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VALIDATION STUDY OF IMMUNOCHEMICAL ELISA ASSAY FOR OCHRATOXIN A QUANTIFICATION IN DESSERT WINES FROM SUN-DRIED GRAPES

ESTUDO DA VALIDAÇÃO DO MÉTODO IMUNOQUÍMICO ELISA PARA A QUANTIFICAÇÃO DE OCRATOXINA A EM VINHOS DOCES DE SOBREMESA ELABORADOS COM UVAS DESIDRATADAS AO SOL

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ABSTRACT

Ochratoxin A (OTA) contents have implications on consumers safety and wine quality. Immunological methods are widely used to determine OTA in various matrices. Solid-phase extraction with immunoaffinity columns containing antibodies specific to ochratoxin A were used to clean-up the samples by two different methods in alkaline medium. Competitive enzyme-linked immunosorbent assay (ELISA) commercial kit was utilized for OTA quantification in dessert wines samples. This ELISA assay uses an instrumental response based on absorbance measurements at 450 nm. The method was performed with standard solutions (provided with the kit), in spiked and naturally contaminated wine samples. The detection limit was 0.054 ìg/L and recoveries of OTA from spiked wine samples, at levels from 1 to 3 ìg/L, ranged from 94 to 102%, with relative standard deviations less than 3%. This ELISA method was checked against an official instrumental method like HPLC with tandem mass spectrometric and with fluorescence detection. ELISA kit method resulted effective for measuring OTA ranging from 0.25 to 9 ìg/L in dessert wines.

RESUMO

Os conteúdos do ocratoxina A (OTA) têm implicações para as qualidades dos vinhos, revelando-se um parâmetro importante para a segurança alimentar e consecuentes implicações para a saúde dos consumidores. Os métodos imunológicos são amplamente utilizados para determinação da ocratoxina A em várias matrizes. A extracção fase-sólida com colunas de imunoafinidade contendo anticorpos específicos para ocratoxina A foram utilizados para a limpeza de amostras por dois métodos diferentes, em meio alcalino. O kit comercial ELISA utilizado na quantificação de OTA em amostras do vinhos doces mostrou-se adequado. Este ensaio ELISA utiliza uma resposta instrumental baseada em medições do absorvência a 450 nm. Foram usadas as soluções padrão provenientes do kit, amostras de vinho doce reforçado e amostras naturalmente contaminadas. O limite de detecção foi de 0.054 ìg/L e as taxas de recuperação de OTA de amostras vinho reforçado de 1 a 3 ìg/L, com variações entre 94 e 102%, e desvios padrão relativos inferiores a 3%. O método ELISA foi comparado com o método cromatografia líquida de alto desempenho e detecção por espectrómetro de massa (MS/MS) e com detector de fluorescência. O kit ELISA revelou-se o método mais eficaz para medir OTA numa gama de 0.25 a 9 ìg/L em os vinhos doces.

Keywords: Ochratoxin A; ELISA; Wine; Mycotoxins; Immunochemical method.
Palavras Chave: Ocratoxina A; ELISA; Vinho; Micotoxinas; Método imunoquímico.

INTRODUCTION

Ochratoxin A (a derivative of isocumarin linked to *L*-β-phenylalanine) is a secondary metabolite produced by *Aspergillus* sp. (*A. ochraceus, A. carbonarius* and *A. niger*) and *Penicillium* (*P. verrucosum*, *P. nordicum* and *P. viridicatum*) species on several agricultural and animal commodities (feed, food and beverages) (Abarca *et al.*, 2001). OTA (Figure 1) is a highly toxic mycotoxin which nephrotoxic, carcinogenic, immunotoxic, and teratogenic activities are well documented (López de Cerain *et al.*, 2000). The presence of OTA on grapes, wines and dried vine fruits is mainly due to the presence of *Aspergillus* section *Carbonarius* and *Niger aggregate* (Abarca *et al.*, 2001; Bellí *et al.*, 2004). The range of OTA contents detected in wines produced in Europe varied between 0.01 and 3.4 ìg/L (Battilani and Pietri, 2002). In fact, OTA has become a seriously and restrictive factor for the export and commerce of wines, and as a consequence a regulation including maximum levels for table wines (2 ìg/L) and raisins (10 ìg/kg) has been adopted in the European Community (Commission Regulation, 2002 and 2006).

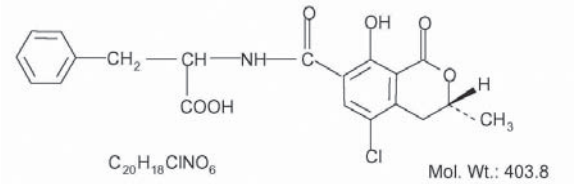

Fig. 1 - Chemical structure of ochratoxin A.
Estrutura química da ocratoxina A.

Several studies reported the detection of higher OTA contents in raisins and dessert wines from sun-dried

and overripe grapes than those found in white and red table wines (MacDonald *et al.*, 1999; Bellí *et al.*, 2002). Mold growth and toxin production depends on several factors like geographical and climate conditions (rain and relative humidity), training vineyard or fungal strain (Pardo *et al.*, 2005). Moreover, Bau *et al.* (2005) revealed the risk of OTA production increases sharply with the ripening of berries, which can explain high levels of OTA found in these special sweet wines, probably associated with the particularities of its process of elaboration. However, considering that the overall consumption of sweet wines is occasional the contribution to daily intake of OTA could be considered very small.

Many different analytical methods have been employed to determine OTA content in wines and in a variety of matrices (Van Egmond, 1996; Valenta, 1998; Monaci and Palmisano, 2004). Recently, the combination of HPLC with selective detection system as mass spectroscopy (MS) is used for OTA determination in multiple foodstuffs and commodities (Lindenmeier *et al.*, 2004; Timperio *et al.*, 2006). However, high-performance liquid chromatography with fluorescence detection (HPLC-FD) and clean-up with immunoaffinity column (IAC) or solid-phase extraction (SPE) is the technique most commonly used (Visconti *et al.*, 1999; Leitner *et al.*, 2002; Gozález-Peñas *et al.*, 2004; Sáez *et al.*, 2004; Hernández *et al.*, 2006). Reversed-phase HPLC-FD with sample dilution (PEG and $NaHCO_3$) and cleaned-up using IAC has been adopted by Organisation Internationale de la Vigne et du Vin and AOAC International like official analysis method (Bezzo *et al.*, 2000; Visconti *et al.*, 2001; AOAC, 2002). These chromatographic methods requires very expensive equipment, with high maintenance needs, being time consuming and necessitate qualified and trained personnel, all factors which make them unsuitable for routine use in the food industry.

The immunochemical methods are a valid alternative to analyzed OTA. Furthermore, especially enzyme linked immuno-sorbent assay (ELISA) proved to be sensitive, selective, and cost effectiveness for the routine use and screening analysis of mycotoxins in most of the matrices, including ochratoxin A (Morgan, 1989; Kawamura *et al.*, 1989; Barna-Vetro *et al.*, 1996; Solti *et al.*, 1997; Kwak and Shon, 2000). This kind of method is applicable to several classes of samples, so it will be necessary to validate in this matrix. Although chromatographic methods are more accurate and sensitive than ELISA techniques, for its simplicity, high specificity and speed, as well as the number of samples that can be analyzed at the same time ELISA method has advantages. Moreover, it requires minimal sample preparation and equipment and does not involve organic solvents. However, the current ELISA kits should be used as quantitative method taking some precautions, since the technique is susceptible to physicochemical factors such as pH, selectivity (cross-reactivity) of antibody used, to the clean-up procedure or to the matrix interferences coming from the samples (Crowther, 2001). In both techniques it is necessary to extract the analyte from the sample by applying a clean-up procedure in order to remove interferential substances. The solid-phase immunoaffinity columns, despite its high economic cost, they contain monoclonal antibodies specific for OTA which are indispensable in the ELISA assays if the goal is to quantify OTA contents.

The aim of this study was to validate in-house commercial ELISA kit for ochratoxin A in sweet dessert wines produced from sun-dried grapes. This ELISA method has not been validated in this type of matrix until now. The method is evaluated according to selectivity, limits of detection and quantification, linearity, recovery and precision (intra-day and inter-day). Finally, some appropriate extraction methods were examined to extract OTA from these sweet wines.

MATERIALS AND METHODS

Samples

Natural sweet wines were made from sun-dried grapes cv. Pedro Ximenez white variety from the denomination of origin "Montilla-Moriles" area (Cordoba, Spain). Healthy grape bunches were dried post-harvest by direct exposition to sun. Once transported the grapes to the winery, these are crushed and pressed in horizontal pneumatic presses. After, the must is fortified with ethanol at 9% (v/v) alcoholic strength. These wines have a natural dark color and the total soluble solids around 40 ºBrix degrees. The pH values ranging from 3.9 to 4.4 and titratable acidity are between 3 and 4 g/L (in tartaric acid).

Analytical reagents

All reagents used in this work were of analytical grade or higher. Sodium phosphate 0.4 M buffer prepared with Na_2HPO_4 and NaH_2PO_4 was adjust to pH 7.5. Phosphate-buffered saline PBS 10 mM with 0.05% Tween 20 was purchased from (Sigma-Aldrich Quimica, Madrid, Spain). Ethanol absolute, sodium hydrogen carbonate, sodium dihydrogen phosphate 1-hydrate and disodium hydrogen phosphate 2-hydrate were provided by Panreac (Barcelona, Spain). Methanol (HPLC grade) and polyethylene glycol 8000 (PEG) were supplied by Scharlau (Barcelona, Spain). Distilled-deionised water was prepared in the laboratory using water desmineralize equipment previous to distillation system (Pobel, Madrid, Spain). For ELISA assay was used purified water HPLC grade (Panreac, Barcelona, Spain). All the glassware used was rinsed with methanol and dried for 24 hr.

Standard solution preparation

Stock standard solution of OTA (200 ìg/mL) was prepared by dissolving 1 mg OTA powder standard (Sigma-Aldrich Quimica, Madrid, Spain) in 5 mL of absolute ethanol.

Working standard solution was prepared by dilution of the stock solution: 1 mL up to 25 mL with absolute ethanol at 8 ì g/mL concentration. The concentration was calculated by UV spectrophotometry assuming a molar absorption coefficient of 5500 M^{-1} cm^{-1} at 333 nm (Xiao *et al.*, 1995).

Wine control samples containing OTA 1.00, 1.50, 2.00, and 3.00 ìg/L were prepared derived from the working standard solution, which was added to each samples prior to extraction and incubated overnight in dark and then assayed in triplicate. All the solutions were stored refrigerated at 4 ºC.

Ochratoxin A Extraction

Sample extraction with sodium hydrogen carbonate and PEG (method A).

A variation of the methodology described by Serra *et al.* (2004) to grapes was followed. 30 mL of sample was poured into a glass beaker and adjusted to pH 7.8 with NaOH 4 M. 20 mL of this neutralized sample was measured and adding 20 mL PEG solution (5% $NaHCO_3$, 1% PEG 8000). The sample was homogenized and prepared during 15 min in a magnetic stirrer and then centrifuged (15 min to 1479 × *g*). The supernatant was added in a syringe and filtered with a glassfiber filter with retention rating of 1.6 ì m (Millipore, Bedford, USA). An aliquot of 12 mL was taken and passed through the immunoaffinity column. In our validation study extraction method with PEG-$NaHCO_3$+IAC was used.

Sample extraction with sodium phosphate 0.4 M buffer (method B).

The samples were centrifuged at 524 × *g* for 10 min before analysis only if is presented cloudy. The wine was adjusted to pH 7.8 using NaOH 4 M. A 6 mL of this wine was mixed with a 6 mL of 0.4 M sodium phosphate buffer and then passed through the immunoaffinity column.

Immunoaffinity clean-up

Samples were cleaned-up by using RIDA® immunoaffinity columns purchased from R-Biopham AG (Darmstadt, Germany). 12 mL volume of diluted wine, prepared as previously described, was cleaned up through an AIC at a slow flow-rate of about one mililiter per minute. The columns were washed with PBS-Tween buffer and 20 mM sodium phosphate buffer, and finally dried up in an air stream. The final elution of OTA from the IAC was carried out using 1 mL of methanol through the column by gravity and collected in a glass vial. The extract eluted from the IACs was diluted with purified water and 0.13 M sodium hydrogen carbonate buffer before ELISA assay.

Competitive ELISA

Ochratoxin A was quantified by using RIDASCREEN® Ochratoxin A kit purchased from R-Biopham AG (Darmstadt, Germany). This analysis was carried out according to the method described by the application note (Ridascreen, 1999). Assays were performed in 96-well microtitre plates Nunc (Roskilde, Denmark). Standard solutions and prepared samples (50 ìL) were added with the same amount of diluted enzyme conjugate to each precoated well. After 30 min incubation at room temperature in the dark, the plate was washed with 3 x 250 ìL washing buffer (PBS with 0.05% v/v Tween 20) per well. 100 ìL of substrate chromogen solution (tetramethylbenzidine) was added to wells and allowed to incubate for 15 min. When the stop solution (100 ìL for each well) was added the color changed from blue to yellow. The measurements of optical density for 96-well microtitre plates were performed on an automated microplate reader model ELx800™ (Bio-Tek Instruments, Winooski, VT, USA) at 450 nm. For data processing was used RIDA®SOFT Win software. The results were corrected with the percentage of recovery of the columns. All the samples were done in duplicate.

The kit standard solutions (0, 0.05, 0.1, 0.3, 0.9 and 1.8 ìg/L) were subjected to analysis to give an OTA calibration curve, by plotting the percentage of optical density, OD B of standard / OD B_0 of blank values (*y*-axis) against the OTA concentration (*x*-axis). The resulting absorbance values are inversely proportional to the OTA concentration of samples; and the cubic spline method was used to fit the calibration curve.

HPLC-MS/MS and HPLC-FD analysis

These analyses were performed by two external public official Laboratories. For HPLC-MS/MS OTA control, the samples were not cleaned-up previous to analysis (Timperio *et al.*, 2006). The HPLC-FD method was performed according to the official method (Bezzo *et al.*, 2000; CEN, 2003).

Statistical methods

The statistics programme SPSS® 12 for Windows and Microsoft Excel® 2003 were used. The analytical results represent an average ± standard deviation of two determinations obtained for each parameter. The t-paried test statistical analysis was used for significant differences and simple correlation coefficients. Different criteria were followed for the validation, gathered by Miller (2000) and OIV (2005).

RESULTS AND DISCUSSION

Working range

The commercial kit range of ochratoxin A quantification was from 0.25 ìg/L to 9 ìg/L, data shown by the specifications of kit. With the standards kit at 5 different concentration levels (+ blank) a non-linear calibration curve (n = 5) was obtained, each time the method was carried out. Repeated standard curves resulted in curves very similar in shape and parameters (Figure 2a). Moreover, the linearity and repeatability were evaluated with these standard solutions. The principal results of the regression line study are presented in Table I.

The standard deviation and the coefficient of variance were calculated for each concentration (data not showed). The standards displayed a coefficient of variation below 1% and standard deviations between 0.02 and 5.44 ìg/L, the values being higher when the standard concentration increases (Figure 2b).

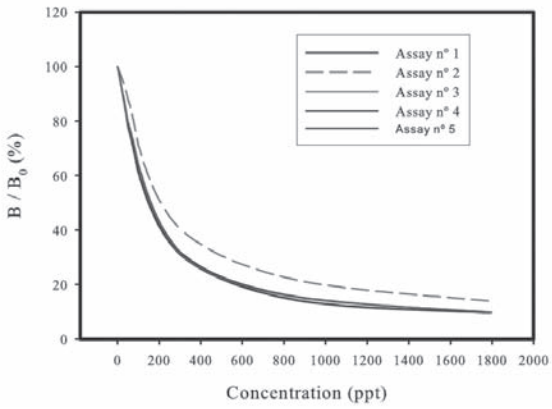

Fig. 2.a. - Calibration curves obtained at five different days and with two ELISA kits.
Curvas de calibração obtidas em cinco dias diferentes e com dois kits ELISA.

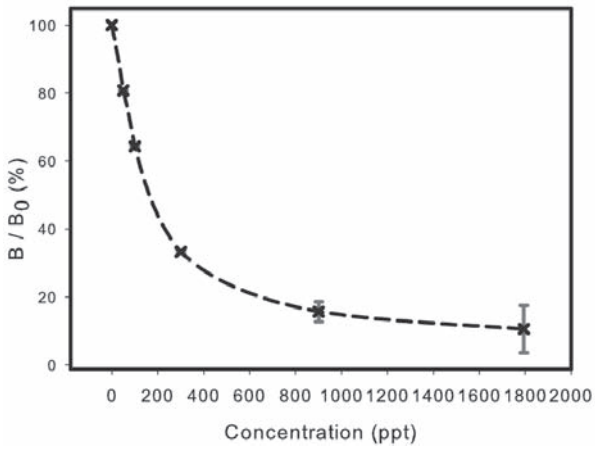

Fig. 2.b. - Mean of five calibration curves with interval of confidence of 99%. Each data point represents the mean of duplicate analysis for the specific standard curve calibration.
Média de cinco curvas de calibração com intervalo de confiança de 99%. Cada ponto representa a média de dados duplicados de análise para a curva de calibração específica.

TABLE I

General data results of validation for the method
Resultados gerais de validação para o método.

Parameter	Procedure	ELISA Data
Linearity	Spiked recovery tests $Y_{found} = a + b\ x_{added}$ Values ± SD	Slope (b) = 1.034 ± 0.017 Intercept (a) = - 0.062 ± 0.038 r = 0.988
	Standard curves (n = 5) Values ± SD	Slope (b) = 0.997 ± 0.001 Intercept (a) = 1.071 ± 1.222 r = 1.000
Accuracy	Paired t-test of HPLC-FD values and ELISA*a* values of 9 wine samples (P = 0.05)	Avg. Diff. = 0.151 Desv. Est. Dif. = 0.227 t exp = 0.949 t critical = 2.31
Repeatability	1 sample analyzed by the same operator (n = 5)	mean = 2.962 µg/L Std. dev.= 0.106 % CV = 3.58%
Intralaboratory reproducibility	1 sample analyzed by 2 operators in 10 different days (n = 10)	Mean = 2.882 µg/L Std. dev. = 0.135 % CV = 4.68%
Repeatability	Range levels 0 to 1 µg/L (n = 30) 1 to 3 µg/L (n = 30) 3 to 8 µg/L (n = 30)	 r = 0.179 ; % CV mean = 9.10 r = 0.380 ; % CV mean = 5.57 r = 0.990 ; % CV mean = 6.05

Selectivity

The use of immunoaffinity techniques assured the selectivity of this method. When OTA standard was added to OTA free-samples, an increase of OTA was observed. As reported in the kit insert, the ELISA test is very specific towards OTA (100%), only demonstrating a small amount of cross reactivity with ochratoxin *á* (< 0.1%), ochratoxin B (14%) and ochratoxin C (44%). Cross-reactions can affect results by indicating a false positive or by elevating the predicted concentration.

Limit of detection and quantification

According to OIV (2005) we calculated a limit of detection of 0.054 ìg/L with the values of the lowest standard close to the blank (n = 10) for a level of confidence of 99%. The instructions of the manufacturer show the limit of detection of approximately 0.050 ìg/L in beer. The uncertainty of the LOD is defined by the uncertainty of the standard deviation (Huber, 2003), being 0.04 and 0.10 ìg/L the lower and upper limits of confidence, respectively, with a level of confidence of 95%. The study of sensibility indicated that the limit of quantification (LOQ) of the method was 0.062 ìg/L. On the other hand, a result greater than 0.131 ìg/L indicates, 99 times in 100, the presence of OTA in the sample.

Recovery analysis and Specificity

Separation procedure must be applied to wine samples prior to immunoassay by immunoaffinity column. The recovery experiments were performed in triplicate on spiked wine samples at different concentrations. The range level of recovery was elected to fill the usual OTA concentration levels of these sweet wines.

In parallel, the wine used to verify the content of previous OTA contamination (0.20 ìg/L) was analyzed. The average of recovery obtained by the described method for OTA was 99.7% (average coefficient of variation of 2.57%). The results are shown in Tables I and III. Analysing data of the recovery test, we verify the absence of any interference and an acceptable specificity. The calculation of the Student critical bilateral value shows that the slope of the regression line is equivalent to 1 and the intercept point is equivalent to 0, at a 99% confidence level. This method meets with the requirements of CEN (Recoveries of 70% to 110% and RSD < 20% for levels from 1 to 10 (ìg/Kg) (CEN, 1999). The recovery results are comparable to the published ones in the official method, from 88% to 105% and 84% to 93% for white and red wines, respectively (Visconti *et al.*, 2001).

Accuracy

As it can be observed previously the recovery values were between the 94% and the 102% (Table I), showing the good accuracy of the method used. Applying the Student´s t-test for a confidence level of 95% it is possible to affirm that significant differences do not exist between the obtained values and the awaited ones.

On the other hand, the accuracy of the ELISA kit method in relation to reference method (HPLC-FD) is based on a series of wine samples with concentration values in analyte covering the range level. Data were analyzed using t-test (Table I) and calculating the Z_{score} that was lower than 2. So, ELISA can be considered accurate in relation to the chromatography method in the range level under consideration, with a risk of error $\alpha = 5\%$.

Precision

The precision of the ELISA assay was determined by assaying data from calibration curves of kit standards in duplicates and with quality control samples (OTA spiked or naturally contaminated wines). The results

TABLE II

Precision data at five levels with the OTA standards.

Determinaçao da concentraçao de OTA nos padrões do kit ELISA.

Standards Concentration	Kit 1 (n = 4)		Kit 2 (n = 4)		Kit 3 (n = 5)		Inter-assay Precision (n = 3)		Total Precision (n = 13)	
	X	CV	X	CV	X	CV	Xi	CV	Deviation	CV
(ppt)	(ppt)	(%)	(ppt)	(%)	(ppt)	(%)	(ppt)	(%)	(%)	(%)
50	50.09	0.18	50.07	0.04	50.03	0.06	50.06	0.09	0.12	0.09
100	99.74	0.17	99.74	0.04	99.84	0.11	99.77	0.11	0.23	0.11
300	300.49	0.10	300.74	0.03	300.55	0.07	300.57	0.07	0.19	0.07
900	901.37	0.17	901.03	0.05	899.26	0.61	900.55	0.28	0.06	0.27
1800	1792.7	0.16	1792.91	0.07	1792.67	0.19	1792.76	0.14	0.40	0.14

X: mean of the replicas; Xi: mean of means of each kit; CV: coefficient of variation

TABLE III

Recovery data in wine sample spiked at five levels with OTA standard

Dados de recuperação em amostras de vinho reforçado em cinco níveis com padrão do OTA

Fortification level (µg/L) (n = 3)	Amount recovered Mean ± SD (µg/L)	Recovery mean (%)	Coefficient of variation (%)
1.00	1.144 ± 0.02	94.4	2.12
1.50	1.717 ± 0.04	101.1	2.49
2.00	2.201 ± 0.06	100.1	2.91
2.50	2.763 ± 0.07	102.5	2.52
3.00	3.207 ± 0.09	100.2	2.81
Mean of means		99.7	2.57

obtained for the precision test using different kits are show in Table II. The intra-assay precision coefficients of variation were below 1% for OTA kit standards analyzed. For each concentration, individual samples showed very similar calculated concentrations when measured on the same microplate and at different times showed a slight increase in variability (Table II). The little variability in the data for standards ensures good precision of the results for the wine samples analyzed.

In another precision study in sweet wine with high OTA content naturally contaminated, for the analysis of this wine all the process was repeated each time including extraction, on the same or different microplate with different curves. So, the same sample was extracted ten times on different days by two analysts. The value of intralaboratory reproducibility (R) means that in the 95% of the cases, the difference between two values obtained by the method, under the conditions defined, will be lower than or equal to 0.213 ìg/L.

The results obtained by the method under study will have a repeatability rate lower than 0.173 ìg/L with a probability of 95%. Repeatability is the closeness of agreement between mutually-independent analysis results obtained on the same sweet wine, with the same operator within a short period of time (OIV, 2005). The coefficient of variation values for intra and inter-day precision were less than 5% (Table I).

Another repeatability test was calculated for the values obtained of each duplicate analysis of multiple different sweet wines samples. It is not necessary for the repeatability conditions to be maintained from one wine to another, but all the replicas (in this case two) carried out on the same wine must be carried out under repeatability conditions. It defined several range levels to calculate repeatability values (Table I). In this case, the well-to-well average CV values go up to 9% for the lower interval concentration, the repeatability values being greater when OTA concentration of the wines analyzed increased.

The results showed that this ELISA had a good repeatability, and inter-assay/total precision for the sweet wines analyzed and satisfies minimum performance criteria established by OIV.

Extraction method

Wines containing high levels of phenolics produces a decrease of recovery, moreover especially if higher sample volumes where loaded onto columns (Castellari *et al.*, 2000). The sweet wines utilized contain high values of proteins and reducing sugars, and in some cases contained elevated phenolic compounds. Ethanol and sugar wine content did not inference in the clean up by immunoaffinity columns (Ratola *et al.*, 2004). The correction of pH samples is critical before the extraction clean-up (Valenta, 1998; Visconti *et al.*, 1999), in our case the values of pH were those that Castellari *et al.* (2000) proposes. The volume of sample used is comparable to those used in other works. For the study, nine wine samples were extracted by the two methods. The preparation of the sample with sodium phosphate buffer made it necessary to pass the eluate twice to obtain a clear liquid from the IACs. In contrast if the polymer PEG-$NaHCO_3$ is used, the result is a totally clear eluate in one go. So, in general both extraction methods produce results similar in comparison with HPLC-FD data (Figure 3).

Comparison to reference HPLC-MS/MS and HPLC-FD methods

The results of nine different naturally contaminated wine samples were confirmed by two independent high-performance liquid chromatography techniques. The same samples were tested in two laboratories using different detection methods for quantifying OTA. This confirmation increases confidence in the ELISA technique here reported. There was good correlation data (R^2 = 0.788 and 0.960) between ELISA assay and HPLC-MS/MS and FD results, respectively (Figure 3). The squared r correlation coefficients are very large since the small number of samples analyzed by the diverse methods. Furthermore, analysis of variance showed that there is a statistically significant relation between the results

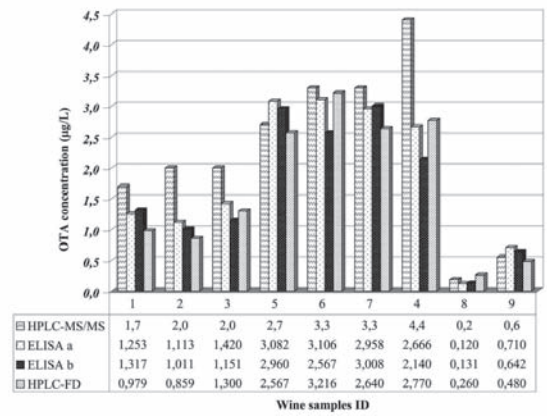

Fig. 3 - Vertical bar chart of the ELISAs vs. HPLC methods for the determination of ochratoxin A of PX dessert wine samples. ELISA **a**: extraction with sodium hydrogen carbonate and PEG; ELISA **b**: extraction with sodium phosphate buffer. *Diagrama de barras verticais dos métodos ELISA versus HPLC para a determinação de ocratoxina A em amostras de vinho doce PX. ELISA **a**: extracção com hidrogenocarbonato de sódio e PEG; ELISA **b**: extração com tampão fosfato de sódio.*

obtained by HPLC and ELISAs with a 95% confidence level (Table I).

For the two methods of clean-up samples, the method A (with PEG solution) showed better correlation results than method B with the HPLC-MS/MS. It is possible that the diluted sample dirties the AIC in the method B reduce the interaction between analyte and antibody. Systematic light overestimates for OTA has been determined in these wines using ELISA with respect HPLC-FD results. This circumstance was reported previously by Sáez *et al.* (2004).

The only significant discrepancy was the high value of sample nº 4 (Figure 3) by HPLC-MS/MS, that possibly is an outlier, but if applied the Grubbs´s test (P = 0.05) the suspected measurement can not be rejected. The lack of purification of the samples, in the HPLC technique with MS detector, can lead to an overestimation of the results.

CONCLUSIONS

The special analytical features present in sweet wines made from raisins, as a consequence of their winemaking process, requires in-house validation of the immunological method used for OTA control. The joint use of immunoaffinity columns with the ELISA commercial kit utilized in this work produces good results, and the data obtained are consistent with HPLC results. OTA can be quantitatively determined from sweet wine samples using this analytical methodology. The immunochemical (ELISA) method utilized to control OTA is sufficiently accurate, simple and specific for rapid routine analysis control of dessert wines winemaking.

Two clean-up procedures were applied and both showed to be effective for OTA extraction, but the PEG method gave cleaner extracts and better extraction results. Overestimated results are sometimes possible mainly due to the matrix effects in the sweet wines studied or by cross-reactivity of antibody used in the kit. The detection limit of the kit is considered very good below the typical OTA ranges in these sweet wines. On the other hand, the use of the relatively high cost immunoaffinity columns is necessary to obtain the better results by this technique.

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