INVESTIGATING THE BEST STRATEGY TO ELIMINATE MATRIX EFFECTS FOR EGG-ALLERGENS DETECTION IN WINES BY SURFACE PLASMON RESONANCE IMMUNO-SENSOR

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Abstract–In this communication, we investigated the feasibility to develop an SPR based method tailored to the detection of egg residues in wines, the final goal being the elimination of matrix-effect on the analytical response. Two model wines matrices were selected, subjected to various purification procedures, and compared pair-wise with the standard curve both in terms of specific analytical responses and calibration curve slopes. Different statistical tools were usedfor significant comparisons.

Keywords:biosensor, matrix effect, egg white, allergen, wine

1. INTRODUCTION

The clarification or fining of wine is a very common procedure, which allows removing undesired substances, mainly proteins, phenols and tannins,accounting for wine bitterness and astringency. Egg-derived products have optimal fining properties, thus they can be used in winemaking,with various commercial preparations thanks to the ability to interact and promote precipitation of wine polyphenols and other undesirable compounds. However, due to its allergenic potential any residue of egg white proteins remaining in wine could represent a risk for sensitized consumers. As a fact, a recent opinion issued by the European Food Safety Agency (EFSA) highlighted that traces of fining proteinscan likely survive in the end-product [1]. As from July 1st 2012 the European Union obliges European wine producers to indicate the use of allergenic aids of animal origin whenever added for fining purposes [2,3].

Within this European legislative frame, reliable and sensitive analytical approaches for the detection of egg proteins in foods and beverages are urgently demanded. No official analytical method for the determination of fining agent proteins are prescribed, the only reference being the article 120g of the European regulation 1234 of 2007 concerning analytical methods to detect wine composition [4]. In the latter, a direct reference to the International Organization of Vine and Wine (OIV) resolution was stated, who in the resolution 427-2010 [5] modified by the OIV/COMEX 502-2012 [6] set up the analytical requirements to be fulfilled by immunoassays based methods under development.The most common choice among immunoassayswas enzyme-linked immunosorbent assays (ELISAs). For the specific case of allergens detections in wines several ELISA kit are already commercially availableand recently some collaborative tests have been performed to validate the performances of the assay according to the OIV resolution [7,8].

Biosensors represent a potential alternative to ELISAs, and provide probably one of the most promising way to achieve simple, fast, reproducible, and cheap multi-analyte detection [9].Among the various optical transduction mechanisms, surface-plasmon resonance (SPR) based technique plays an important role.In 2015, we reported on the development of the first SPR based biosensor tailored to the fast detection of residual egg-based fining agents in white and roseé wines [10].Recently, such investigation was updated with the modification of the analytical procedure in

order to widen the method applicability to the analysis of red wines [11].

The main open issue of our works was that we resorted to a different purification procedure as a function of the vinification (either white/roseé or red) and/or grapes selection;from the practical perspective, this demands to perform matrix-matched calibration curves for quantitative purposes, which hampered the SPR biosensordefinitively affirmation asroutinedetection tool.

In this communication, we open the perspective to devise an analytical method that could be considered with a certain probability, free from matrix-effect. Two wines matrices (one white and one red), were selected and subjected to variouspurification procedures, given by the combination of size exclusion chromatography, chemo-physical adsorption and dilution.The specific analytical signals in purified wine matrices weretested against the relevant standard curve, by means of a Student's t-test of the regression linesslopesfor a statistically relevant comparison. In addition, a paired t-test on the averaged data sets was applied for definitive decision, the final goal being to select the best purification procedure that allowed a matrix-matched calibration curve not significantly different from the relevant standard curve.

2. MATERIALS AND METHODS

2.1. Reagents

Policlonal anti-egg white antibody was provided by Euroclone spa. CM5 Chips, amine coupling kit andrabbit anti-β2microglobulin polyclonal antibody (neg-Ab) and PD-10 desalting columns were purchased from GE Healthcare Life Sciences. All other chemicals were purchased from Sigma Aldrich.

2.2. Surface Plasmon Resonance (SPR) detection

A Biacore® X apparatus (GE Healthcare Life Sciences) with CM5 sensor chips (carboxymethylated dextran matrix) were usedfor SPR experiments. Details about the apparatus, the sensor chip functionalization and the immunoassay experimental parameters were described elsewhere [10]. All SPR measurements were carried out at 25 °C with a flow rate of 10µL/min.

All buffer solutions employed were preliminary filtered through 0.20 µm cellulose acetate filters. Wine samples were subjected to centrifugation at 10000 rpm for 10 min.

2.3. Stock solutions and wine matrices

Stock solutions of egg white (EW) were freshly prepared by dissolving 1 mg and 5 mg of powder, respectively, in 1 mL of ammonium bicarbonate 50 mM. For standard calibration curve, serial dilutions of the stock egg white in 10 mM sodium acetate buffer (pH 4.8) were prepared in the range 0.06–400 µg/mL for EW.

The white and red wines produced by Trebbiano and Nero d'Avola grape varieties, respectively, were purchased from a local retailer. Both wine matrices were subjected to several purification protocols based on various combination of (i) size exclusion chromatography (SEC), (ii) chemico-physical adsorption with PVPP for 120 min (70 mg_{PVPP}/mL_{wine}, according to our previous results [11]), (iii) dilution in 10 mM sodium acetate buffer (pH 4.8). After each purification procedure, the matrix was split into aliquots and spiked with variable amount of EW to achieve a final concentration level ranging from 5 to 200 µg/mL, matching the assay operating range(six levels, two replicates for each level).

3. RESULTS AND DISCUSSION

In this communication, we investigated the prospect to overcome the main issue affecting our previously developed biosensor concerning the dependence of the final analytical response on the wine matrix. A direct assay based on the immune-recognition by anti-egg white antibodies covalently linked to the sensor chip surface was used for tracing fining agent residues in wines.

Aiming at this, we design highly targeted experiments in which artificially contaminated wine matrixes (white and red), were subjected to different purification procedures, and compared,in terms of specific response and regression-line slope, withthe relevantstandard curve. Since we are only interested into the evaluation and suppression of the matrix effect (ME), we decided to post-pone the allergen addition to the matrix purification.With an unambiguous control onthe egg white amount dissolved in solution (either buffer or purified matrix), the contribution of the ME to the analytical

recovery can be isolated and quantified, the final goal being its suppression.

Three main purification stepswere taken into consideration, either individually or in combination, to reduce the ME: (i) size exclusion chromatography (SEC) on disposable cartrdge, (ii) chemo-physical adsorption on polymeric insoluble microparticles of poly-vinyl-polypyrrolidone (PVPP), and (ii) dilution with buffer. Each of these procedures could provide the potential to simplify the matrix complexity and differently reduce the contribution of interfering components on the immuno-recognition event and specific analytical signal detection.The disposable columns for SEC purification provided a fast and efficient toolto remove low molecular weight matrix components(<5 kDa).In addition, water-insoluble PVPP can specifically interact with polyphenols via H bonds, removing them from the sample by centrifugation The latter step can be particularly relevant for application to red wines matrices, since polyphenols canhamper the achievement of the required sensitivity and reliabilityin the detection of EW,by target analyte masking and as well as sensor life-time impairing.

In Fig. 1, the different experimental protocols designed and tested were schematically represented, and named with capital letters from *A* to *I*. After each purification procedure, the matrix wassplit into aliquots and spiked with variable amount of EW to achieve a final concentration level ranging from 5 to 200 µg/mL (six levels) thus matching the assay operating range. In parallel, progressive dilution of EW were prepared in acetate buffer within the same range and analysed for standard calibration curves.

As first step, the comparison between matrix-matched and standard curves was performed by a Student's t-test on the slopes of the two regression lines. Comparing the slopes of two regression lines is a quite commontask in analytical laboratories, e.g. for qualitycontrol, for method comparison studies and method development,however, literature differs in how to calculate the pooled standard error for the t-test statistic [12]. As for this issue, we referred to the review of Andrade and Estévez-Pérezi from 2014, which clearly settled the ambiguity [12].

The analytical signals recorded were fitted with a regression-line ($y=a+bx$, in semi logarithmic scale as for x-axis), and the experimental fitting parameters, i.e. the slopes, were compared by a Student's t-test, with the standard curve regression line. The null hypothesis testwas H_0: $b_{Matrix}=b_{Standard}$(equivalent to H_0: $b_{Matrix}-b_{standard}=0$), against the alternative hypothesis H_1: $b_{Matrix}\neq b_{Standard}$(equivalent to H_1: $b_{Matrix}-b_{Standard}\neq 0$).

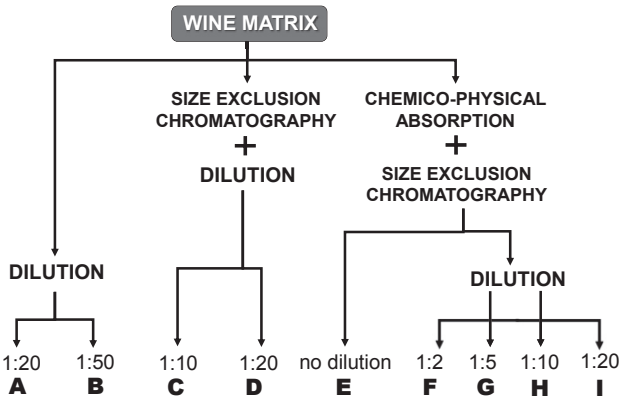

Fig. 1. Scheme of the sample preparation protocol designed and tested in order to reduce the matrix effect on the final analytical response.

In order to proceed, an estimate as accurately as possible of the standard error on the difference $b_{Matrix}-b_{standard}$andthe effective degrees of freedom were required. Aiming at this, a first preliminary screening of the similarity of the regression variances by the Fisher–Snedecor's F-testwas carried out. This test consists of dividing the largest by the lowest variance and comparing it to unity, (one-tail tables to get the upper critical levels):

$$F_{(n_1-2,\ n_2-2,\ 1-\alpha)} = \frac{S^2_{(y/x),1}}{S^2_{(y/x),2}} \qquad (1)$$

For all the tested procedures (from *A* to *I*) the null hypothesis of the F-test cannot be rejected, namely theregression variances $S^2_{(y/x),Matrix}$ can be considered equal to the $S^2_{(y/x),Standard}$, at 1% significance level [12]. Therefore, we can pool the estimates of the error variances, weightingeach by their degrees of freedom:

$$S^2{}_{(y/x)pool} = \frac{(n_1-2)S^2_{(y/x),1}+(n_1-2)S^2_{(y/x),1}}{n_1-2+n_2-2} =$$

$$= \frac{\sum(Y_{i_1}-\hat{Y}_{i_1})^2+\sum(Y_{i_2}-\hat{Y}_{i_2})^2}{n_1-2+n_2-2} \qquad (2)$$

Consequently, the t-teststatistic given below as Eq. (3), follows a $t_{(n_1+n_2-4)}$ distribution:

$$t_{(n_1+n_2-4,1-\frac{\alpha}{2})} = \frac{b_1-b_2}{S_{(y/x)pool}\sqrt{\left(\frac{1}{\sum(X_{1i}-\bar{X}_1)^2}+\frac{1}{\sum(X_{2i}-\bar{X}_2)^2}\right)}} \qquad (3)$$

In Table 1, the results of the Student's t-testat the 5% significance, on the slopes comparison of purified matrix regression line with the standardregression line, were itemized. Compared with the critical t value $t_{(20,\ 0.975)} = 2{,}09$, only the statistic on procedures A and B resulted in the rejection of the null hypothesis, whereas all other procedures allowed building up calibration curves whose slopes did not differ significantly, at the confidence level 97.5%, from the slope of standard curve. Notably, the two procedures A and B in which the ME cannot be considered negligible correspond to the direct matrix dilution, namely the typical approach employed in ELISA kit.

Table 1. Results of Student's t-test on the slopes of the two regression linespurified wine matrix vs standard.

Wine matrix	Procedure	$t_{(n_1+n_2-4,1-\frac{\alpha}{2})}$	H_0: b_{Matrix}-b_{Stand}=0
White	A	2.15	Rejected
White	B	2.70	Rejected
White	*C*	*1.79*	*Accepted*
White	*D*	*0.25*	*Accepted*
White	*E*	*1.92*	*Accepted*
White	*F*	*1.23*	*Accepted*
White	*G*	*1.03*	*Accepted*
White	*H*	*0.55*	*Accepted*
Red	*D*	*1.44*	*Accepted*
Red	*H*	*0.23*	*Accepted*
Red	*I*	*0.31*	*Accepted*

As an example, Fig. 2 showed an overlap of the calibration curves (matrix-matched vs standard), for two different purification protocols, resulting in rejection (panel A, protocol: direct dilution 1:50) and acceptance (panel B, protocol: PVPP + SEC + dilution 1:10) of the null hypothesis, respectively.

The quite conservative results obtained with the Student's t-test on slopes, rejecting the null hypothesis only for two procedures, could be likely ascribed to the limited number of data points available for the statistic calculation. In order to confirm and eventually improve the discriminant factor for a more definitive conclusion, further statistic tools were implemented for fit-for-purpose protocol selection.

In particular, since the same concentration levels were used for egg white standard curve and matrix-matched curves, apaired t-test for the comparison of the averaged data sets was applied. With this statistical approach the ME could be considered negligible, if the differences in analytical responsebetween two observations at each paired concentration level, $D_i = y_{i,matrix} - y_{i,standard}$, are normally distributed around the true mean difference μ_D=0. Therefore, after calculation of the experimental differences (D_i) and mean difference ($\overline{D}$), the null hypothesis test was H_0: $\overline{D} = \mu_D$(equivalent to H_0: $\overline{D} = 0$), against the alternative hypothesis H_1: $\overline{D} \neq \mu_D$ (equivalent to H_1: $\overline{D} \neq 0$).

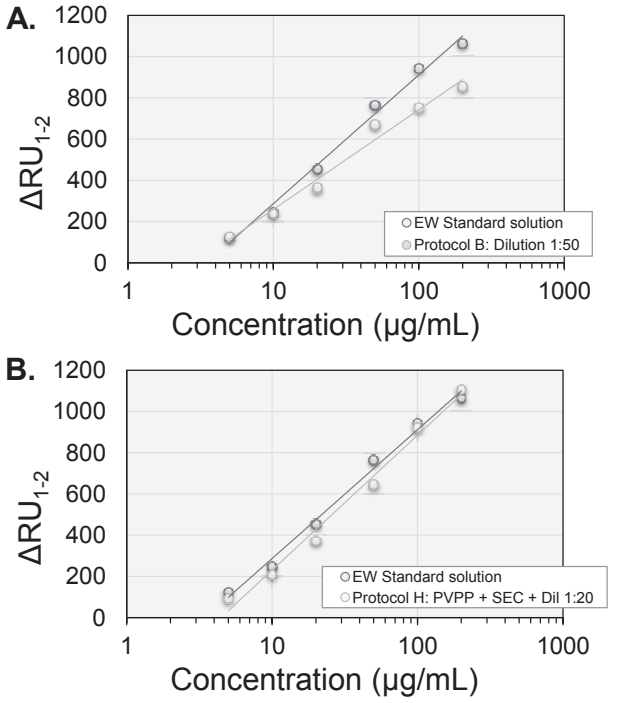

Fig. 2. Overlap of the calibration curves (matrix-matched vs standard), for two different purification protocols, resulting in rejection (panel A) and acceptance (panel B), respectively, of the null hypothesisH_0: b_{Matrix}-$b_{Standard}$=0.

The t-teststatistic given below as Eq. (4), follows a $t_{(n-1)}$ distribution (where $n=n_1=n_2$):

$$t_{(n-1,1-\frac{\alpha}{2})} = \frac{\overline{D}}{S_D/\sqrt{n}} \qquad (4)$$

Table 2. Results of paired t-test on the analytical responses of the each pairedcalibration curves, purified wine matrix vs standard solution.

Wine matrix	Procedure	$t_{(n-1,1-\frac{\alpha}{2})}$	H_0: $\overline{D} = 0$
White	A	4,56	Rejected
White	B	2,69	Rejected
White	C	5,22	Rejected
White	D	7,19	Rejected
White	E	3,71	Rejected
White	F	5,67	Rejected

White	G	3,68	Rejected
White	*H*	*1,85*	*Accepted*
Red	D	3,11	Rejected
Red	*H*	*2,16*	*Accepted*
Red	*I*	*1,94*	*Accepted*

In Table 2, the results of such statistic for each matrix purification protocol was reported. Compared with the critical t value $t_{(5,\ 0.975)} = 2{,}57$, only the most complex purification procedures H and I, given by the combination of all three steps,chemo-physical adsorption,size exclusion chromatography and dilution resulted in the acceptance of the null hypothesis, namely the contribution of the ME on the final response can be deemed negligible.

4. CONCLUSIONS

In this communication, we investigated the perspective to devise an SPR based analytical method that could be considered free from matrix-effect,with a certain confidence. Two wines matriceswere selected and subjected to variouspurification procedures, given by the combination of size exclusion chromatography, chemo-physical adsorption and dilution.The comparison between matrix-matched and standard curves was performed,firstly, by a Student's t-test on the slopes of the two regression lines, resulting in a quite conservative approach, which allowed rejecting the null hypothesis only for two procedures, related to the direct dilution. Furtherstatistical investigations were carried out by means of a paired t-test on each experimental observations obtaining a more restrictive selection of the best matrix purification procedure, which highlight the need for a quite complex protocol for matrix purification in light of significant suppression of the matrix effect on the final response.

Further efforts will be directed to increase sensitivity of this approach by introducing in this system gold-nanoparticles capable of enhancing the SPR signal.

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REFERENCES

[1] EFSA Panel on Dietetic Products, Nutrition and Allergies,"Scientific Opinion related to a notification from the International Organisation of Vine and Wine (OIV) on casein/caseinate/milk products to be used in the manufacture of wine as clarification processing aids pursuant to Article 6, paragraph 11 of Directive 2000/13/EC – for permanent exemption from labelling",*EFSA Journal,*vol. 9, nº 10, pp.2384-2397, October 2011.

[2] European Commission,"Commission Regulation No 1266/2010". *Official Journal of European Union* vol. nºL347, pp. 27-28, December 2010.

[3] European Commission,"Commission Implementing Regulation No 579/2012". *Official Journal of European Union,* vol. nºL171, pp. 4-7, June 2012.

[4] European Commission,"Council Regulation (EC) No 1234/2007"*Official Journal of the European Union* nºL299, pp. 1-343, October2007.

[5] International Organisation of Vine and Wine (OIV), "Criteria for the methods of quantification of potentially allergenic residues of fining agent proteins in wine. Resolution OIV/OENO 427/2010", http://www.oiv.int/en/technical-standards-and-documents/resolutions-oiv/oenology-resolutions. Accessed 26 July 2016.June 2010.

[6] International Organisation of Vine and Wine (OIV),"Criteria for the quantification of potentially allergenic residues of fining agent proteins in wine. OIV-OENO 427–2010 modified by OIV–Comex", http://www.oiv.int/en/technical-standards-and-documents/resolutions-oiv/oenology-resolutions. Accessed 26 July 2016.2012.

[7] M. Lacorn, R. Ristow, T. Weiss and U. Immer,"Collaborative tests of ELISA methods for the determination of egg white protein and caseins used as fining agents in red and white wine",*Food Anal Methods,* vol. nº7, pp. 417–429, February 2014.

[8] P. Restani, F. Uberti, C. Tarantino, C. Ballabio, F. Gombac, E. Bastiani, L. Bolognini, F. Pavanello and R.Danzi,"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",*Food Anal Methods,* vol. nº7, pp. 706–712, March 2014.

[9] R. Pilolli, L. Monaci and A. Visconti,"Advances in biosensor development based on integrating nanotechnology and applied to food-allergen

management",*Trends in Analytical Chemistry,* vol. nº47, pp.12-26, June 2013.

[10] R. Pilolli, A. Viscontiand L. Monaci,"Rapid and label-free detection of egg allergen traces in wines by surface plasmon resonance biosensor", *Analytical and Bioanalytical Chemistry*, vol.nº407, pp. 3787-3797, May 2015.

[11] R. Pilolliand L. Monaci,"Challenging the Limit of Detection for Egg Allergen Detectionin Red Wines by Surface Plasmon Resonance Biosensor". *Food Anal. Methods*, in press, DOI 10.1007/s12161-016-0464-z, March 2016.

[12] J.M. Andrade and M.G. Estévez-Pérez, "Statistical comparison of the slopes of two regression lines: A tutorial*". Analytica Chimica Acta*, vol. nº838,pp. 1–12, May 2014.