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FLUOROMETRIC VALIDATION PROCEDURE FOR DETERMINATION OF OCHRATIOXIN A IN WINE

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ABSTRACT

Fluorometry with previous immunoaffinity column clean-up is a method for determination of ochratoxin A in wine which is validate in order to evaluate its performances. The linearity of the method was checked, and a good coefficient of correlation (0,9814) was found. The limit of detection was satisfactory (0,199 ng/ml). Repeatability, as measurement of the precision, estimated through RSD values showed acceptable value only for the concentration level of 0,1 ng/ml (6,47%), but too high for concentration level of 1,0 ng/ml (17,86%). This is a big deviation from true value, especially when red wine samples are analysed. It is a due to the red colour present in the final eluat, which gives high readouts and it can produce false positive results when fluorometry was applied. However, due to the factors that influence fluorometric analysis, it was found that it presents relatively accurate, precise and selective, quantitative method in the field of determination of ochratoxin A in wine. Fluorometry can be applicable only as a screening method for the prediction of ochratoxin A contamination in wine, especially in the laboratories who are dealing with a big number of samples for mycotoxins analysis.

Keywords: method validation, ochratoxin A, immunoaffinity columns, fluorometry, wine.

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin mainly produced by some species of the genera *Aspergillus* and *Penicillium*. It might contaminate agriculture commodities (cereals and cereals products, wine, beer, grape juice, coffee, cocoa and cocoa products). OTA receives increasing attention as there is growing evidence that this mycotoxin might be responsible not only for intoxication in livestock after consumption of contaminated food and feed, but may also be involved in the etiology of Balkan endemic nephropathy (1). OTA is nephrotoxin to all animal species (kidney is the most sensitive target organ). It also exerts immunotoxic, teratogenic, genotoxic, mutagenic and carcinogenic effects at higher dose levels (2); therefore, presents serious risks for the human health. The International Agency for Research on Cancer (IARC) evaluated OTA as a possible carcinogen in humans (group 2B). The intake of different contaminated food and drinks might provide a total amount of OTA near 100 ng per kg body weight that presents a PTWI (provisional tolerable weekly intake) set by the World Health Organization (3). A maximum residual level (MRL) for OTA has been established by the Commission Regulation (EC) No. 123/2005 and it is in the range from 0,5 to 10 μg/ kg for different commodities. Our country has adopted the EU regulations since December 2005 (4).

Wine OTA contamination has been reported all over the world (5) considered the fact that it is the second major source of OTA intake (13 %). Wine is a product significantly important for the European economy and population and therefore it requires from each member or EU exporter country to carry out systematic surveys to assure that the wine is OTA-free and safe.

Different analytical methodologies have been established for OTA determination (6). Fluorometry is an analytical method for determination of ochratoxin A and mycotoxins in general, which has no such selectivity, accuracy and sensitivity as LC-FD, but it can be used as a screening method, especially in the laboratories who are dealing with a big number of samples for mycotoxin analysis. The use of immunoaffinity columns (IAC) for the clean-up procedure is highly recommended, allowing the isolation of the analyte from most matrix interferences, due to its selectivity.

The aim of this work was to evaluate the method performances for the fluorometry and to carry out the method validation. Those parameters would be used to set a fluorometry as a screening method for prediction of ochratoxin A in wine. Then, thirty wine samples were analysed with this method.

MATERIALS AND METHODS

Apparatus

The fluorometer (Vicam V1, series 4) was delivered from Vicam (Watertown, MA, USA). It is necessary to check purity of chemicals before employing the analysis. One (1) ml of water should give zero fluorescence (blank sample) and 1 ml OchraTest™ Eluting Solution should also give zero value for fluorescence. In this manner the quality of the cuvettes was also checked. Fluorometric calibration was done according to the manufacturer (7) with mycotoxins calibration standards: red calibration standard with maximum value of 36 ng/ml and green

calibration standard with minimum value of -3 ng/ml. The performed calibration was checked with yellow calibration standard which readings should be in the limited range.

Reagent and standard solutions

HPLC grade solvents (methanol, acetonitrile, water) benzene, NaCl, NaOH, $NaHCO_3$ (pro-analysis grade chemicals) and glacial acetic acid 100% (suprapur) were delivered from Merck (Darmstadt, Germany). PEG 8000 was purchased from Biochemica, Fluka. OchraTest™ Eluting Solution was from Vicam (Watertown, MA, USA).

Immunoaffiity OchraTest® columns (Vicam, USA) were used for clean-up procedure. OTA standard was purchased from Supelco (50 µg/ml, dissolved in benzene:acetic acid (99:1)). A stock solution (2 µg/ml) was prepared from this solution by diluting an aliquot with solvent mixture and was further kept at + 4°C. 1,5 ml of stock solution was transferred into a silanized vial and evaporated under a stream of nitrogen. The content was redissolved in a vial with 1,5 ml LC mobile phase (filtered through a 0,20 µm filter) and quantitatively transferred into a volumetric flask of 25 ml and diluted to volume with the filtered mobile phase. The final OTA concentration in this solution was 100 ng/ml. According to the official AOAC method (8), five working standards in a range from 0 to 1,0 ng OTA/ml were prepared and used for calibration.

Wine samples

The samples were originated from wine producing region in Macedonia and were purchased from a local store. The samples were kept sealed in the refrigerator at + 4°C, in their original bottles until analyses. All samples were analyzed to find OTA level and they were run in duplicate. For the recovery experiment, OTA-free red and white wine samples were spiked with known amount of OTA solutions at two levels (0,1 ng/ml and 1,0 ng/ml).

Analytical procedure

All wine samples were always degasified in ultrasonic bath for 20 min before treatment. 10 ml of wine was diluted and mixed vigorously with a solution containing 5 % $NaHCO_3$ and 1 % PEG 8000. The pH was adjusted to 8,5 with 1 M NaOH. Then, solution was filtered through glass microfiber filter and 10 ml of filtrate were applied onto an IAC (OchraTest™ column) at a flow rate of about 1 drop/sec. The washing step was performed with 5 ml of washing solution (2,5 % NaCl and 0,5 % $NaHCO_3$) and then with 5 ml water at a flow rate of 1-2 drops/sec. The column was dried by passing air through it, and afterwards, OTA was eluted with 2 ml of OchraTest™ Eluting Solution at a flow rate of about 1drop/sec in a glass cuvette. Reading of ochratoxin A concentration was after 60 sec. The OchraTest™ Eluting Solution contain 0,1 N NaOH instead of methanol, because this solution increase the signal of fluorometer in its operating range (excitation 360 nm and emission 450 nm).

RESULTS AND DISCUSSION

Method validation procedure was performed according to the manufacturer instruction (7). The range of the fluorometer was determined by spiking OTA-free (LC-FD determined) white wine samples at concentrations of 0; 0,1; 0,2; 0,5 and 1,0 ng/ml. Each sample was run in duplicate. The results are presented in the Table 1. We used lower concentration range because maximum measurement level of the fluorometer was 36 ng/ml. The choice of using white wine samples was made concerning the fact that red wine samples have complex matrix and the red colour was present in the final eluat (gives high readouts and false positive result).

Table 1. *Range of the method*

spiked concentration (ng/ml)	detected concentration (ng/ml)	mean concentration (ng/ml)
0	0 0	0
0,1	0,164 0,296	0.23
0,2	0,396 0,52	0.458
0,5	0,44 1,28	0.86
1,0	2,28 2,28	2.28

Linearity of the method was checked performing calibration curve as correlation between spiked and detected concentrations. The correlation coefficient was 0,9814 which means a good and satisfactory linearity, but as can be seen from the Table 1, there is a big deviation from a true value, probably as a result of others interfering fluorescence substances.

For this study limit of detection (LOD) was defined as follows: LOD = mean + 3 SD, where the mean is determined from readouts of ten (10) OTA-free samples (determined by LC-FD) and SD is the standard deviation of those 10 readouts. LOD using this protocol was 0,199 ng/ml.

Repeatability, as measurement of the precision, estimated through RSD values was determined using spiked OTA-free white wine samples with two concentration level (0,1 and 1,0 ng/ml). Each sample was tested six (6) times. The mean, standard deviation and RSD were determined and they are shown in the Table 2.

As can be seen from the Table 2, there is a big deviation from true values as in the case of range determination. Especially high percent of RSD was found for concentration of 1,0 ng/ml which is 17,86 %.

Thirty wine samples were examined employing fluorometry and compared with LC-FD in order to make comparison between two methods. All wine samples were with OTA concentration level bellow LOD, but there are differences between results obtained from bought methods. Those differences are coming especially at red wine samples. It is due to the red colour present in the final eluat, which gives high readouts and it can produce false positive result when fluorometry was performed (9).

CONCLUSIONS

As we can see from the results, fluorometry present relatively accurate, precise and selective quantitative method for determination of OTA in wine. The method show good coefficient of correlation (0,9814) and satisfactory limit of detection (0,199 ng/ml). The RSD value (as measurement of precision) was satisfactory only for the concentration level of 0,1 ng/ml (6,47 %), but too high for concentration level of 1,0 ng/ml (17,86 %). The other disadvantage was high readouts and false positive results when red wine samples were analysed. On the other hand the method is safe, simple, easily performed in less than 10 minutes and requires no special skills and there is no need for expensive instrumentation.

For those reasons, immunoaffinity column clean-up followed by fluorometric determination can be used only as a screening method for a prediction of ochratoxin A contamination in wine and other methods (LC-FD) should be applied as method of choice for the determination of ochratoxin A in wine.

Table 2. *Repeatability of the method*

number of samples	**0,1 ng/ml**	**1,0 ng/ml**
	detected concentration (ng/ml)	
1	0,22	2,28
2	0,208	3,36
3	0,208	2,64
4	0,216	3,0
5	0,2	3,4
6	0,24	2,28
mean	**0,215**	**2,82**
STD	**0,013**	**0,505**
RSD (%)	**6,47 %**	**17,86 %**

REFERENCES

1. Creppy E.E. (2002). Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicology Letters 127, 19-28.
2. FAO FOOD AND NUTRITION PAPER. (1990). Manuals of food quality control. Training in mycotoxins analysis. No. 14/10. Rome
3. FAO/WHO (Food and Agriculture Organization/World Health Organization) (1996). Toxicological evaluation of certain food additives and contaminants. Joint FAO/WHO Expert Committee on Food Additives (JECFA). WHO Food Additives Series 35. Geneva, Switzerland.
4. Regulation for general requirements in food safety (2005). Official Gazette in R. of Macedonia No. 118/2005.
5. Pacin A., Rasnik, S., Vega, M., Saelzer, R., Ciancio Bovier. E., Rios, G., and Marinez, N. (2005). Occurrence of ochratoxin A in wines in the Argentinian and Chilean mar-

kets. ARKIVOC (XII) 214-223.
6. N.Ratola, P.Barros, T. Simões, A.Cerdeira, A.Venâncio, A.Aves. (2006). Worldwide interlaboratory study on the determination of ochratoxin A in different wine type samples. Talanta 70, p. 720-731.
7. OchraTest Instruction Manual, (1999). Vicam (Watertown, MA, USA).
8. AOAC Official Methods of Analysis (2005). Chapter 49, p.66. AOAC Official Method 2001.01. Ochratoxin A in wine and beer. Immunoaffinity colum clean-up/liquid chromatographic analysis (First action 2001, Final action 2005).
9. Biljana Stojanovska-Dimzoska, Zehra Hajrulai-Musliu, Elizabeta Dimitrieska-Stojkovic, Pavle Sekulovski. (2010). Comparison of two different analytical methods for determination of ochratoxin A in wine. 32nd Mycotoxin Workshop, Demark, poster presentation p.25

ФЛУОРОМЕТРИСКА ВАЛИДАЦИОНА ПРОЦЕДУРА ЗА ОПРЕДЕЛУВАЊЕ НА ОХРАТОКСИН А ВО ВИНО

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АПСТРАКТ

Флуорометријата со претходно пречистување со примена на имуноафинитетни колони е користен метод за определување на охратоксин А во вино, валидиран со цел да се евалуираат неговите перформанси. Линеарноста на методот е проверена и утврдена е добра вредност на коефициентот на корелација (0,9814). Лимитот на детекција беша задоволителен (0,199 ng/mL). Повторливоста, како мерка за прецизноста, е проценета преку RSD, чии вредности беа прифатливи само за концентрациското ниво од 0,1 ng/mL (6,74 %), но беа премногу високи за ниво на концентрации од 1,0 ng/mL (17,86 %). Ова е значително отстапување од средните вредности, особено за анализа на примероци од црвено вино. Тоа е заради присутната црвена боја во конечниот елуат, кој дава високи отчитувања а со тоа и лажно позитивни резултати при примена на флуорометријата. Меѓутоа и покрај факторите кои влијаат на флуорометриското определување, утврдено е дека таа претставува релативно точен, прецизен и селективен метод за определување на охратоксин А во вино. Флуорометријата може да биде применлива само како скрининг метод за проценка на контаминацијата на вино со охратоксин А, особено за лаборатории кои имаат голем број на примероци за анализа на микотоксини.

Клучни зборови: валидација на метод, охратоксин А, имуноафинитетни колони, флуориметрија, вино.