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Short communication

Investigation on Ochratoxin A stability using different extraction techniques

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Abstract

The stability of Ochratoxin A during its extraction using different extraction techniques has been evaluated. Microwave-assisted extraction and pressurised liquid extraction, in addition to two other reference methods of extraction, i.e. ultrasound-assisted and magnetic stirring-assisted extraction, were evaluated. The effect of extraction temperature using the cited techniques was checked.

The results show that Ochratoxin A can be extracted using microwave-assisted extraction at temperatures up to 150 °C without degradation. Pressurised liquid extraction can be used at temperatures up to 100 °C, for extraction times of less than 30 min. Further, both ultrasound-assisted extraction and magnetic stirring extraction can be applied at temperatures up to 65 °C.

High-performance liquid chromatography combined with fluorescence detection using a Chromolith RP-18e column at a flow rate of 5 mL min^{-1} was used to quantify the Ochratoxin A. The retention time for the Ochratoxin A was 1.3 min. The limits of detection (LOD) and of quantification (LOQ) were 0.03 and 0.10 μg L^{-1}, respectively.

Keywords: Ochratoxin A; Stability; Microwave-assisted extraction; Pressurised liquid extraction; Ultrasound-assisted extraction

1. Introduction

The compound 7-carboxyl-5-chloro-3,4-dihydro-8-hydroxyl-3*R*-methylisocoumarin-7-L-phenylalanine ($C_{20}H_{18}ClNO_6$) usually known as Ochratoxin A or OTA, can be found in several food and drink commodities, including cereals, coffee and wine, at different levels [1]. OTA is a mycotoxin produced by several species (fungi and moulds) of *Aspergillus* and *Penicillium* [2].

OTA has been widely studied so far since considered a potential human health hazard [3,4]; its toxic properties in fact include DNA damage, nephrotoxicity, immunotoxicity, and carcinogenicity. It has been included by the International Agency for Research on Cancer in the list of compounds that are possibly carcinogenic to humans [5].

The chromatographic determination of OTA and the extraction from liquid samples have been widely studied [6]. Most validated methods applied for the extraction of Ochratoxin A are based on the solubility of OTA in organic solvents [7]. Most procedures are based on liquid–liquid extraction using either extraction with organic solvent [8] or alkaline solutions [9]. Therefore they are suitable for liquid but not solid samples. When dealing with solid samples, organic solvent mixtures are frequently used even if procedures typically employed result to be long, time consuming and the interaction of OTA with the matrix could lead to poor recovery yields [10,11].

Therefore, there is an increasing demand for new extraction techniques, with shortened extraction times and reduced organic solvent consumption, to reduce sample preparation costs. Microwave-assisted extraction (MAE), pressurised liquid extraction (PLE) and ultrasound-assisted extraction (UAE) could be suitable techniques for developing new methods for the determination of OTA in solid samples. However, the first step in evaluating these methods should be the determination of the stability of OTA during the extraction process.

Pressurised liquid extraction is a sample preparation technique where temperature and pressure are used to accelerate extraction of compounds from solid and semi-solid samples. Pressure is used to increase the contact between the extract-

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ing fluid and sample, as well as for maintaining the solvent in liquid state; the technique works at temperatures higher than the boiling point of the fluid. Temperature is used to break the analyte–matrix bonds; moreover, temperature can dramatically modify the relative permittivity of the extracting fluid, thus increasing selectivity [12]. An application of PLE to the extraction of OTA from rice has been reported recently [13]; however no study of the stability of OTA during PLE has been found in the literature.

Microwave-assisted extraction (MAE) in a closed system under pressure is one of the techniques developed to reduce the volume of solvents required, improve the precision of analyte recoveries, and decrease the costs [14]. No reports on the application of MAE to the extraction of OTA have been found in the literature.

The aim of the present work is to investigate about the effects of temperature and pressure on the stability of OTA when PLE and MAE are applied to a standard solution. The analyses of OTA are carried out using a fast HPLC method coupled to fluorescence detection that provides a limit of detection of 0.032 μg L^{-1}, due to the fact that OTA shows an intense natural fluorescence [15]. Using these extraction techniques the main drawbacks offered by conventional extraction techniques such as long time requiring and poor recoveries could be overcome.

2. Experimental

2.1. Chemicals and solvents

Ochratoxin A was obtained from Sigma (Sigma–Aldrich, St. Louis, MO, USA) and stored at −8 °C. OTA purity was 98%. All the reagents used were of analytical grade: methanol (Merck, Darmstadt, Germany). HPLC grade water was obtained from a Milli-Q Ultrapure water system (Millipore, Bedford, MA, USA). Celite 545 (particle size 0.02–0.1 mm) (Merck) was used as the supporting material for PLE.

2.2. Preparation of standard solutions

A stock of the OTA standard solution 50 mg L^{-1} was prepared in methanol and stored at −32 °C.

After each extraction, the volume of extract was made up to 25 mL with the solvent. All samples were filtered through a 0.45 μm nylon syringe filter (Millipore) before chromatographic analysis. All extractions were performed in triplicate.

2.3. Microwave-assisted extraction

An ETHOS 1600 microwave-oven (Milestone, Shelton, CT, USA) equipped with 10 perfluoroalkoxy Teflon® closed vessels was used. Extractions were performed at 500 W using magnetic stirring at 50% of nominal power. Temperatures ranging from 50 to 175 °C were evaluated. Higher temperatures were not evaluated as degradation of OTA was found to occur at 175 °C. The extraction protocol used was the following: 0.5 mL of OTA standard solution (50 μg L^{-1}) in approximately 23 mL of the extraction solvent, methanol, for 20 min.

Table 1
Parameters of the calibration curves of OTA in the HPLC method

Calibration range (μg L^{-1})	0.7–0.025
Regression equation	$y = 56723x - 2686$
Regression coefficient	0.9984
Detection limit (μg L^{-1})	0.032
Quantification limit (μg L^{-1})	0.104

2.4. Pressurised liquid extraction

An ASE-200 pressurised liquid extractor (Dionex, Sunnyvale, CA, USA) was used for the extractions. The extraction cell volume was 11 mL and the collection vial volume was 40 mL.

For the extraction, the Celite 545 inside the cells was spiked with 0.5 mL of the OTA solution (50 μg L^{-1}). One cycle of 20 min was used at a pressure of 100 atm. After each extraction, the cell was rinsed with 5 mL of methanol and finally purged with nitrogen for 1 min. Temperatures assayed ranged from 50 to 150 °C.

2.5. Reference extraction methods

UAE was performed in a water bath of 400 W (J.P. Selecta, Barcelona, Spain). The extraction protocol used 0.5 mL of OTA standard solutions (50 μg L^{-1}) in approximately 23 mL of methanol for 20 min. Four temperatures were assayed: 40, 50, 60 and 65 °C. The same protocol and temperatures were used as for the magnetic stirring-assisted extraction (MSAE).

2.6. High-performance liquid chromatography

Chromatographic analyses were performed using a RP-HPLC (Dionex) with a fluorescence detector (RF-2000, Dionex). The excitation wavelength used was 333 nm and the emission wavelength 460 nm. The software for control of equipment and data acquisition was Chromeleon version 6.60.

OTA was separated on a monolithic column (Chromolith TH Performance RP-18e, 4.6 mm, 100 mm, Merck). Two solvents were used: solvent A (10% methanol, 2% acetic acid in water) and solvent B (90% methanol, 2% acetic acid in water) at a flow rate of 5.0 mL min^{-1}. The gradient applied was as follows: 0 min, 40% B; 1 min, 90% B; 4 min, 90% B; 6 min, 100% B. The retention time for OTA was 1.3 min ($k' = 3.1$). The injection volume was 50 μL.

A series of working standard solutions from 0.025 to 0.7 μg L^{-1} OTA was prepared by dilution. These were used to prepare the calibration curve. Table 1 presents the properties of the calibration curves of OTA. The limits of detection and of quantification have been calculated using the ALAMIN software [16].

3. Results and discussion

3.1. HPLC method

Since the samples to be analysed were those that only contained OTA and, where applicable, its degradation compounds,

it was decided to employ a method of determination by HPLC using a monolithic column and a rapid elution method. Initially an isocratic method was employed, but the appearance in the fluorescence detection system of a second peak with signal in some assays made it necessary to devise a system in gradient that would permit the separation of this second compound of OTA.

The definitive method is presented in Section 2. Under these conditions, the elution time of OTA was found to be only 1.31 min.

The repeatability of the analysis by HPLC was determined by the assessment of the standard deviation of the areas of peak and of the retention times of a total of 10 analyses of a sample of OTA of concentration 2 $\mu g\,L^{-1}$. A R.S.D. < 0.20% for the retention time and a R.S.D. < 1.18% for the area of the peak were found. These results confirm the reliability of the analysis by HPLC.

3.2. Stability in a system of extraction by magnetic stirring

This technique is employed because it is a simple and low cost method. A standard solution of OTA was utilised (50 $\mu g\,L^{-1}$)of which 0.5 mL dissolved in an approximate volume of 23 mL of methanol was employed. The decrease of volume due to the evaporation of the methanol during the assays was corrected by replacement with the same solvent. Thus the stability of OTA in solution was determined at different temperatures between 25 and 65 °C, for a total time of 20 min.

The values of OTA found in the stirred solutions at different temperatures are given in Fig. 1. These values have been calculated with respect to the reference prepared daily in duplicate.

As can be confirmed, any temperature would be valid for performing the extraction from real samples, since OTA was found to be stable over the whole range assayed.

The results were compared statistically and did not show significant differences, to a 95% confidence level.

3.3. Stability in a system of ultrasound-assisted extraction

The ultrasound waves facilitate the contact between the sample containing the analytes and the solvent acting as extractor. This is therefore a technique offering considerable advantages

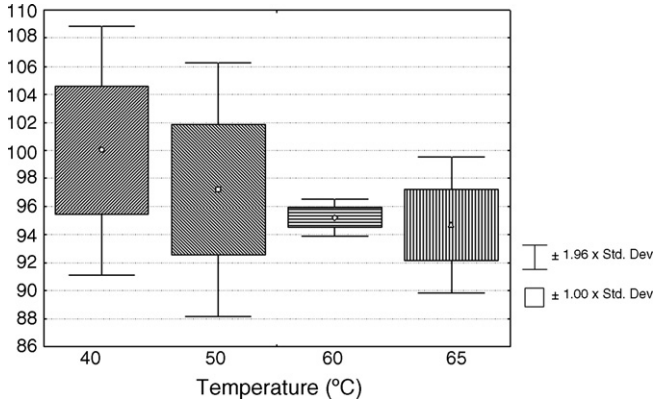

Fig. 1. Box and whisker plots of recoveries for OTA during the MSAE at different temperatures.

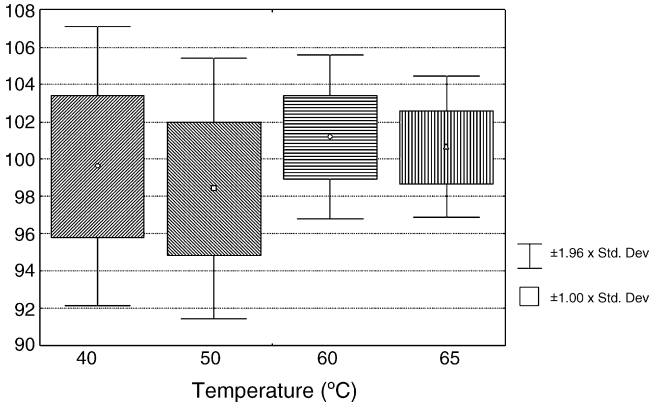

Fig. 2. Box and whisker plots of recoveries for OTA during the UAE at different temperatures.

over extraction by magnetic stirring, mainly in respect of time. It is necessary to check the stability of OTA in these conditions, since the extraction takes place more energetically and the consequence of this could be the degradation of the OTA. For this a quantity of 0.5 mL dissolved in an approximate volume of 23 mL of methanol was employed. The decrease of volume due to the evaporation of the methanol during the assays was corrected by replacement with the same solvent.

Fig. 2 shows the results for the values of OTA found in the solutions of the extractions performed at temperatures between 25 and 65 °C.

As occurs in the case of magnetic stirring, apparently OTA is perfectly stable in these conditions, up to the highest temperature tested (65 °C). Statistical comparisons were made between the results and no significant differences between them have been found. Temperatures higher than 65 °C not were considered because the intense evaporation of the methanol would prevent the experiments being carried out in a reliable way.

3.4. Stability in a system of microwave-assisted extraction

This extraction technique allows the sample to be heated by a process different from the usual convection. In this case the sample receives the energy directly and it is essential to study the stability prior to devising a method of extraction.

Of the two options that can be applied, under atmospheric pressure or in a closed system, the latter was selected since it permits the extractions to be performed at temperatures above the boiling point of the solvents employed. This procedure usually means more extraction efficiency or reduced extraction times. Therefore closed extraction vessels were employed.

A quantity of 0.5 mL (50 $\mu g\,L^{-1}$) dissolved in an approximate volume of 23 mL of methanol was introduced. The power of the microwave system was set at 500 W, which allows the working temperature to be reached in a time that ranges between 1 min 21 s in the case of 50 °C and 2 min 39 s in the case of the highest temperature assayed (175 °C). The system was maintained under those conditions for 20 min.

Fig. 3 shows the results of the extractions performed at temperatures between 50 and 175 °C. As can be observed, there is no degradation apparent at temperatures of up to 150 °C.

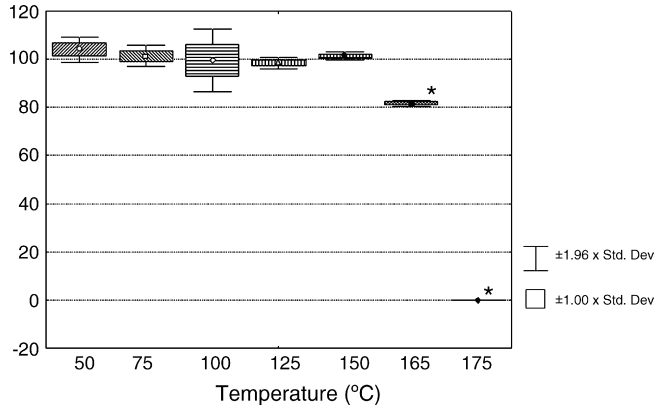

Fig. 3. Box and whisker plots of recoveries for OTA during the MAE at different temperatures. *Significant difference at 95% level of confidence.

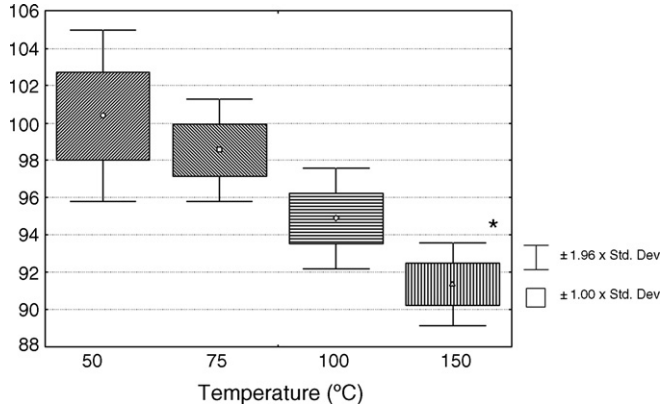

Fig. 4. Box and whisker plots of recoveries for OTA during the PLE at different temperatures. *Significant difference at 95% level of confidence.

In contrast, at 165 °C, there is already an appreciable amount of degradation of this compound, and at 175 °C, this degradation becomes intense and affects up to 100% of the compound; no signal is detected in the analysis by HPLC, which indicates that the concentration of OTA has dropped below 0.032 μg L^{-1}, which is the detection limit of the chromatographic method employed. This means that at least 0.024 μg of the 0.025 μg initially introduced into the system has been degraded.

Therefore, MAE would be a viable technique for extraction at temperatures up to 150 °C, with a duration of up to 20 min. Statistical comparison was applied to determine if there were significant differences between the recoveries obtained, and these were only found for the values at 165 and 175 °C.

3.5. Stability in a system of extraction by pressurised liquid

This technique has two great advantages over conventional extractions: first, the higher working pressure facilitates contact between the sample and the solvent, and second, the higher working temperature should also facilitate the extraction process. However, as in the previous case, the high extraction temperatures must be evaluated for their influence on the stability of the compounds to be extracted. Unlike MAE, PLE allows extractions to be performed in the complete absence of air, i.e. to take place in an atmosphere of nitrogen, which could be an advantage in preventing oxidation.

A quantity of 0.5 mL of standard solution of OTA (50 μg L^{-1}) was employed; this was placed on a support of hydromatrix material (Celite, Merck) customarily utilised in this system of extraction. An ASE-200 automated extraction system (Dionex) was employed, but in this case, it was controlled manually. When the system works in automatic mode the sample is washed prior to the extraction; this however must be avoided in studies of stability, since when standards are extracted these are carried over with the elution. Experiments were conducted at temperatures from 50 to 150 °C for 20 min at 100 atm. After this time methanol was pumped over the system for the elution, to allow the collection of the OTA and its possible degradation products. The volume eluted was made up to 50 mL and was analysed by HPLC.

Fig. 4 shows the results obtained. It can be confirmed that, at successively higher temperatures, the intensity of the OTA degradation increases; this exceeds 5% at 150 °C, but at 100 °C a degradation below this percentage is obtained. Only the results obtained at 150 °C were found to be significantly different from the reference.

It must be taken into account that, in the case of real samples, extractions in several cycles are utilised, to facilitate the extraction; hence the solvent is subject to renewal and this can also affect the stability. Given these considerations, it is important that, at a temperature of 100 °C, the stability should be determined taking into account the duration of the method; this should be less than 20 min, which is the time studied here.

4. Conclusions

From the experiments performed, it can be concluded that various different temperatures can be utilised for the extraction of OTA with different extraction systems. In the case of magnetic stirring and ultrasound-assisted extraction, extractions of 20 min of duration can be performed at temperatures up to 65 °C. On the other hand, utilising MAE for the same period of time, temperatures of up to 150 °C can be reached without degradation of OTA taking place. In the case of PLE, the maximum working temperature is 100 °C, also for 20 min.

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