PROFICIENCY-TESTING SCHEME FOR ALLERGENIC PROTEINS ANALYSIS IN WINE

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INTRODUCTION

Fining agents are commonly used in the winemaking process to clarify and stabilize wines. They have different origins (animal, vegetal or mineral) and are added to wines to remove certain elements that would cloud the wine or affect its aroma, color and/or bitterness. These agents should not be present in the final product but even the presence of low amount of residual fining proteins can represent a **risk for allergic consumers** [1,2].

Reliable detection and quantification of the residual allergenic agents is necessary to ensure compliance with food labelling, as the EU Commission Implementing Regulation No. 579/2012 of 29 June 2012 establishes that wines treated with allergenic additives or processing aids are subjected to specific labelling if their presence can be detected in the final product [3,4]. According to the OIV-COMEX 502-2012 resolution, wines are considered free of presence of residues if allergens are not detected using techniques with a detection and quantification limits of 0.25 mg/L and 0.50 mg/L respectively [5,6]. To meet this requirement, different analytical approaches such as immunological tests, genomic tests (PCR) and several methods based on mass spectrometry were developed. Among them, **ELISA test** (Enzyme-Linked-Immuno-Sorbent-Assay) is routinely used to detect allergens in wines, because of its specificity and sensitivity, easy application and since the equipment required is not expensive. However, commercial kits available are likely to estimate diverse forms of the researched protein. To respond to increasing demand of laboratories that need to evaluate their performances, Bipea organizes regular proficiency testing schemes (PTS) for detection and quantification of residual fining proteins in wines. The aim of this study is to describe the setting up of the tests and show the results obtained in 3 different trials for **casein, ovalbumin** and **lysozyme** analyses on **white, red** and **rosé wines**.

EXPERIMENTAL

Sample production and shipment

From October 2019 to June 2020, three different wines (white, red and rosé) were spiked with casein, ovalbumin and lysozyme at different spiking levels (from 0 to 1 mg/L):

- White wine : Graves blanc wine, alcoholic strength by volume: 13,5%.
- Rosé wine: IGP Sable de Camargue, alcoholic strength by volume: 12,5%.
- Red wine: IGP, Pays d'Oc, Cabernet Sauvignon, alcoholic strength by volume: 13,5%.

The procedure for the preparation of the samples varies according to the allergens added in wine. For lysozyme and ovalbumin, a batch of wine was spiked with the target allergens and then homogenized and divided into series of samples. This operation was performed using a homogenization tun. The principle of a quick successive production, which involves a quasi-simultaneous filling, ensures the homogeneity of the product between all the samples.

Concerning casein, samples of wine were individually spiked using a calibrated solution.

Nine batches of samples were prepared at different concentrations (see Table 1).

The homogeneity and stability of the samples were verified according to the requirements of ANNEX B of the ISO 13528 standard [7].

Samples were shipped at (5±3) °C to the laboratories participating to the test (20 on average) together with a standard sample for monitoring the temperature. Given the stability of the product, the participants were invited to analyze the samples as soon as possible after the reception.

Results collection and data statistical treatment

Reply forms were made available to allow the laboratories to return their analysis results. Participants are invited to enter in the reply form some complementary information such as the date of the beginning of the analysis, Elisa kit performed and its detection and quantification limits. **Statistical treatments** of the returned results are conducted according to ISO 13528 [7]. The **assigned values** (x_{pt}) are estimated using the robust means of all results (except incoherent ones) from the application of robust algorithm A. **Performances** of each laboratory are evaluated using robust standard deviations (s*) set as the standard deviation for performance assessment (σ_{pt}), with a tolerance value minimal at 0.20 mg/L. This value is used to identify an interval around the assigned value. Results in this range are considered as satisfactory. Laboratory results (x) are also evaluated through **z-scores** (z). The z-score for a result x_i is calculated as:

$$z_i = ((x_i - x_{pt}))/\sigma_{pt}$$

Laboratories with a "z score $\leq|2|$" or "z score $>|3|$" are considered having reported "**Satisfactory**" or "**Unsatisfactory**" results, while the remaining laboratories (which z score is $>|2|$ but $\leq|3|$) reported "**Questionable**" results. Results are published in a specific interlaboratory comparison report distributed to all participants who can then classify their results and implement some corrective and/or preventive actions if necessary.

RESULTS & DISCUSSION

Results of the proficiency tests of October 2019, February and June 2020 (Rosé, white and red wine respectively) are examined in detail. Table 1 summarizes the statistical data of each test for each allergen. Assigned values (x_{pt}) were estimated for all tests except for not spiked wines, for which most of the results were expressed as quantification limits. Standard uncertainties, $u(x_{pt})$, that allow quantification of the confidence that can be given to the assigned value, were calculated as indicated in paragraph 7.7 of the ISO 13528 standard [7].

Laboratory results are acceptable, with only few unsatisfactory ones, however, data examination allowed to note that, in general, results are dispersed, as coefficients of variation are ≥ 21% for all PT. These data are not startling, considering the uncertainty of measurement related to the method of analysis and the variety of ELISA kits used by the laboratories that may differ in operating method for allergen extraction and quantification. For the casein analyses, some immunosorbent kits that are likely to estimate diverse forms of casein, and, likewise, commercial kits for ovalbumin cannot be specific for this protein but take into account other forms of albumin.

Nevertheless, dispersion may be caused also by other factors as the storage temperature of the samples before analysis and laboratories sampling protocol. Samples must be well-maintained at around (5±3) °C to preserve the stability of the allergen in wine. A blank sample was sent to all laboratories to check the temperature since the reception and to make them aware about this point. Finally, the sampling protocol can be different from a laboratory to another. An instruction was added in the reply form to alert the laboratories about the importance of the sample homogenization before analysis.

Compound	Wine	Spiking value (mg/L)	X_{pt}[1] (mg/L)	$u(x_{pt})$[2] (mg/L)	σ_{pt}[3] (mg/L)	$p(x_{pt})$[4]	CV (%)	$u(x_{pt})/\sigma_{pt}$	p_{NQ}[5]	p_S[6]	p_Q[7]	p_U[8]
Casein	White	0,80	0,28	0,03	0,06	8	21	0,50	7	10	2	0
	Rosé	0,00							14	-	-	-
	Red	0,60	0,49	0,06	0,22	20	45	0,27	1	20	0	0
Lysozyme	White	1,00	1,01	0,10	0,23	8	23	0,43	2	8	2	2
	Rosé	0,75	0,66	0,07	0,19	13	29	0,37	0	12	3	0
	Red	0,00							19	-	-	-
Ovalbumin	White	0,00							11	-	-	-
	Rosé	0,54	0,35	0,04	0,12	16	34	0,33	1	14	1	1
	Red	1,00	0,95	0,13	0,45	19	47	0,29	0	20	0	1

1. x_{pt}: Assigned value
2. $u(x_{pt})$: Standard uncertainty of the assigned value
3. σ_{pt}: Standard deviation for proficiency assessment: σ_{pt}=s*, with a minimum at 0.10 mg/L
4. $p(x_{pt})$: Number of results taken into account for the estimation of the assigned value
5. p_{NQ}: Number of non quantitative results
6. p_S: Number of satisfactory results
7. p_Q: Number of questionable results
8. p_U: Number of unsatisfactory results

Table 1. Main statistical parameters of the proficiency tests of October 2019 (Rosé wine), February 2020 (White wine) and June 2020 (Red wine)

Histograms in Figures 1 to 3 show the distribution of quantitative laboratories' results of 3 PT. On these graphs, assigned value and tolerance interval are indicated in the x-axis and the results of the laboratories are shown in different colors as a function of the performed Elisa kit. Some statistics by kit are also shown in Tables 2 to 4.

These data show that the lower standard deviation is observed for major kits used by laboratories, even if a lack of data, particularly for some kits, lead to a difficult conclusion. Considering the uncertainties of on the estimated means, there is no significative difference between the results obtained by different kits.

Considering the tests where no allergens were added in the wine, all laboratories returned results < 0.25 mg/L, that corresponds to the detection limit of the OIV-COMEX 502-2012 resolution for which wines are considered allergen free. These data are comforting as false positive results may lead to unnecessary product withdrawal.

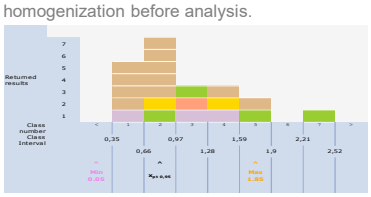

Figure 1. Red wine – Ovalbumin: PT results represented as a histogram, mg/L

	AlerTox ELISA Ovalbumin	BioSystems Ovalbumin	Novakits – Elisa ovalbumine	R-Biopharm–Ridascreen Fast Ei / Egg Protein	Other kit
Means, x^*_m	0,97	1,51	1,05	0,82	
Standard uncertainty of the means, u^*_m	0,44	0,55	0,35	0,12	
Standard deviation of the results, s^*_m	0,61	0,88	0,4	0,32	
Number of the results, p_m	3	4	2	11	1

Table 2. Red wine – Ovalbumin: Means by used ELISA Kit, mg/L

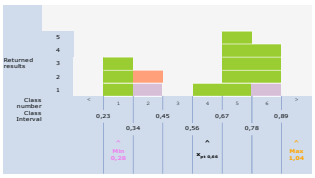

Figure 2. Rosé wine – Lysozyme: PT results represented as a histogram, mg/L

	BioSystems Lysozyme	Novakits – Elisa lysozyme	R-Biopharm–Ridascreen Fast Lyzozyme
Means, x^*_m	0,61		0,61
Standard uncertainty of the means, u^*_m	0,26		0,1
Standard deviation of the results, s^*_m	0,3		0,27
Number of the results, p_m	2	1	12

Table 3. Rosé wine – Lysozyme: Means by used ELISA Kit, mg/L

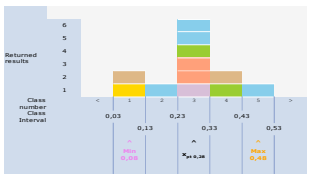

Figure 3. White wine - Casein: PT results represented as a histogram, mg/L

	AlerTox ELISA Casein	BioSystems Casein	ELISA SYSTEM – Casein	Novakits – Elisa caseines	R-Biopharm–Ridascreen Fast Casein	Other
Means, x^*_m	0,29	0,33		0,24	0,32	
Standard uncertainty of the means, u^*_m	0,01	0,1		0,21	0,12	
Standard deviation of the results, s^*_m	0,02	0,11		0,24	0,19	
Number of the results, p_m	2	2	1	2	4	1

Table 4. White wine – Casein: Means by used ELISA Kit, mg/L

CONCLUSION

These PTS enable the participating laboratories to draw up a general inventory of their analytical skills and improve their analytical performances in detection and quantification of residual fining proteins in wines. This program, approved and accredited by COFRAC (Comité Français d'Accréditation / French Accreditation Body), has been further developed to include beta-lactoglobulin and gluten analyses to allow laboratories to demonstrate their performances for analyses of these allergens too. Laboratories can now monitor punctually and/or continuously through time the reliability of their results and obtain recognition of their analytical procedures by the accreditation bodies according to ISO/IEC 17025 [8] for allergens analyses in wines.

REFERENCES

(1) Peñas E., Lorenzo C., Uberti F, Restani P., Allergenic Proteins in Enology: A Review on Technological Applications and Safety Aspects, Molecules, 2015, 20, 13144-13164

(2) Rizzi C., Mainente F., Pasini G, Simonato B, Hidden Exogenous Proteins in Wine: Problems, Methods of Detection and Related Legislation – a Review, Czech J. Food Sci., 34, 2016 (2): 93–104

(3) Commission of the European Communities. 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, labeling and presentation of certain wine sector products. Off. J. Eur. Union 2009, L 193, 60–80

(4) Commission Implementing Regulation (EU) No 579/2012 of 29 June 2012 amending Regulation (EC) No 607/2009 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, labeling and presentation of certain wine sector products Off. J. Eur. Union 2012, L171, 4–7

(5) OIV. Criteria for the Methods of Quantification of Potentially Allergenic Residues of Fining Agent Proteins in Wine. Resolution OIV/OENO 427/2010

(6) OIV. Criteria for the Quantification of Potentially Allergenic Residues of Fining Agent Proteins in Wine. OIV-OENO 427-2010 Modified by OIV-COMEX 502-2012

(7) ISO 13528:2015 - Statistical methods for use in proficiency testing by interlaboratory comparisons

(8) ISO/IEC 17025:2017 General requirements for the competence of testing and calibration laboratories.