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The assay of Ochratoxin A based on the use of its diastereoisomer as internal standard

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Mohammed Attya, Leonardo Di Donna, Fabio Mazzotti, Alessia Fazio, Bartolo Gabriele, Anna Napoli and Giovanni Sindona*

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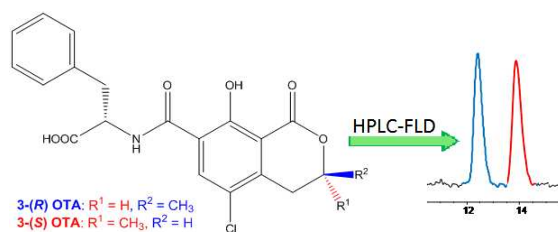
A new methodology for the determination of Ochratoxin A (OTA) was developed using a diastereoisomeric internal standard approach and HPLC-FLD.



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The assay of Ochratoxin A based on the use of its diastereoisomer as internal standard

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Abstract

The excellent separation achieved by HPLC of the diastereomers *3-(R)* and *3-(S)* of ochratoxin A and their identification by fluorimetric detection has prompted the development of a new method for the assay of ochratoxin A, one of the most abundant food-contaminating mycotoxins in the world. The new method is based on the use of N-[(5-chloro-3,4-dihydro-8-hydroxy-3-(S)-methyl-1-oxo-1H-2-benzopyran-7-yl)carbonyl]-L-phenylalanine, as internal standard. The latter was synthesized by a coupling reaction between commercially available phenylalanine and ochratoxin α. The goodness of the method is confirmed by the very low limit of detection and quantitation values achieved in the assay of ochratoxin A in the two fortified matrixes (0.002 and 0.005 μg L^{-1} for wine; 0.029 and 0.072 μg Kg^{-1} for the flour), and is further supported by the observed accuracy

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values. The new approach meets the requirement for the assay of sub-picogram amount of toxin contaminations in complex matrixes as foods. Moreover the method is affordable to any laboratory since it is based on relatively inexpensive separation and detection methodologies.

Keywords

Foods/Beverages, mycotoxins, diastereoisomer, internal standard

1. Introduction

Currently, the modern analytical food chemistry is based on reliable methodologies for the assay of organic molecules; regulations and codex imposes, in fact, more and more severe limits regarding the presence of pollutants, such as pesticides and toxins, in foodstuff; in addition, in order to asses the quality of food, a deeper investigation is required for the characterization of each market product. In the last decades the development of new analytical tools and devices has improved the performance on the quantification of organic analytes, by lowering the detection limits in the low ppb or even in the low ppt range. The use of the chromatographic systems in conjunction with a variety of detection techniques allowed sensitivity, reproducibility and in some cases excellent levels of specificity. Furthermore, the use of internal standards in addition to the latter instrumentation improved the repeatability and the accuracy of the measurements. The internal standard method is generally used to avoid systematic errors in the measurement. The internal standard (IS) is a molecule that matches as closely, but not completely, the chemical species of interest in the samples; consequently, the influence of sample preparation on the signals of IS and analyte should be equivalent. However, it cannot be trivial to find an appropriate IS that will elute in a position of the chromatogram that does not interfere or merge with any of the components of the mixture. Isotopomers are often used as internal standards in the isotope dilution mass spectrometry: the main advantages of this method rely in the fact that the IS virtually behaves in the

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same way of the analyte either in the sample preparation step or in the gas phase, i.e. during the analysis.[1-4]

Ochratoxin A, (OTA, (–)-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-(R)-methyl-1-oxo-1H-2-benzopyran-7-yl)carbonyl]-L-phenylalanine, **1**, chart), is a mycotoxin produced by some fungi of the Aspergillus and Penicillium species (such as Aspergillus ochraceus and Penicillium verrucosum); it is found in raw and improperly stored food products.[5-7] OTA has been shown to be nephrotoxic, mutagenic, genotoxic, teratogenic, hepatotoxic, neurotoxic, immunotoxic, in both animals and humans,[8,9] and in 1993 was classified as a possible carcinogen to humans (Group 2B) by the International Agency for Research on Cancer (IARC).[10] OTA is normally and easily detected by fluorescence (FL) detection connected to a HPLC system, which is, in general, equipped with a C_{18} reversed phase column. This is made possible by the particular structure (chart) of the analyte that if excited by a radiation at 333 nm wavelength, emits photons at 460 nm. To avoid interferences,[11-12] the matrixes are in all cases subjected to previous steps of clean up and purification. The most common type of purification provides the use of an immunoaffinity column which is based on the molecular recognition of the analyte.[13-16] Other methods of clean up rely on the exploitation of SPE cartridges packed with normal phase (silica),[17-18] reversed phase (C_{18} derivatized silica),[19-21] or ion exchange.[22] The clean up procedures are useful also as pre-concentration step, and allow lowering the detection limit to a concentration of few ppt. In addition to the FL detector, the OTA has been assayed using ultraviolet and mass spectrometry detection; in the latter case it is convenient to use an internal standard to avoid error on the determination of the analyte. In particular, ochratoxin B and C,[23-25] have been often added as internal standard, for their structural similarities to OTA; furthermore, the mass spectrometric detection offers the advantage of measuring the signal due to the mass to charge values, so applications of isotope dilution may be found in literature.[26-28] There are few reports on the use of fluorescence detection coupled with

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internal standard methods, all utilizing ISs with strong differences in chemical structure and detection response. [29] Nevertheless, OTA is usually and easily detected and quantified by external standard methodology.[30-33] This paper describes a new method for the assay of ochratoxin A (**1**) based on the use of its 3-(S)- diastereoisomer (**2**) as internal standard which is virtually absent in the natural environment except for some particular situation.[34] The internal standard can be easily separated from the final diastereomeric mixture obtained in the synthesis of **1**;[35] the measurements were carried out using a HPLC system connected to a fluorescence detector after immunoaffinity clean up. To our knowledge there is only one paper regarding the use of a diastereomer as internal standard in a quantitative analysis, which, however, deals with the assay of a peptide.[36]

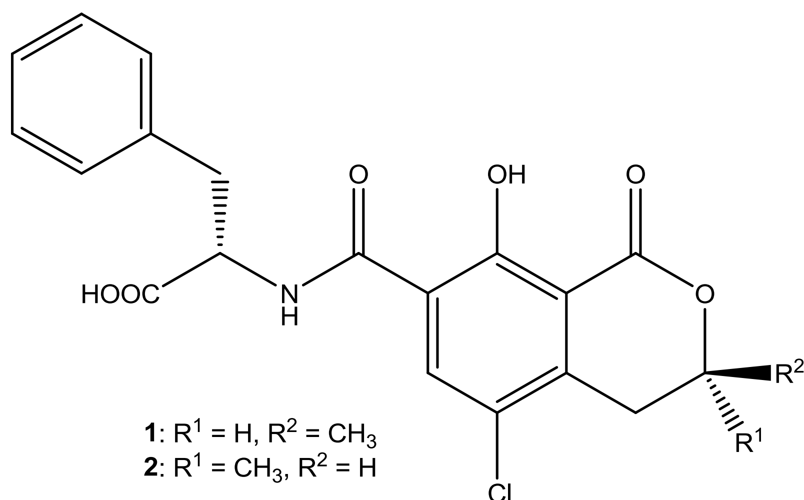

Chart: Chemical structures of *3-(R)*-ochratoxin A (**1**) and *3-(S)*-ochratoxin A (**2**).

2. Experimental

2.1 Chemicals

Solvents were obtained commercially from Carlo Erba (Rodano, Italy); OTA (**1**) and other reagents were purchased from Sigma-Aldrich (St. Louis, MO); 3-(S)-OTA (**2**, 99% purity), used as internal standard, was obtained by a coupling reaction between L-Phenylalanine tert-butyl ester and Ochratoxin α.[35] The tert-butyl moiety was removed using TFA in CH_2Cl_2 under N_2 at 25°C. The

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crude mixture of **1** and **2** diastereoisomers was submitted to the purification step using preparative TLC; SiO_2 plates were used as stationary phase (20 plates, 20 × 20 cm, 0.25 mm thickness), while a mixture of 79:20:1 benzene-acetone-formic acid was utilized as eluent. Pure **1** (R_f 0.47; yield: 46 mg, 46%, mp 110-112 °C, $[\alpha]^{25}_D$ ($CHCl_3$, c = 5 mg/mL) = – 31.5°) and **2** (R_f 0.43; yield: 34 mg, 34%, mp 182-183 °C, $[\alpha]^{25}_D$ ($CHCl_3$, c = 3 mg/mL) = + 66.7°) were obtained as colorless solids.

OchraTest™ Immunoaffinity columns were purchased from Vicam (Milford, MA). Certificated wheat Wheat (ochratoxin A, medium level, BCR® certified Reference Material) were purchased from Sigma-Aldrich (St. Louis, MO).

The PBS buffer was prepared by dissolving 8.0 g NaCl, 1.2 g Na_2HPO_4, 0.2 g KH_2PO_4 and 0.2 g KCl in 990 mL of purified water.

Both OTA and 3-(S)-OTA are suspect carcinogenic and should be handled with all safety precaution.

2.2 Sample Preparation

a) Wine: 50 µl of the internal standard (**2**) solution at 10 µg L^{-1} were added to 10 mL of wine and vigorously mixed with 10 mL of diluting solution (1% PEG + 5% $NaHCO_3$, pH 8.3) into a 100 mL flask. 10 mL of the latter solution were passed onto the immunoaffinity column at a flow rate of 1 drop per second. The column was rinsed with 10 mL of washing solution (2.5% NaCl + 0.5% $NaHCO_3$) and then with 10 mL of water at the same flow rate until dryness; compound **1** and **2** were eluted with 2 mL of CH_3OH: The solution was evaporated to dryness at 50 °C under N_2. The residue was dissolved in 250 µL of HPLC mobile phase (see below) and then injected into HPLC.[26]

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b) Flour: 500 μL of a 100 μg Kg^{-1} solution of internal standard (**2**) were mixed to 50 g of flour. The mixture was homogenized and then extracted with 100 mL of acetonitrile/water (60/40, v/v) for 5 minute. The residue was filtered onto a filter paper, and 10 mL of filtrate were added to 40 mL of PBS buffer. The resulting solution was filtered and 10 mL were poured into the immunoaffinity column at a flow rate of 1 drop/second. The column was then washed with 10 mL of PBS buffer and 10 mL of water at the same flow rate. The analytes were recovered from the column using 1.5 mL of CH_3OH. The solution was evaporated to dryness at 50 °C under N_2, dissolved in 1 mL of H_2O:CH_3OH (1/1, v/v) and then injected into HPLC.[26]

2.3 HPLC conditions

The analyses were performed using a HPLC 1100 from Agilent Technologies (Waldbronn, Germany) equipped with a fluorescence detector and a Luna C_{18} column 5μm particle size, 25 cm × 4.6 mm (Supelco, Saint Louis, MO). The flow rate was set at 1 mL/min and the following eluents and isocratic conditions were used: 50% H_2O, 50% acetonitrile, 2% formic acid. 60 μl of samples were injected into the loop. The run time was 20 min, while the excitation and the emission wavelength of the fluorescence detector were set to 333 nm and 460 nm, respectively.

2.4 Analytical Parameters

Standard solutions at 10 μg L^{-1} of OTA (Sigma-Aldrich, St. Louis, MO) and 3-(S)-OTA (IS) were diluted to obtain the calibration solutions at 0.1, 0.25, 0.5, 1, 2 and 5 μg L^{-1}. The IS in the standard solutions was used at a concentration of 1 μg L^{-1}.

Accuracy tests were performed on wine and flour samples (blank matrixes) obtained from local markets. To determine the content of ochratoxin A in the above mentioned matrixes the standard addition method was used; once known the level of the mycotoxin in the blanks, different samples

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of flour and wine were spiked with known amount of OTA standard and submitted to the procedure of analysis. Repeatability tests were conducted by analyzing each sample in triplicate. Another accuracy test was performed on certified wheat obtained from Sigma Aldrich, repeatability tests were conducted by preparing five different samples and analyzing each sample in triplicate.

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated by applying the equations 1 and 2, following the directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry.

$$S_{LOD}=S_{RB} + 3\sigma_{RB} \qquad \text{(eq. 1)}$$

$$S_{LOQ}=S_{RB} +10\sigma_{RB} \qquad \text{(eq. 2)}$$

Where S_{LOD} is the signal at the limit of detection, S_{LOQ} is the signal at the limit of quantitation, S_{RB} is the average area of the noise taken immediately before the elution of compound **1** in the chromatogram obtained from flour and wine and σ_{RB} is the standard deviation.

3. Results and discussion

The internal standard used in the assay of OTA is the synthetic compound **2** (chart) which presents an inverted chiral center on position 3; It may be easily obtained by a simple coupling reaction between ochratoxin α and phenylalanine.[35] The fluorescence chromatogram of a standard mixture of **1** and **2** shows two peaks at different retention times separated by approximately 1.6 minutes. The elution of the compounds was confirmed by LC/MS analyses.[35] A matching behavior should be expected by the two isomers towards immunoaffinity purification and detection response. In fact, the molecular recognition is based on the chirality of the phenylalanine moiety,[37] whereas fluorescence detection depends on the ochratoxin α moiety which,[12] on the contrary, should be not affected by the different chirality at position 3. To check the latter hypothesis, a solution of both of

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them (**1**, **2**) at the same concentration (1 μg L^{-1} each) has been submitted to the purification step on the immunoaffinity column; the recovered solution, injected into the HPLC afforded two peaks having the same area (figure 1b), thus confirming either that the antibody of the immunoaffinity column acts in a similar way on the two substrates and that the fluorescence response is equivalent. A similar experiment was conducted using different concentration of **1** and **2** (figure 1a).

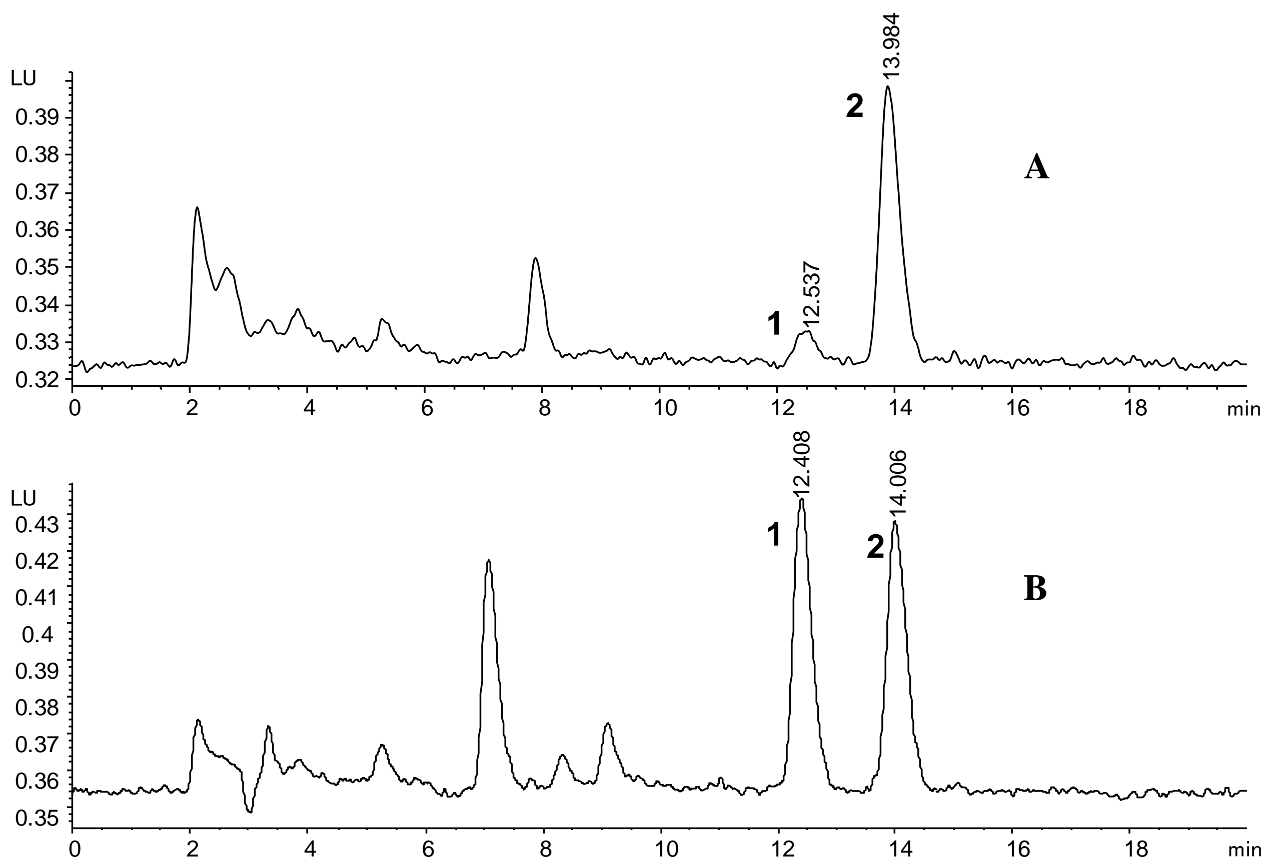

Figure 1. HPLC-FL chromatogram of a standard solution of 0.1-1 μg L^{-1} (**A**) and 1-1 μg L^{-1} (**B**) of **1** and **2**, respectively, after immunoaffinity clean-up.

The calibration curve ($y = 1.004x + 0.0096$, $R^2 = 0.9995$) has been derived using standard solutions of **1** in the concentration range from 0.1 to 5 μg L^{-1} containing the internal standard (**2**) at a fixed concentration of 1 μg L^{-1}. The excellent correlation coefficient (R^2) shows that the linearity of the interpolated values is maintained in the range monitored.

The methodology for the determination of OTA has been applied for the determination of OTA in certificated wheat and was able to determine OTA in the limit of the reported concentration, with

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RDS% 2.47 for different 15 measurements. The methodology was also applied either to wine and flour; a preliminary assay of **1** on a sample of wine and flour from the market has been conducted using the standard addition method. Four samples of flour spiked with 0.400, 0.800, and 3.000 μg Kg^{-1} of **1** and wine spiked with 0.030, 0.045, and 0.150 μg L^{-1} of **1** were used to extrapolate the content of OTA in these matrixes.

The latter experiments were conducted to know the exact amount of the analyte in those foods, in order to treat them as blank matrixes. The analyses revealed the presence of **1** at 0.298 ± 0.003 μg Kg^{-1} and 0.037 ± 0.001 μg L^{-1} in flour and wine respectively. To ensure the consistency of the method some accuracy tests were performed. The same food samples, analyzed with the standard addition method, were spiked with a known amount of OTA, extracted and then submitted to the analyses with the internal standard methodology. The flour was spiked with **1** to reach a concentration of 0.400, 0.800 and 3.000 μg Kg^{-1} and with **2** at a final concentration of 1.000 μg Kg^{-1}. The results of the assay gave 101.60%, 102.38% and 103.67% of accuracy for the three spiked samples (table 1). Figure 2 shows the chromatogram relative to the flour sample spiked at 0.800 μg Kg^{-1}. The replication of the measurements gave a precision value (RSD %) below 4%. LOQ and LOD values were extrapolated using equations (1) and (2) using as signal of reagent blank (S_{RB}) the signal of the baseline of the chromatogram immediately before the elution of **1**. The value of LOQ and LOD for the flour was found to be 0.072 μg Kg^{-1} and 0.029 μg Kg^{-1}, respectively (table 1).

fortified flour samples (μg Kg^{-1})	found value (μg Kg^{-1})	Accuracy %	RSD %
0.40	0.41 ± 0.02	101.60	3.69
0.80	0.82 ± 0.01	102.38	0.85
3.00	3.11 ± 0.03	103.67	1.03
LOD (μg Kg^{-1})	LOQ (μg Kg^{-1})		
0.029	0.072		

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fortified wine samples ($\mu g\ L^{-1}$)	found value ($\mu g\ L^{-1}$)	Accuracy %	RSD %
0.030	0.030 ± 0.001	99.81	3.33
0.045	0.047 ± 0.001	104.44	2.13
0.150	0.158 ± 0.004	105.33	2.53
LOD ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1}$)		
0.002	0.005		

Table 1. Accuracy, LOQ and LOD measurements; repeatability of the method.

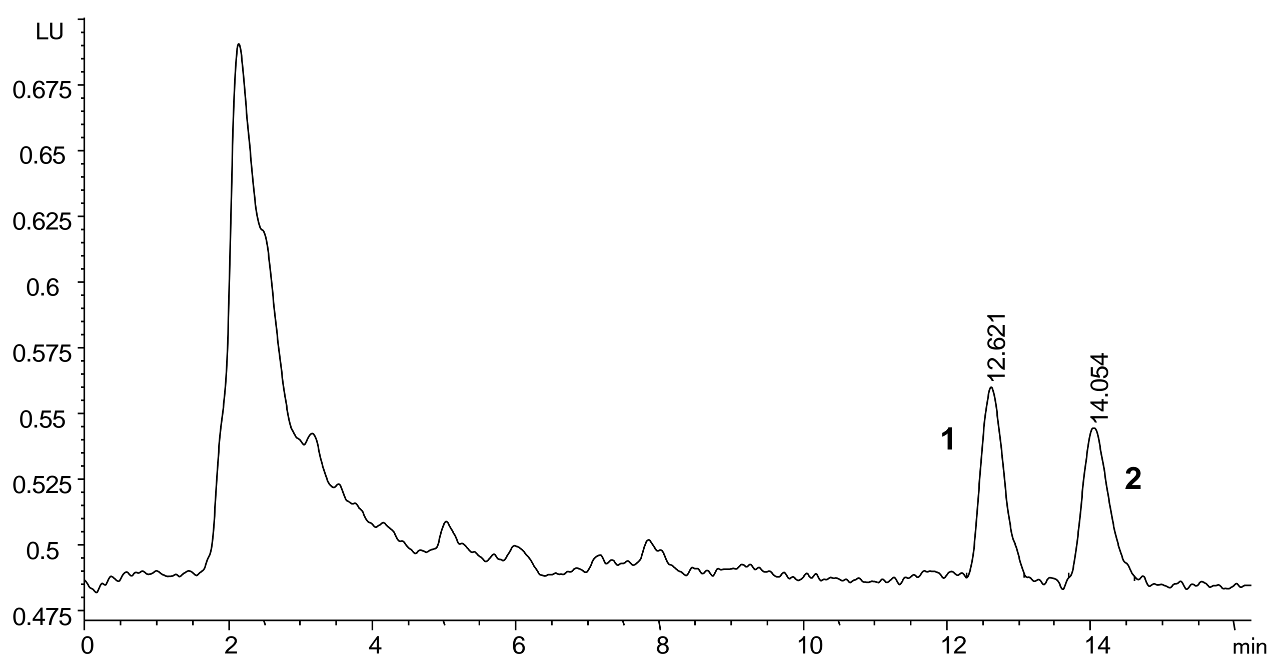

Figure 2. HPLC-FL chromatogram of a flour sample spiked at 0.800 $\mu g\ Kg^{-1}$.

Similar accuracy tests were conducted for wine. Three samples of wine were treated by adding **1** to reach a concentration of 0.030, 0.045 and 0.150 $\mu g\ L^{-1}$. The concentration of the IS in the wine was 0.050 $\mu g\ L^{-1}$. The latter value was chosen because there is a 20 times concentration step of the final solution in the sample preparation. Table 1 shows that the accuracy value, achieved from the analyses with the internal standard; in particular 99.81%, 104.44% and 105.33% accuracy were obtained for 0.030, 0.045 and 0.300 $\mu g\ L^{-1}$ spiked matrixes, respectively; the repeatability was

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below 3.5%. The values of LOQ and LOD calculated at the same way of the flour were 0.005 and 0.002 μg L^{-1} respectively (table 1). The limit of detection and quantitation was considerably lower than those found in literature, in particular in those cases in which an internal standard has been used.[27,29]

4. Conclusions

A new methodology for the assay of Ochratoxin A was developed using a diastereoisomeric internal standard. This new class of ISs, which behaves exactly at the same way of the analytes, may improve the reliability of the measurements in those methodologies in which external standards are usually employed. In particular, IS diasteromers may be used in conjunction with all those detectors, like fluorimeter, ultraviolet, refraction index etc., that cannot be used in the presence of labeled internal standards. A further advantage may be represented by the relatively inexpensive technique of detection.

Acknowledgment

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