW-E2	RW-E3	RW-E4
Total number of accepted laboratories (<i>p</i>)	15	16	16	16	16
Total number of outlying laboratories	3	2	2	2	2
Total number of replicates (<i>n</i>)	30	32	32	32	32
Grand mean [mg/L]	0.11	0.26	1.06	5.25	7.64
Repeatability standard deviation (<i>s_r</i>)	0.07	0.15	0.13	0.54	0.94
Reproducibility standard deviation (<i>s_R</i>)	0.07	0.18	0.23	0.94	1.01
Repeatability relative standard deviation (RSD _r %)		55.5	12.3	10.3	12.3
Reproducibility relative standard deviation (RSD _R %)		67.5	21.3	18.0	13.2
HORRAT value		3.45	1.34	1.44	1.12
Assigned value [mg/L]	0	0.33	1.13	5.5	7.6
Recovery [%]		78.8	94.2	95.5	100.5

Table 4 Performance statistics for the RIDASCREEN®FAST Ei/Egg R6402 in WW

Sample	WW-E0	WW-E1	WW-E2	WW-E3
Total number of accepted laboratories (<i>p</i>)	13	18	18	18
Total number of outlying laboratories	5	0	0	0
Total number of replicates (<i>n</i>)	26	36	36	36
Grand mean [mg/L]	0.11	0.59	3.59	6.45
Repeatability standard deviation (<i>s_r</i>)	0.03	0.22	0.40	1.12
Reproducibility standard deviation (<i>s_R</i>)	0.08	0.3	0.62	1.43
Repeatability relative standard deviation (RSD _r %)		37.4	11.1	17.3
Reproducibility relative standard deviation (RSD _R %)		52.1	17.2	22.1
HORRAT value		3.01	1.30	1.83
Assigned value [mg/L]	0	0.82	5.1	7.5
Recovery [%]		71.4	70.4	86.0

easily to give the coefficients of the polynomials (Weisstein 2012). The third derivative is used as a smoothing factor to determine the extent of interpolation. Lower factors lead to more approximations, higher ones (>100) lead to more interpolations of the curve function. RIDA®SOFT Win uses a factor of 10,000. To minimize boundary effects and to allow extrapolation, two more control points are added to the set of control points as starting and end points where the first one is near zero and set to $x(0)=0.001$ and $y(0)=OD(STD1)$ and the virtual end point is determined by calculating the linear regression of the other control points by assuming that $x(n)$ has the same distance to $x(n-1)$ as $x(1)$ has to $x(0)$.

Thus, the cubic spline curve is very suitable for the calculation of concentrations within the calibration range; however, the extrapolation of the cubic spline curve beyond the calibrator containing the lowest measurand concentration is mathematically problematic for concentrations close to zero and impossible for concentrations at zero or below zero. Therefore, in this study, a third-order polynomial curve fitting was used during preparation of the descriptive statistical analysis to calculate the blank samples and samples with concentrations below the lowest measurand-containing calibrator (calibrator 2; also marking the LOQ for both assays).

The test kit for the determination of egg is calibrated to a whole egg powder (NIST RM 8445), so that a conversion factor of 0.26 is needed to calculate egg white protein. The casein ELISA expresses the result as milligrams casein per liter. The final data from the laboratories were sent to Dr. Reinhard Ristow, the organizer of the collaborative study.

Overall Results from the Collaborative Study

Eighteen laboratories provided full sets of data for both assays. However, due to the inconsistent calibration curve for casein run 1 from laboratory E (Fig. 1), only casein run 2 could be used and some values are missing

in Table 6. Similarly, the calibration curve for casein run 2 from laboratory R was inconsistent (Fig. 1), but in this case, only calibrator 1 was affected and it was decided to add the resulting data to the set of raw data (Table 6). All sets were obtained using the manufacturer's software, in which the calibration was carried out using a cubic spline function for samples at or above the LOQ stated by the manufacturer. To calculate values for the blank samples (RW-C0, WW-C0, RW-E0, and WW-E0) and the samples with very low concentrations (WW-C1 and RW-E1), a third-order polynomial function was used as an alternative curve fitting (the cubic spline curve fitting is not able to calculate values near or below zero).

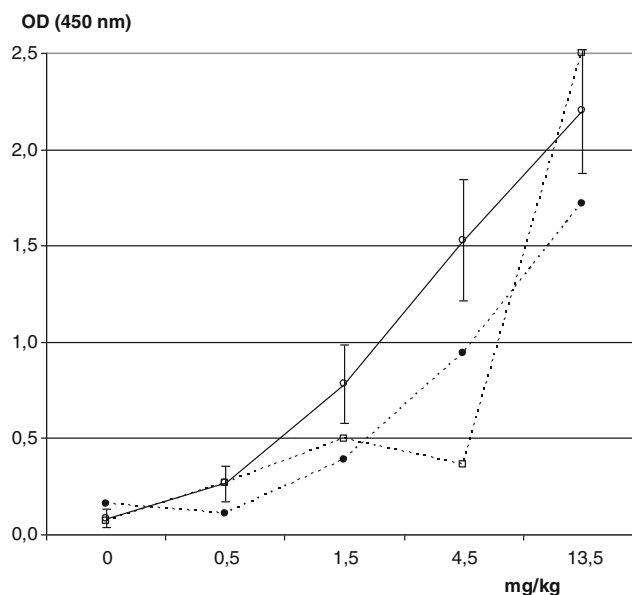


Fig. 1 Mean casein calibration values (with standard deviations) of all laboratories, except laboratory E run 1 and laboratory R run 2 (open circles); calibration values of laboratory E run 1 (open squares) and laboratory R run 2 (closed circles) are given for comparison; no curve fitting in any case

Table 5 Whole egg powder concentrations determined by all participating laboratories using the RIDASCREEN®FAST Ei/Egg R6402

Laboratory	RW-E0	RW-E1	RW-E2	RW-E3	RW-E4	WW-E0	WW-E1	WW-E2	WW-E3								
A	0.14	0.08	0.30	1.2	0.88	6.4	5.3	8.0	7.0	0.06	0.08	0.40	0.51	3.6	4.0	5.6	6.6
B	0.32	0.10	0.29	0.93	0.96	4.8	3.6	9.0	8.9	0.27	0.33	0.34	0.64	3.4	3.5	4.3	5.7
C	0.17	0.14	0.30	0.33	1.1	5.8	5.3	7.3	7.5	0.10	0.05	0.61	0.57	3.6	4.1	6.9	6.7
D	0.09	0.14	0.29	0.35	1.6	5.6	5.5	7.5	7.5	0.13	0.10	0.61	0.55	3.7	3.7	6.7	6.3
E	0.16	0.06	0.24	0.18	1.0	6.2	5.1	7.5	7.8	0.00	−0.01	0.60	1.1	3.9	3.4	6.7	5.3
F	0.24	0.10	0.30	0.34	1.4	5.7	6.7	9.3	6.9	−0.02 c	2.1 c	1.0	0.69	3.9	4.0	7.6	8.0
G	0.22	0.22	0.28	0.35	0.96	5.2	5.4	6.8	8.3	0.22	0.21	1.0	0.89	4.1	4.1	6.1	9.6
H	0.11	0.04	0.31	0.14	1.0	4.8	4.9	7.1	6.3	0.09	0.04	0.60	0.55	3.5	2.9	5.6	6.3
I	0.06	−0.01	0.28	−0.18	0.64	4.7	5.5	9.8 g	10.8 g	0.04 c	−0.24 c	0.36	0.41	3.7	2.7	5.0	3.7
J	0.03	0.09	0.34	0.32	1.1	4.4	5.0	8.2	5.7	0.01 c	3.2 c	0.54	0.57	3.6	4.2	6.7	6.8
K	1.12 g	1.19 g	1.14 g	1.2	1.0	4.0	4.1	5.9	6.9	1.2 g	1.1 g	1.1	1.4	2.0	3.1	3.9	7.2
L	1.04 c	0.31 c	0.41	0.63	1.2	8.2 c	3.6 c	5.9	7.6	0.18	0.11	0.14	0.97	3.0	2.0	8.2	7.9
M	0.10	0.12	0.10	0.11	1.1 c	5.6 c	2.2 c	7.6	7.2	0.12	0.10	0.35	0.10	3.4	3.1	7.8	8.2
N	0.00	0.17	0.35	0.21	0.99	1.1	7.5	9.7	7.7	0.15	0.10	0.52	0.56	4.0	4.5	7.1	7.9
O	0.09	0.13	0.35 c	−0.59 c	0.96	5.1	5.2	8.5	6.6	0.09	0.16	0.60	0.18	4.6	4.1	5.4	5.6
P	0.06	0.07	0.25	0.24	1.3	6.2	5.3	8.0	8.6	0.05	0.06	0.80	0.35	3.1	3.0	7.4	6.9
Q	0.14	0.00	0.25	0.32	1.0	5.9	5.8	8.6	8.9	0.05	0.08	0.55	0.64	4.3	4.2	6.9	7.1
R	0.77 c	−0.13 c	0.16	−0.28	−0.04 c	3.1	3.8	9.1 g	12.9 g	0.21 c	−0.31 c	0.01	0.24	3.3	4.1	6.2	2.5

The calculation of the values of the samples RW-E0, RW-E1, and WW-E0 was done on the basis of a third-order polynomial function, while the other values were obtained by using the cubic spline function of the RIDA®SOFT Win software. Italicized values were below the LOQ and, therefore, also calculated by the third-order polynomial function

c a Cochran outlier, g a Grubbs outlier

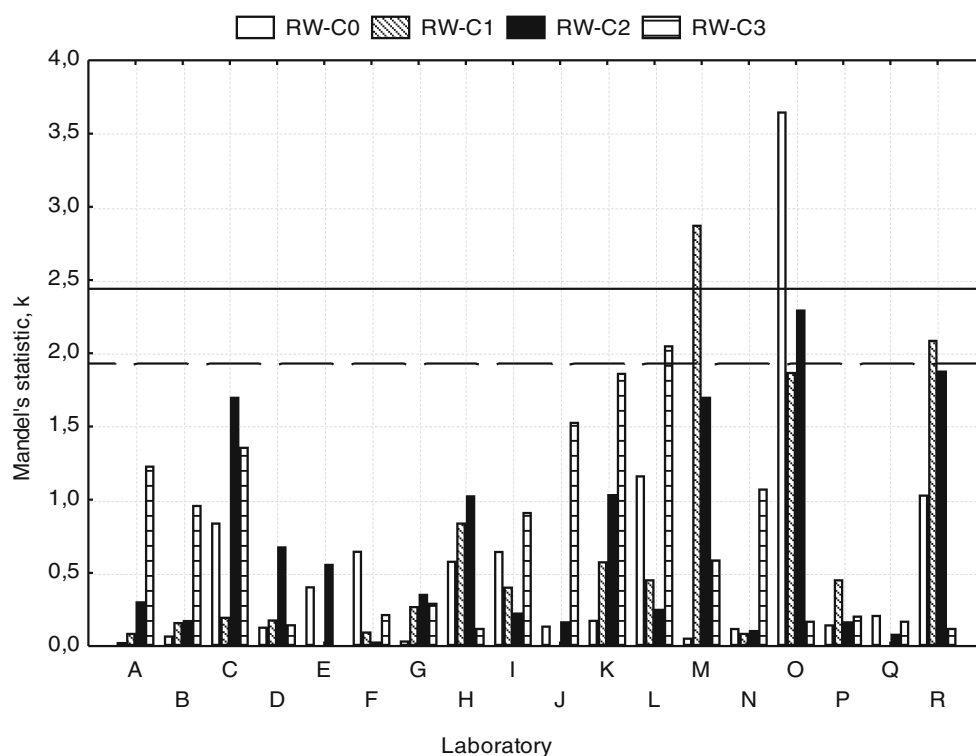
Table 6 Casein concentrations determined by all participating laboratories using the RIDASCREEN®FAST Casein R4612

Laboratory	RW-C0	RW-C1	RW-C2	RW-C3	WW-C0	WW-C1	WW-C2	WW-C3	WW-C4									
A	-0.01	-0.02	1.1	1.1	6.4	6.0	12.8	9.3	-0.03	-0.03	0.11	0.10	0.79	0.87	5.7	7.0	4.1	3.9
B	-0.07	-0.05	0.99	0.95	4.9	5.1	8.7	6.0	0.00	0.01	0.13	0.16	0.73	0.96	5.7	4.7	3.5	3.4
C	-0.05	0.12	1.0	0.98	8.6	6.6	10.9	6.9	0.01 c	0.67 c	0.20	0.17	0.83	0.92	4.8	5.9	4.1	5.7
D	-0.04	-0.07	1.1	1.0	4.9	5.7	7.4	7.9	-0.05	0.00	0.18	0.17	0.78	0.77	5.5	6.3	3.5	4.0
E	-0.08	0.00		1.1	7.4	6.8			-0.09	0.00	0.09	0.14	0.87			4.1		
F	-0.25 g	-0.38 g	0.95	0.98	5.9	5.9	8.2	8.8	-0.38 g	-0.40 g	-0.05	0.11	0.87	0.74	6.1	6.3	4.0	4.4
G	0.05	0.06	1.3	1.2	4.4	4.0	12.9	13.7	0.01	0.15	0.10	0.07	1.0	0.80	4.4	4.2	3.0 c	5.7 c
H	-0.05	-0.17	0.94	0.75	4.0	5.2	5.9	5.6	-0.06	-0.10	0.10	0.20	0.75	0.74	5.5	4.8	2.9	3.6
I	-0.15	-0.02	1.0	0.95	5.4	5.6	6.1	8.8	-0.03	-0.20	0.41 c	0.16 c	0.78	0.77	-0.03 c	6.0 c	3.3	2.9
J	0.03	0.00	1.2	1.2	6.6	6.4	13.7	9.3	0.06	0.01	0.35 g	0.44 g	0.94	0.83	8.3 g	8.9 g	5.1	5.0
K	0.43 g	0.39 g	0.51	0.63	4.4	3.2	5.9	0.51	4.3 c	2.9 c	4.3 c	0.45 c	0.64	0.52	3.7	6.4	2.1 g	0.89 g
L	0.75 g	0.99 g	1.2	1.1	5.3	5.6	5.6	11.5	0.85 g	0.82 g	0.92 c	1.42 c	0.75	0.67	4.1	5.4	4.3	3.9
M	0.07	0.08	0.94 c	0.31 c	5.9	3.9	9.5	7.8	0.06	0.10	0.11	0.13	0.67	0.65	4.6	4.4	3.7	3.8
N	0.00	0.02	1.1	1.1	6.4	6.6	6.0	9.1	0.02	0.00	0.05	0.03	0.75	0.74	6.2	6.2	4.8	4.4
O	0.01 c	0.75 c	1.1	0.69	7.5	4.8	8.1	8.6	1.5 c	-0.03 c	0.11	0.18	0.87	0.84	7.4	7.1	3.9	3.8
P	-0.17	-0.14	1.2	1.1	5.1	5.3	7.9	8.5	-0.15	-0.13	0.09	0.06	0.80	0.80	4.8	5.5	4.1	3.4
Q	0.02	-0.02	1.3	1.3	8.2	8.1	13.3	12.8	0.05	-0.03	0.25	0.23	1.0	1.0	9.7 g	9.8 g	5.8 g	6.0 g
R	-0.36 g	-0.15 g	1.1	0.6	2.3	4.5	5.1	4.7	-0.18	-0.19	0.08 c	0.44 c	0.72	0.85	4.3 c	12.8 c	5.3	4.4

The calculation of the values of the samples RW-C0, WW-C1, and WW-C0 was done on the basis of a third-order polynomial function, while the other values were obtained by using the cubic spline function of the RIDA®SOFT Win software. Italicized values were below the LOQ and, therefore, also calculated by the third-order polynomial function

c a Cochran outlier, g a Grubbs outlier

Fig. 2 Determination of casein in RW: Mandel's within-laboratory consistency statistic, k , grouped by laboratories. Dashed line $p<0.05$, solid line $p<0.01$



Resulting data for egg are given in Table 5, whereas values for casein are shown in Table 6. A few other samples were recalculated using the third-order polynomial because the values were below the LOQ. They were designated with an asterisk in Tables 5 and 6.

Statistical Analysis and Discussion

To evaluate the consistency of the data sets in a graphic chart, Mandel's h and k statistics are presented for casein (Figs. 2, 3, 4, and 5) and for egg (Figs. 6, 7, 8, and 9).

Fig. 3 Determination of casein in WW: Mandel's within-laboratory consistency statistic, k , grouped by laboratories. Dashed line $p<0.05$, solid line $p<0.01$

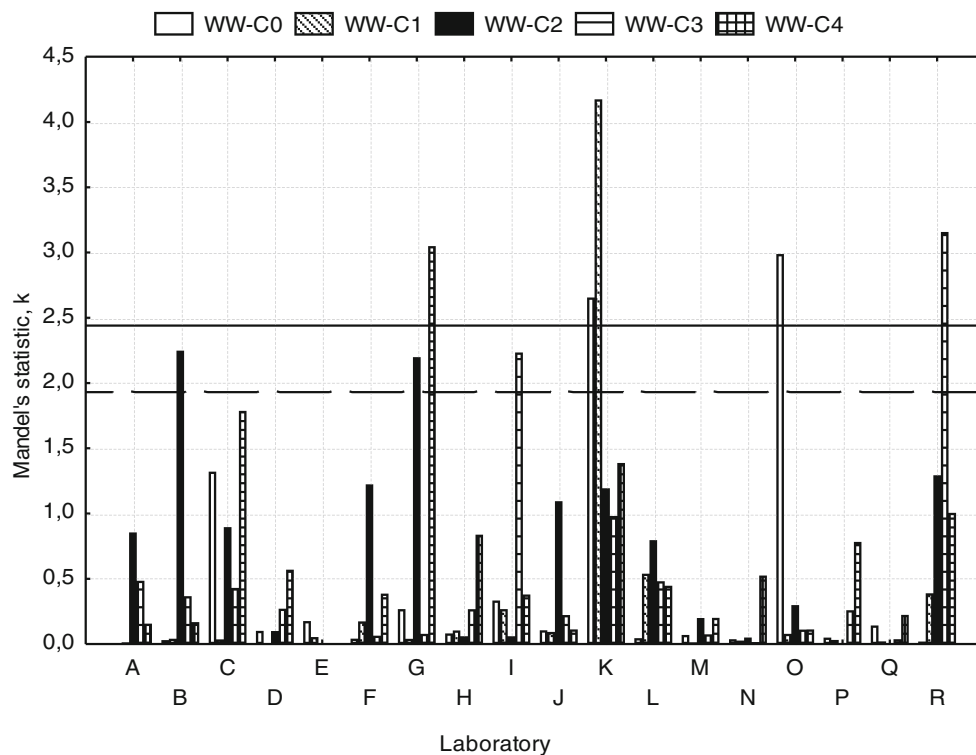
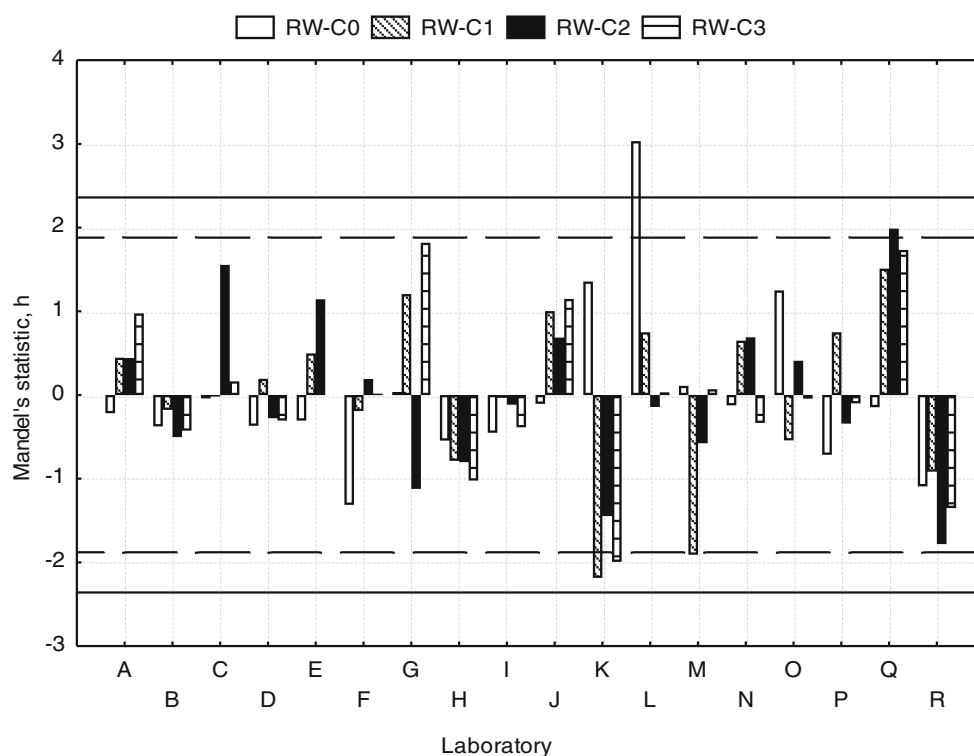


Fig. 4 Determination of casein in RW: Mandel's between-laboratory consistency statistic, h , grouped by laboratories. Dashed line $p<0.05$, solid line $p<0.01$



Mandel's k describes the within-laboratory consistency and Mandel's h describes the between-laboratory consistency. The calculation was done according to ISO 5725-2:2002-12. For casein, laboratories O, M, R, and L showed the highest within-laboratory variation in RW (Fig. 2), while laboratories K, G, R, and O were identified for WW (Fig. 3).

It is quite obvious that laboratories K, Q, and L show a higher between-laboratory variation for both types of wine compared to the other laboratories (Figs. 4 and 5). Since the within-laboratory variation for laboratory Q is very low, it should be noted that this laboratory determined consistently higher concentrations for some samples close to the assigned

Fig. 5 Determination of casein in WW: Mandel's between-laboratory consistency statistic, h , grouped by laboratories. Dashed line $p<0.05$, solid line $p<0.01$

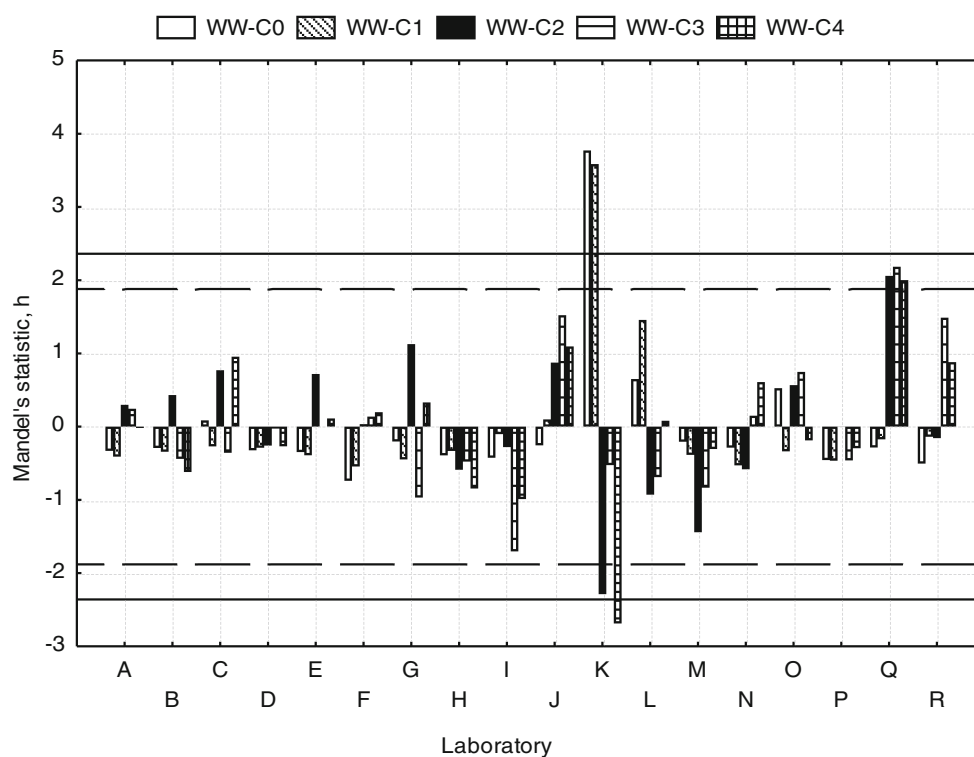
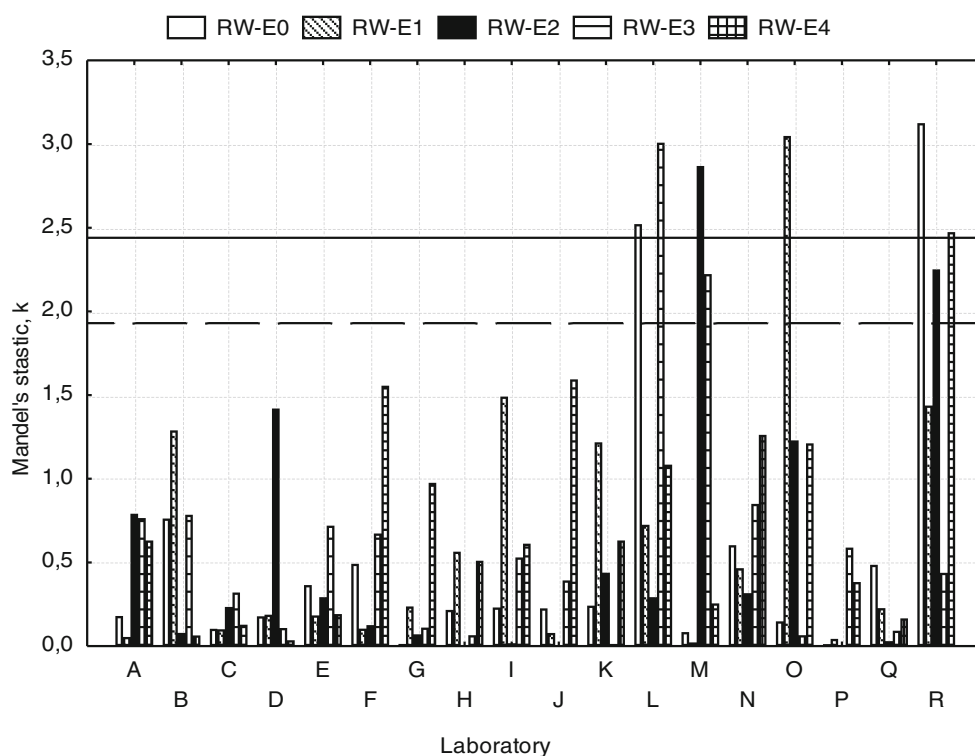


Fig. 6 Determination of egg in RW: Mandel's within-laboratory consistency statistic, k , grouped by laboratories. Dashed line $p<0.05$, solid line $p<0.01$



values. It could be speculated that the transport or time between preparation of the sample and measurement of the sample is different from the other laboratories. As for casein in RW, laboratories O, M, R, and L showed the highest within-laboratory variation for egg in RW (Fig. 6), while besides a

few others, laboratories K, L, and R were in the group of laboratories with the highest within-laboratory variation for egg in WW (Fig. 7). Finally, the most important contributors to the between-laboratory variation for egg in both types of wine are laboratories K, L, and R (Figs. 8 and 9).

Fig. 7 Determination of egg in WW: Mandel's within-laboratory consistency statistic, k , grouped by laboratories. Dashed line $p<0.05$, solid line $p<0.01$

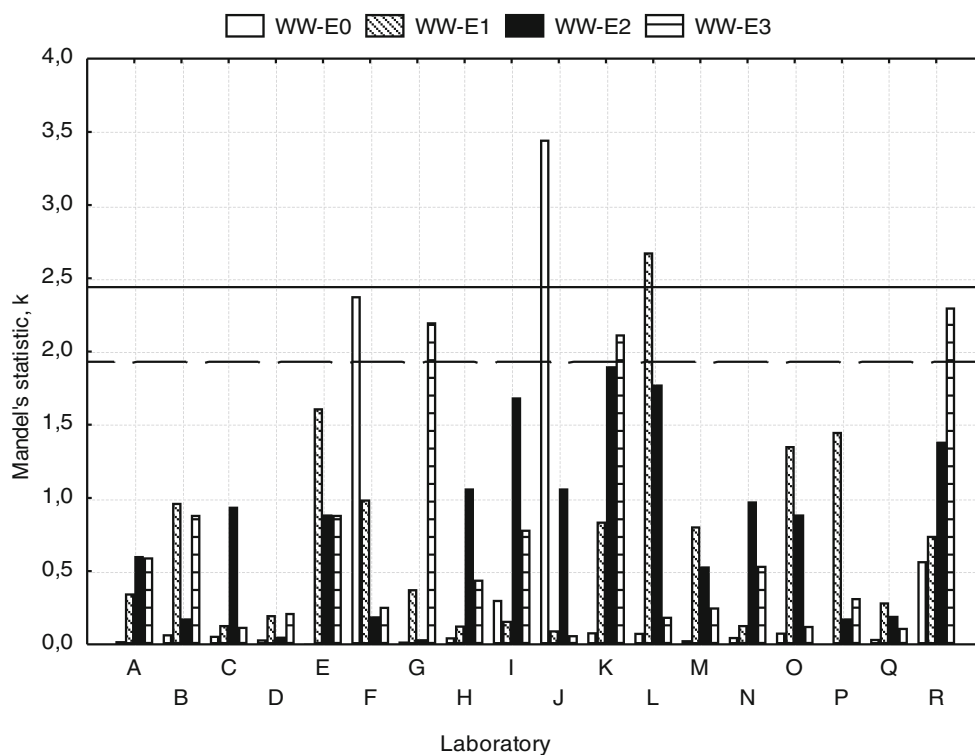
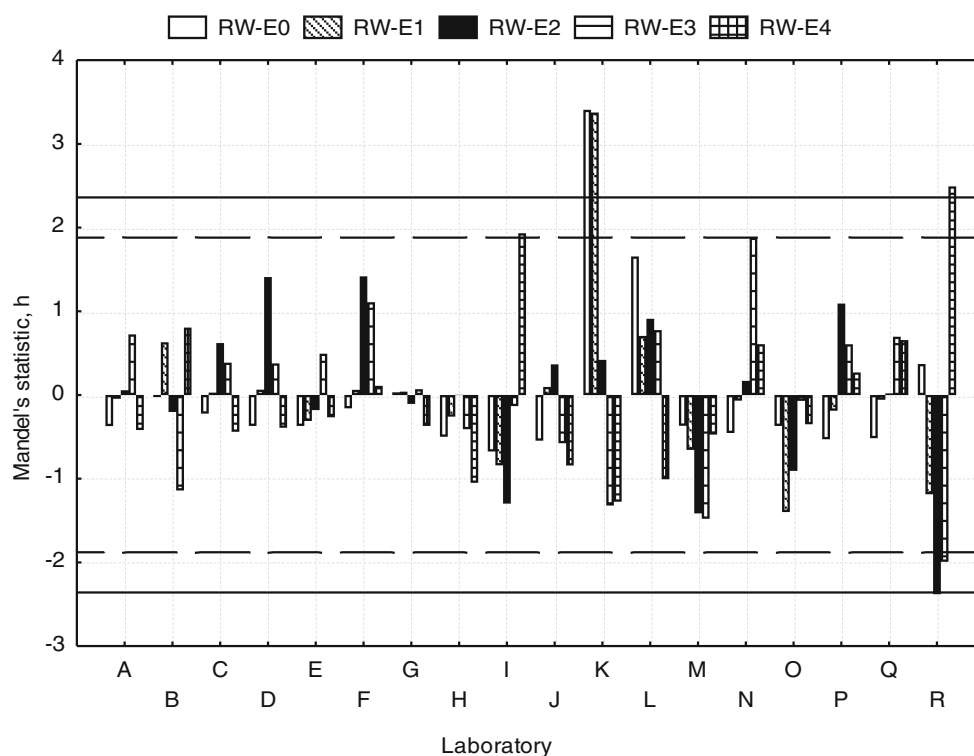


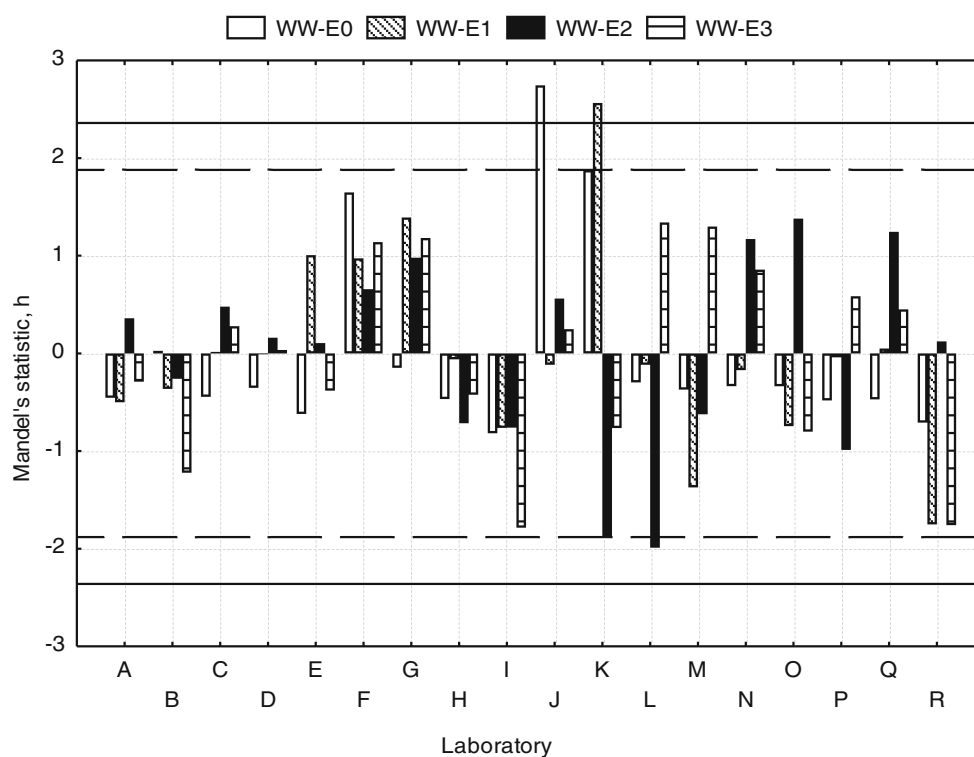
Fig. 8 Determination of egg in RW: Mandel's between-laboratory consistency statistic, h , grouped by laboratories. Dashed line $p<0.05$, solid line $p<0.01$



Outliers were identified by using the Cochran and the single and double Grubbs tests according to the AOAC Guidelines Appendix D (AOAC International 2002). Outliers are designated in Table 5 (egg) and Table 6 (casein) with “c” for a “Cochran” outlier and “c” for a “Grubbs”

outlier. The number of outliers is shown in Tables 1, 2, 3, and 4. By analyzing these data, it is clear once again that laboratories K, L, and R contribute to a significant part to the identified outliers. For the blank samples RW-E0, WW-E0, RW-C0, and WW-C0, nearly 60 % of all outliers are due to

Fig. 9 Determination of egg in WW: Mandel's between-laboratory consistency statistic, h , grouped by laboratories. Dashed line $p<0.05$, solid line $p<0.01$



these three laboratories. The same is true for samples RW-E1 and WW-C1 with low amounts of fining agents.

Tables 1, 2, 3, and 4 summarize the assessment of repeatability, reproducibility, LOD, and recovery for both assays and both wine categories. For the calculation of the LOD, two different approaches are compared in this paper: According to ISO 5725-2 (2002) and OIV-MA-AS315:23 R2012 (2012), the s_r of the blank samples should be used, whereas AOAC recommended the use of s_R (Abbott et al. 2010). Both approaches use the grand mean derived from all laboratories plus the threefold standard deviation (s_r or s_R).

For casein in wine, the LOD is between 0.15 and 0.27 mg/L, depending on the standard deviation used for this calculation (s_r or s_R). Since the mean of the blank samples was not significantly different from zero, it was not included in the LOD calculation (Tables 1 and 2). Casein in RW shows a peculiar behavior: the Horwitz ratio (HORRAT) values increase with increasing spiking amounts of the fining agents, whereas the recovery decreases. A possible explanation could be the poor solubility or instability of casein in RW at higher concentrations. Nevertheless, these concentrations could be clearly quantified with a sufficient recovery. For WW, acceptable HORRAT values were obtained for all samples above the LOD. The only exception is sample WW-C1 (assigned value of 0.37 mg/L; grand mean of 0.12 mg/L) with a HORRAT value of 2.46. This is not unusual because the relative standard deviations RSD_r and RSD_R are higher than 35 % and, therefore, more comparable with a blank sample. The recoveries of the other samples are between 72 and 76 % which is somewhat low, but it should be borne in mind that casein is not well soluble in wine and that an assigned value only reflects a single point of measurement.

For egg in RW, the LOD is 0.32 mg/L, irrespective of the standard deviation used for the calculation. For WW, values between 0.20 and 0.35 mg/L are calculated, depending on the standard deviation used for calculation (for the explanation, see previous paragraphs). In the case of egg, the grand mean was 0.11 mg/L for both types of wine and significantly different from zero. Therefore, it was included in the calculation of the LOD for egg in wine. These values are calculated as whole egg powder to which the assay is calibrated to. For recalculation of this value to egg white protein, a factor of 0.26 is necessary, as indicated by the test kit manufacturer. Therefore, the “true” LOD values are quite lower and the assay is fit-for-purpose. Spiking egg white protein in RW resulted in very good recoveries of more than 94 % for samples with concentrations of more than 0.5 mg/L. The highest HORRAT value is observed for the low concentrated sample RW-E1 with an assigned value of 0.33 mg/L and a grand mean of 0.26 mg/L. For WW, the recovery is lower but maybe also attributable to the instability of the spike in WW. With the exception of the lowest

concentrated sample WW-E1, all HORRAT values are acceptable.

Recommendations

As for all allergens, the HORRAT value should be taken with care because nearly all commercial assays that are available on the market include a dilution step of the sample before measurement due to the extraction procedure. Therefore, the concentration of the extracted solution should be taken as a basis for the estimation of HORRAT values and not the concentration of the allergen in the sample. For a concentration of 1 mg/L, we could expect an RSD_R of 16 %. Compared to this, the lowest calibrator in both ELISA systems is 0.025 mg/L. Furthermore, Albert and Horwitz (1997) discussed a limit for calculations for c smaller than 10^{-9} (=0.001 mg/L), where results become uninterpretable because of the appearance of excessive numbers of false-positive and false-negative values.

It is unnecessary to say that the reproducibility standard deviations for samples with low or zero concentrations are high because each value from each laboratory resulted from one calibration curve in a laboratory. Since every calibration curve is very flat at low concentrations, only minor changes in OD values which are derived from pipetting or incubation errors will result in high variations of calculated concentrations.

It should also be mentioned from a practical point of view that the calculation of results below the LOQ of a method is, per definition, impossible. The extrapolation of the cubic spline curve fitting to OD values close to calibrator 1 (zero concentration) or the usage of an alternative curve fitting as described here is not recommended under practical conditions. The alternative third-order fitting was only used to calculate values for an estimate of the LOD of the method. An experienced laboratory using this method regularly should build its own “intralaboratory” LOD from repeated measurement of blank wines which reflects the true situation in one laboratory. If a measurement shows OD values between the determined LOD and calibrator 2 (equivalent to the LOQ), then the results could be given as “positive but below LOQ.” If a laboratory wants to repeat the spiking experiments, care should be taken due to the instability of the spiked samples.

Conclusions

The whole collaborative study has shown that both allergen ELISA systems are capable of analyzing casein or egg white protein in WW and RW with sufficient sensitivity and precision. The determination of casein in WW and egg white

protein in RW fits the performance criteria set by the OIV resolutions. However, it is clear from the data set of this collaborative study that a few laboratories struggled when using this assay, but most of the participants showed a variation of results that could be expected for this kind of analytes.

Both assays can be used for systematic control during the single steps of a fining process as, e.g., filtration. As mentioned previously, filtration of wines should remove egg and milk proteins almost completely and result in residual concentrations below the LODs of analytical tests and far below allergy-eliciting concentrations. However, insufficient, incomplete, or erroneous filtration will result in measurable allergen concentrations (Lacorn et al. 2011) which might harm predisposed individuals.

Conflict of Interest Ulrike Immer has no conflict of interest. Thomas Weiss has no conflict of interest. Markus Lacorn has no conflict of interest. Reinhard Ristow was nominated as the leader of the collaborative tests by the Association of German Wine Analysts and received a cost-covering honorarium from R-Biopharm. This article does not contain any studies with human or animal subjects.

References

- Abbott M, Hayward S, Ross W, Godefroy SB, Ulberth F, van Hengel AJ, Roberts J, Akiyama H, Popping B, Yeung JM, Wehling P, Taylor SL, Poms RE, Delahaut P (2010) *J AOAC Int* 93:442
- Albert R, Horwitz W (1997) *Anal Chem* 69:789
- AOAC International (2002) Appendix D: guideline for collaborative study procedures to validate characteristics of a method of analysis. AOAC official methods of analysis. The Association, Gaithersburg
- Commission Directive 2007/68/EC of 27 November (2007) amending Annex IIIa to 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
- Delfini C, Cersosimo M, Del Prete V, Strano M, Gaetano G, Pagliara A, Ambro S (2004) *J Agric Food Chem* 52:1861
- Gerbaux V, Villa A, Monamy C, Bertrand A (1997) *Am J Enol Vitic* 48:49
- Hu W, Katelaris CH, Kemp AS (2007) *Allergy* 62:709
- ISO 5725-2:2002-12 (2002) Accuracy (trueness and precision) of measurement methods and results—part 2: basic method for the determination of repeatability and reproducibility of a standard measurement method
- Kanny G, Moneret-Vautrin DA, Flabbee J, Beaudouin E, Morisset M, Thevenin F (2001) *J Allergy Clin Immunol* 108:133
- Lacorn M, Goeßwein C, Immer U (2011) *Am J Enol Vitic* 62:382
- Lam HY, van Hoffen E, Michelsen A, Guikers K, van der Tas CHW, Bruijnzeel-Koomen AFM, Knulst AC (2008) *Clin Exp Allergy* 38:995
- Lopez I, Santamaria P, Tenorion C, Garijo P, Gutierrez A, Lopez R (2009) *J Microbiol Biotechnol* 19:1005
- OIV-MA-AS315-23: R2010 (2010) Criteria for the methods of quantification of potentially allergenic residues of fining agent proteins in wine. Compendium of international analysis of methods
- OIV-MA-AS315-23: R2012 (2012) Criteria for the methods of quantification of potentially allergenic residues of fining agent proteins in wine. Compendium of international analysis of methods
- Resolution OIV-COMEX 502-2012 (2012) Revision of the limit of detection and limit of quantification related to potentially allergenic residues of fining agent proteins in wine
- Rolland JM, Apostolou E, Deckert K, de Leon MP, Douglass JA, Glaspole IN, Bailey M, Stockley CS, O'Hehir R (2006) *Nutrition* 22:882
- Rolland JM, Apostolou E, de Leon MP, Stockley CS, O'Hehir R (2008) *J Agric Food Chem* 56:349
- Savage JH, Matsui EC, Skripak JM, Wood RA (2007) *J Allergy Clin Immunol* 120:1413
- Taylor SL, Hefle SL, Bindslev-Jensen C, Bock SA, Burks AW, Christie L, Hill DJ, Host A, Hourihane JOB, Lack G, Metcalfe DD, Moneret-Vautrin DA, Vadas PA, Rance F, Skrypec DJ, Trautman TA, Malmheden-Yman I, Zeiger RS (2002) *J Allergy Clin Immunol* 109:24
- Weisstein EW (2012) Cubic spline. MathWorld, Wolfram Research, Inc., Champaign, IL. Available at <http://mathworld.wolfram.com/CubicSpline.html>