# A Robust Method for Determination of Ochratoxin A in Wine Samples by SPE and HPLC-FLD

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*The aim of this work was the developing of a sensitive, accurate, reproducible and inexpensive HPLC with fluorescence detection method for the analysis of ochratoxin A in wine samples. Mobile phase composition consisted in methanol/aqueous component (0.1% phosphoric acid) in the ratio 60/40 (v/v). Temperature column was set up to 20°C. The sample clean-up and enrichment were achieved using the C-18 solid phase extraction (SPE) cartridges and compared to those achieved with immunoaffinity cartridges. The procedure based on C18 cartridges has been found to be cheaper, more robust and more convenient than the one based on immunoaffinity cartridges. The analytical method was characterized by a linearity domain situated within 0.1 – 10 μg $L^{-1}$ OTA in wine matrix, with a determination limit of 0.06 μg $L^{-1}$, for an injection volume of 50 μL. The method was validated and applied to several Romanian wine samples of various proveniences.*

*Keywords: ochratoxin A, wine samples, HPLC-FLD, C18-SPE, immunoaffinity cartridge*

Ochratoxin A (OTA), [7-(L-β-phenylalanyl-carbonyl)-carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocumarin], is a secondary metabolite produced by several species of *Aspergillus* (*A. carbonarius*) and *Penicillum* (*P. verrucosum*) moulds. Around the world OTA is found more frequently in stored grain. OTA makes its way into a variety of food and beverages, particularly cereal and grain products, but also dried food, wine, coffee, beer, cocoa, juices, spices, pork, poultry and dairy products [1]. Since 1996, OTA contamination has been reported in wine and grapes juices [2]. Wine could account for as much as 15% of the total daily OTA intake [3]. Native fluorescence of OTA allows determination of OTA directly, or after OTA derivatisation [4-6]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was also used for OTA determination in wine [7,8]. Sample preparation is a crucial step in the determination of OTA in wine. Immunoaffinity columns (IA) represent one of the major cleaning techniques in OTA (and other mycotoxins) analysis [9,10]. Due to the high cost of IA columns we preferred the solid phase extraction technique for clean up procedure based on C-18 cartridges.

Immunochemical methods such as commercial ELISA test kits, array fluorescence-based [11], electrochemical [12-14] and impedimetric immunosensors are also alternatives for the determination of OTA in complex samples [15,16].

Nowadays, there is an increasing concern in many countries that OTA may be linked to kidney disease and possibly cancer. In terms of OTA genotoxicity, the toxin promotes oxidative stress, oxidative base damage and single strand DNA cleavage through production of reactive oxygen species. OTA has been found in human blood, tissue and breast milk in several countries. The International Agency for Research of Cancer has classified OTA in group 2B, as a possible human carcinogen. Thus, because of possible health effects, there is an increasing need to monitor this mycotoxin in food samples including wine. In fact, a provisional tolerable weekly intake level of 100 ng $kg^{-1}$ of body weight has been established by the Joint FAO/WHO Expert Committee on Food Food Additives and regulatory limits for OTA content in wine have been established in many countries, for instance the European Union set a maximum allowable concentration of 2 μg $L^{-1}$ (Commission Regulation (EC) No. 123/2005). The International Organization of Vine and Wine (OIV) recommends as analytical method for OTA quantification in wine, its pre-treatment on immunoaffinity column (IAC) and HPLC separation with fluorescence detection (FLD), mostly because a very low detection limit that can be reached, due to the fact that OTA has natural fluorescence. The luminescence based detection (fluorescence, chemiluminescence, electrochemiluminescence) are preferred when complex matrix as food samples are analyzed, due to the high selectivity and sensitivity provided by these detection methods [17, 18].

Sample enrichment is an essential part in analytical methods for determination of low concentration of mycotoxins in real samples. The methods most frequently used in sample enrichment are liquid-liquid and solid-liquid extraction [19]. However, in case of mycotoxins, the literature reports a large number of sample enrichment methods based on immunoaffinity cartridges. Due to the high cost and of the short validity period of immunoaffinity cartridges, more economic alternatives are required. In this context, the enrichment of OTA by using C-18 solid phase extraction cartridges could be a method of choice to the immunoaffinity cartridges.

It is the aim of this paper to develop a cheaper and simpler analytical method based on classic sorbent, which is meanwhile robust and sensitive for the application to the real wine samples.

## Experimental part

### *Materials and methods*

Ochratoxin A (10 mg $L^{-1}$) in acetonitrile was purchased from Fluka. A stock solution of 100 μg $L^{-1}$ was prepared in methanol. A series of working standards in the

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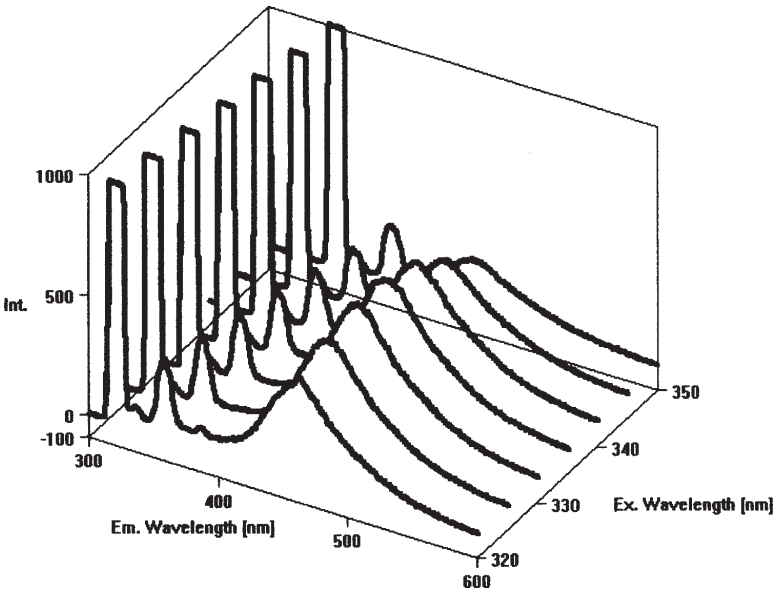


Fig. 1. 3D fluorescence spectrum of ochratoxin A (10 mg $L^{-1}$ OTA in mobile phase)

concentration range 0.1 – 10 μg $L^{-1}$ was prepared using the stock solution. The working standards were prepared in the mobile phase. OTA stock solution was prepared daily, before use. The methanol (HPLC grade) was purchased from Merck, phosphoric acid 85% (p.a) was obtained from Fluka, acetic acid 96% (p.a) and sodium hydroxide from Sigma-Aldrich. Ultrapure water was obtained with a Milli-Q system (Millipore). Extraction was performed with C18 (Isolute Biotage) (200 mg/ 3 mL) cartridges and OchraPrep (R-Biopharm) immunoaffinity columns.

Chromatographic analysis was performed using a modular Thermo Scientific HPLC system model Surveyor Plus equipped with a LC Pump Plus, a Finnigan Autosampler Plus and a Fluorescence detector Plus. Separation was performed on a endcapped, ultrapure, silica-based column Hypersil GOLD 150 x 4.6 mm i.d. (Thermo Scientific) having 5 μm particle size. The column was kept at 20°C by means of the column thermostat included in autosampler. Injection was performed automatically, in different volumes mentioned in text. The final chromatographic method was applied for the injection volume of 50 μL, full loop, and the flow rate was 1 mL $min^{-1}$. Mobile phase consisted in methanol: water acidified with 0.1% phosphoric acid (60:40, v/v). The solvents were degassed using an Elmasonic E30H ultrasonic bath (Elma) for at least 15 min.

Full scan fluorescence spectrum of ochratoxin was recorded with a Jasco FP-6500 fluorimeter.

*Samples*

A total of 9 commercial wine samples (red and white) originating from different regions of Romania (Constanta, Iasi, Husi, Vrancea, Galati) were purchased from a local market. The wine samples were initially filtered through 0.45 μm M.E. Cellulose filters Olimpeak (Teknokroma). In order to estimate the recovery of analyte, in all experiments needed for obtaining the calibration curve, we used a wine sample with no detectable amounts of OTA. Wine sample free of OTA was spiked with various concentration of OTA ranging between 0.1 to 10 μg $L^{-1}$. Each sample was analyzed at least in triplicate.

*Solid Phase Extraction*

Solid phase extraction using immunoaffinity (IA) cartridge

The wine sample was filtered through a 0.45 μm M.E. Cellulose filter and the *p*H was adjusted to 7.2 using 1M NaOH. An aliquot of 10 mL wine sample was then diluted with an equal volume of phosphate buffer saline (PBS) and passed through the IA cartridge under gravity with the flow rate of 1 drop per second. The cartridge was then washed with 20 mL PBS. The mycotoxin retained on the cartridge was then eluted with 1.5 mL MeOH acidified with 0.1% phosphoric acid, followed by 1 mL distilled water. All the elution solvents were collected into a glass vial to give a 2.5 mL total volume. 50 μL sample is then injected onto the HPLC system.

Solid phase extraction using C18 cartridge

The C18 cartridges had been conditioned with 5 mL MeOH, 5 mL ultrapure water and in the end with 2 mL mixture containing methanol : water (600 : 400). The wine samples were initially filtered through 0.45 μm M.E. cellulose filter. An aliquot of 5 mL wine was passed through the C18 cartridge, under gravity with the flow rate of 1 drop per second. The cartridge was then washed with 5 mL of water. The mycotoxin was then eluted from cartridge with 2 mL MeOH.

**Results and discussions**

Full scan fluorescence spectrum of ochratoxin A in the mobile phase consisting in methanol: water acidified with 0.1% phosphoric acid (60:40, v/v) was recorded using a scanning speed of 5 nm $s^{-1}$, the excitation domain of 320-350 nm, and the emission domain of 300-600 nm. Maximum sensitivity was reached for the excitation wavelength of 336 nm and the emission wavelength of 464 nm which were further used for the HPLC-FLD system. The 3D fluorescence spectrum is given in figure 1.

*Reversed-phase HPLC separation*

Different compositions of mobile phase were studied to optimize the chromatographic separation of target analyte towards matrix components. The main chromatographic parameters for method evaluation were separation selectivity, retention time and peak symmetry. In order to avoid the use of the relatively expensive acetonitrile, we checked different mobile phases based on methanol / water compositions with or without acid additives. Mobile phase compositions without acid additives produced large and asymmetric peaks due to the acid character of ochratoxin. Its acidic property influences the peak shape, and in the absence of an acidic additive this compound can be found in mobile phase in both forms, dissociated and undissociated. By bringing the aqueous component of mobile phase to a *p*H close to 2.5, this will shift the

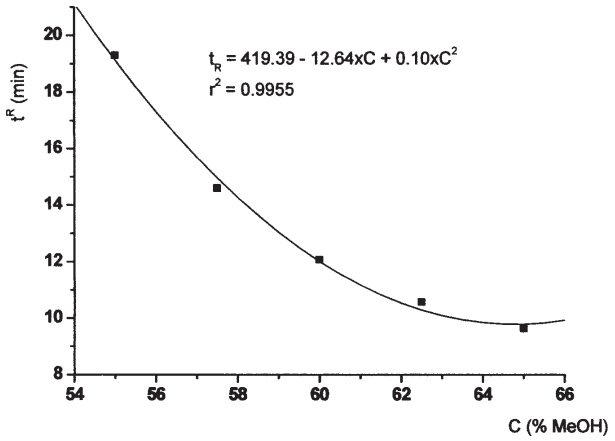


Fig.2. The plot of the dependence of the retention time of ochratoxin on the concentration (%, vol) in mobile phase

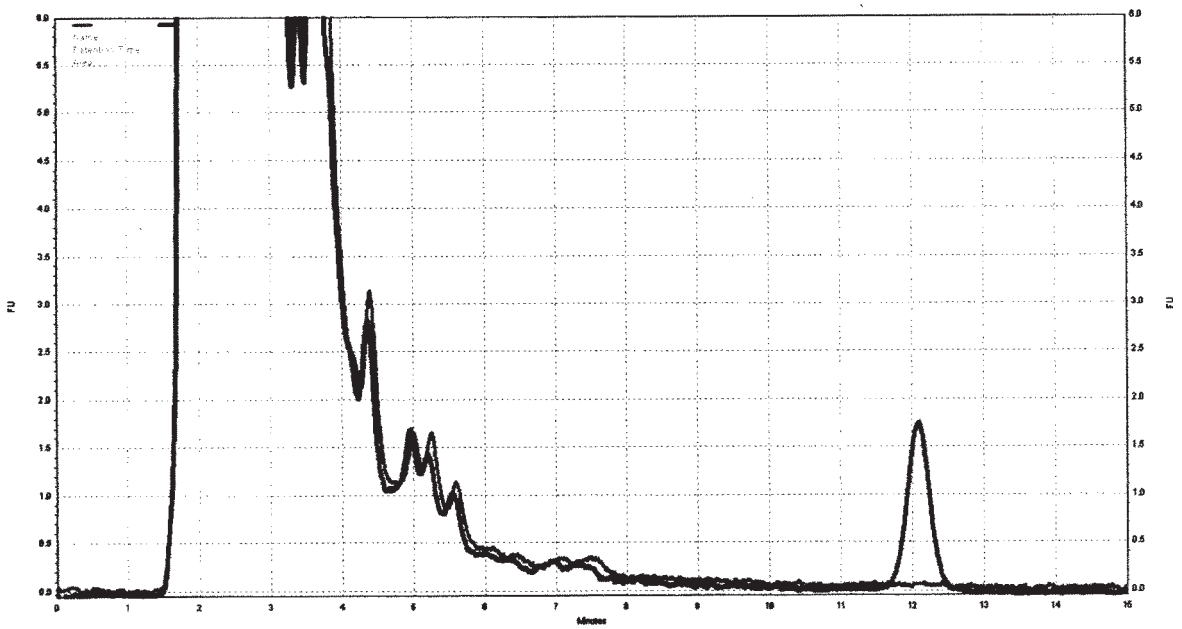

Fig. 3. Two overlaid chromatograms obtained for wine sample with 2.5 $\mu$g L$^{-1}$ OTA (black colour) against blank (grey colour) wine sample, under the conditions detailed in Experimental section

dissociation equilibrium to the undissociated form, which participates to the retention process as a single species. Consequently, the peak symmetry becomes acceptable.

Acetic acid and phosphoric acid were checked as acid additives in aqueous component of mobile phase. An aqueous component containing 0.8% acetic acid (v/v) has a *p*H 3 and the repeatability for the retention time of ochratoxin for 10 consecutive injections was relatively acceptable (1.56%). This value was however higher than that obtained with an aqueous component containing 0.1% $H_3PO_4$, with a pH close to 3 (RSD% for retention time of 10 consecutive injections was 0.44%). This difference can be explained by higher volatility of acetic acid, which modifies in time the *p*H of mobile phase. Although the literature reports many papers based on mobile phase containing acetic acid, we choose phosphoric acid due to its advantages in preparing the aqueous component of mobile phase and better time stability of the chromatographic peak of ochratoxin.

The dependence of the retention time of ochratoxin by the concentration of methanol (MeOH) in mobile phase is given in figure 2. A composition of 60% methanol in mobile phase was set up for method development, due to the highest selectivity obtained at this composition between the peak of ochratoxin and matrix components still present in sample after extraction according to the procedure discussed further. A typical chromatogram obtained for a white wine sample spiked with 1 $\mu$g L$^{-1}$ OTA is shown in figure 3.

Once the optimized HPLC-FLD conditions were set, a calibration curve was performed, using C18 cartridge for solid phase extraction, under the conditions described in section 2 "Materials and methods". The calibration curve for OTA was achieved by plotting peak areas versus concentration of standard solution $C_{OTA}$. Six levels of concentration were tested in triplicate. The calibration curve had the regression equation of y = 116914.7 x $C_{OTA}$ - 6690.8. The calibration curve was linear over the $C_{OTA}$ range from 0.1 to 10 $\mu$g L$^{-1}$, with the correlation coefficient of 0.9928.

Intra-day and inter-day repeatability were checked for three concentration levels of OTA in mobile phase solvent. The values of relative standard deviation for peak area and retention time corresponding to this analyte are given in table 1. This procedure was used in the chromatographic method validation for further application to the OTA determination in wine samples.

It can be concluded that the developed chromatographic method is precise for being applied to the determination of OTA in wine samples using the chromatographic parameters previously mentioned.

*Sample preparation and HPLC-FLD analysis*

Sample preparation for wine samples referred to the choice between two possibilities: analyte isolation and

| | OTA ($\mu g\ L^{-1}$) | RSD% | |
|---|---|---|---|
| | | Retention time | Peak area |
| Intra-day repeatability (n = 5) | 0.5 | 0.41 | 1.80 |
| | 1.0 | 0.45 | 1.24 |
| | 5 | 0.42 | 0.98 |
| Inter-day repeatability (n = 5) | 0.5 | 0.43 | 2.11 |
| | 1.0 | 0.41 | 1.45 |
| | 5 | 0.43 | 1.12 |

**Table 1**
PRECISION OF THE PROPOSED HPLC-FLD METHOD

| Cartridge | Efficiency (%) for | | | Precision as RSD% (n = 3) for | | | Time | Price |
|---|---|---|---|---|---|---|---|---|
| | 0.5 ng $mL^{-1}$ | 1.0 ng $mL^{-1}$ | 2.5 ng $mL^{-1}$ | 0.5 ng $mL^{-1}$ | 1.0 ng $mL^{-1}$ | 2.5 ng $mL^{-1}$ | | |
| C-18 | 90 | 92 | 110 | 2.35 | 1.89 | 1.87 | same | low |
| IA | 72 | 81 | 73 | 3.15 | 2.27 | 2.08 | same | high |

**Table 2**
COMPARISON BETWEEN ANALYTICAL PERFORMANCES OF THE METHODS BASED ON C-18 CARTRIDGES AND IMMUNOAFFINITY CARTRIDGES

| OTA concentration (ng $mL^{-1}$) | Recovery for white wine spiked samples (%) | Recovery for red wine spiked samples (%) |
|---|---|---|
| 0.5 | 93 ± 1.8 | 83 ± 2.1 |
| 1.0 | 101 ± 1.6 | 91 ± 1.4 |
| 2.5 | 95 ± 2.1 | 87 ± 1.6 |

**Table 3**
RECOVERIES FOR THREE LEVELS OF OTA CONCENTRATION (n = 5) IN WHITE VERSUS RED WINE SAMPLES

concentration on C18 cartridges or on immunoaffinity based cartridges. The procedures based on these two types of cartridge were mentioned in section 2. The performances of these methods applied on different concentration levels of OTA in spiked samples are discussed in table 2. As can be observed the analytical procedure based on C18 cartridge is, surprising, more efficient and more precise than that based on immunoaffinity cartridge. Both procedures have the same time consuming, while the price of C18 is however much lower than the price of immunoaffinity cartridge. From these points of view, the C18 cartridges proved to be more convenient for being used in sample preparation of wine samples than the immunoaffinity cartridges.

*Validation of the analytical method for determination of ochratoxin in wine sample*

Red wine samples with no content in OTA were used for obtaining spiked samples with different concentrations of OTA: 0.1; 0.25; 0.5; 1.0; 2.5; 5 and 10 $\mu g\ L^{-1}$. These spiked samples were subject to the sample preparation described previously and analyzed by HPLC-FLD with parameters set up according to the optimized procedure. The dependence of the peak area (A as LU*s) by the concentration of OTA ($C_{OTA}$, $\mu g\ L^{-1}$) in spiked samples is described by the equation: $A = 99761\ C_{OTA} - 10613$; with $r^2 = 0.9990$. The variation coefficient for the calibration slope measured for three consecutive days was 3.88%, which can be considered as acceptable for its validation. The value of the determination limit (LOD) was calculated with the formula:

$$LOD = 6s_B / B$$

where $s_B$ is the standard deviation of the slope B ($s_B$ = 998). The calculated LOD value of 0.06 $\mu g\ L^{-1}$ was situated below the lowest OTA concentration value used in this study. This value of LOD is of the same magnitude with values of the other methods reported in the literature (mentioned in Introduction).

The same procedure was applied to the calibration based on white wine, spiked with OTA at the same concentration levels as described above. The parameters for the calibration curve were following: $A = 104384\ C_{OTA} - 9591$; with $r^2 = 0.9997$. The value of LOD (0.05 $\mu g\ L^{-1}$) was calculated in this case for a slope deviation of $s_B$ = 870.

The wines of different provenience may have different composition patterns. This variability was checked to prove whether it influences or not the analytical results, in what is concerning the chromatographic selectivity and analyte recovery from wine samples (table 3). For the Romanian wines, it was observed that the FL trace of red wine matrix is more complex than that belonging to the white wines. Recovery of ochratoxin seems to be influenced by the sample matrix, because its values were higher for white wine spiked samples than values obtained for red wine.

This method was applied to the determination of OTA in real wine samples including both types of wine samples, i.e. red and white. Excepting one white wine sample from the Iasi region, all the other wines did not contain OTA in a detectable concentration. Even the sample with detectable concentration of ochratoxin (0.7 $\mu g\ L^{-1}$) has such a concentration level that was below the allowable limit of 2 $\mu g\ L^{-1}$. The time required for one analysis is of about 75 min/sample.

## Conclusions

The developed C18-SPE-HPLC-FLD method allows the determination of ochratoxin A in wine samples in the range of 0.1-10 $\mu g\ L^{-1}$, which includes the maximum allowable concentration (2 $\mu g\ L^{-1}$) of this compound in wine. From the analysis of several Romanian wine samples, we have detected ochratoxin just in one wine sample in a concentration of about 0.7 $\mu g\ L^{-1}$, which is however under the mentioned permitted concentration. Because wine samples have a complex matrix it is necessary a previous clean up method, based on solid-phase extraction on C18 silica adsorbent or immunoaffinity principle. The analytical procedure based on the C18 cartridge proved to be more precise than that based on immunoaffinity cartridge. By this choice, the sample clean up is simple, robust and cheap and together with the optimized HPLC-FLD method

may be easy applied in conventional laboratories from the wine industry.

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