JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Ochratoxin A Determination in Beer by Solid-Phase Microextraction Coupled to Liquid Chromatography with Fluorescence Detection: A Fast and Sensitive Method for Assessment of Noncompliance to Legal Limits

ANTONELLA ARESTA, FRANCESCO PALMISANO, ROSA VATINNO, AND CARLO G. ZAMBONIN*

Dipartimento di Chimica, Università degli Studi di Bari, Via E. Orabona, 4, I-70126-BARI, Italy

A solid-phase microextraction-liquid chromatography-fluorescence detection (SPME−LC−FD) method for the determination of ochratoxin A (OTA) in commercial beer samples was developed for the first time using a 60 μm thick poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB) fiber. The procedure required a very simple sample pretreatment, an isocratic elution, and provides a selective extraction. All of the factors influencing fiber adsorption (extraction time, temperature, pH, and salt addition) and desorption of the analyte (desorption and injection time and desorption solvent mixture composition) have been investigated. The linear range investigated in beer was 0.03−2 ng/mL; within-day and between-days relative standard deviation in beer were 4.3 and 5.9%, respectively. The limit of quantification in spiked beer was 53 pg mL^{-1}, well below all European regulatory levels.

INTRODUCTION

Ochratoxins are a group of structurally related secondary metabolites that are produced by some toxic fungi such as *Penicillium verrucosum*, *Aspergillus ochraceus*, and occasionally by some isolates of *Aspergillus niger* (*1*). Ochratoxin A (OTA) or 7-(L-β-phenylalanylcarbonyl)carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3*R*-methylisocumarin is the main mycotoxin in the group of ochratoxins, and it appears to be the one of major toxicological concern. Indeed, OTA has been shown to be nephrotoxic, hepatotoxic, teratogenic, and immunotoxic to several species of animals and to cause kidney and liver tumors in mice and rats (*2*). As far as humans are concerned, the International Agency for Research on Cancer (IARC) classified OTA in Group 2B (possible carcinogen to humans) (*3*). With regard to its nephrotoxicity, OTA is considered to be involved in a severe kidney pathology (the Balkan endemic nephropathy), possibly linked to urinary tracts tumors (*4*).

OTA is generally found in several food commodities such as cereals, oleaginous seeds, green coffee, wine, meat, cocoa, spices, etc., at concentration levels that depend upon both environmental and processing conditions.

A provisional tolerable weekly intake level for OTA of 100 ng kg^{-1} of body weight has been established by the Joint FAO/WHO Expert Committee on Food Additive (*5*). Note that the 2002 report (*6*) on the assessment of dietary intake of OTA by the population of the EU member states reveals that cereals resulted in the main source (50%) of human intake, followed by other commodities such as wine (13%), coffee (10%), spices (8%), beer (5%), etc.

Several countries have issued their own regulatory limits for OTA content in several food commodities; for instance, the maximum allowable concentrations established by the European Union are 5.0 μg kg^{-1} for cereals, 3.0 μg kg^{-1} for cereal-derived products, 5.0 μg kg^{-1} for roasted coffee, and 2.0 μg L^{-1} for wine (*7*). No limit has yet been fixed for beer; however, guidance levels are established in various European countries such as The Netherlands (0.5 μg L^{-1}), Finland (0.3 μg L^{-1}), and Italy (0.2 μg L^{-1}).

In view of the above considerations, sensitive and accurate analytical methods for OTA determination in beer are highly desirable. The state-of-the-art and analytical challenges for OTA determination in foods has been recently discussed in a review paper (*8*). The most widely adopted method for OTA quantitation is reversed-phase liquid chromatography with fluorescence detection (LC−FD) after a suitable sample extraction/cleanup step. Sample treatments for OTA determination in must, wine, and beer have been recently reviewed by Saèz et al. (*9*). Immunoaffinity column (IAC) cleanup is the most widely encountered approach; in the case of beer, the sample can be simply diluted with a solution containing poly(ethylene glycol) (PEG) and $NaHCO_3$, filtered, and then applied to an IAC. After column washing, OTA was eluted with methanol, the eluate was evaporated to dryness, reconstituted in mobile phase, and injected (*10*). Alternatively, OTA in acidified beer could be extracted with $CHCl_3$ and the reconstituted extract could be cleaned-up using IAC (*11*). Apart from the high cost of each column, the main disadvantages of an immunoaffinity cleanup

* To whom correspondence should be addressed. Telephone: +39-080-5442506. Fax: +39-080-5442026. E-mail: zambonin@chimica.uniba.it.

10.1021/jf052666o CCC: $33.50
Published on Web 02/10/2006

for OTA could in some cases be the lack of specificity (because of a cross-reaction with ochratoxin C).

Attempts to avoid IAC cleanup have been described; Medina et al. (*12*) have purified the chloroform extract of beer by using lead hydroxyacetate as a cleanup agent. A preconcentration ratio of 200:1 was achieved, and a limit of detection (LOD) of 5 pg/mL was claimed; however, only chromatograms on sample spiked at 500 pg/mL were shown, and no blank chromatogram was provided for a comparison. Solid-phase extraction on an octadecylsilane cartridge has also been evaluated (*9*) for OTA determination in beer; unfortunately, relative standard deviation (RSD) was found to be concentration-dependent, ranging from 62 to 1.9% at a spiking level of 0.1 and 1 ng/mL, respectively.

Very recently, an automated on-line solid-phase extraction−liquid chromatography−electrospray tandem mass spectrometry (SPE−LC−ESI−MS/MS) method was developed for the determination of OTA in wine and beer (*13*). The method is very sensitive, accurate, and specific, but cost and complexity of the instrumentation involved make it unsuitable for routine use.

On the other hand, several surveys conducted thus far, on OTA occurrence in beer marketed in different countries, have shown that regulatory limit infringement is a rare event. This imply that the major analytical demand is essentially dictated by the need of a rapid screening method to assess noncompliance to guideline (or legal) limits "G" (e.g., 0.2 μg L^{-1} in Italy). To this purpose, a simple and low-cost method possessing a satisfactory precision at an OTA concentration near G (see later) is highly desirable. Only suspected or apparently noncompliant samples could be re-analyzed for confirmatory purposes, by very specific methods based on, e.g., IAC (or SPE) cleanup and fluorescence or mass spectrometry detection.

A possible approach for sample pretreatment simplification could be represented by solid-phase microextraction (SPME), a solventless technique (*14*) that eliminates some disadvantages of conventional extraction methods such as solid-phase extraction (e.g., plugging of cartridges) and liquid−liquid extraction (e.g., use of toxic solvents). SPME has been mainly applied (*14*−*20*) in combination with GC; however, a growing interest for SPME coupled to LC was observed in the past few years as demonstrated by a number of recently published papers (*21*). Surprisingly, examples of SPME application in the field of mycotoxin analysis are limited to few papers from our laboratory (*22*−*24*).

The first SPME−LC−FD procedure for OTA determination in beer is described in the following sections. Figures of merit of this approach are discussed.

EXPERIMENTAL PROCEDURES

Chemicals. OTA was obtained from Sigma (St. Louis, MO). Stock standard solutions were prepared in methanol at approximately 1 mg/mL; the actual concentration was calculated by UV spectrophotometry assuming a molar absorption coefficient of 6640 M^{-1} cm^{-1} at 333 nm. Stock standard solutions stored at −20 °C are stable for several years (*25*). All of the other chemicals used were of analytical grade.

IACs (Ochraprep−Rhone Diagnostics) were obtained from ORSELL (Carpi, Italy).

Apparatus. The SPME interface (Supelco, Bellefonte, PA) consisted of a standard six-port Rheodyne valve equipped with a fiber desorption chamber (total volume of 60 μL), installed in place of the sample loop.

The LC apparatus consisted of a Dionex P680 LPG pump equipped with a vacuum membrane degasser, a Rheodyne 7125 injection valve (connected in series to the SPME interface) fitted with a 50 μL loop, and a Supelcosil LC-18 DB (150 × 4.6 mm) chromatographic column. The fluorescence detector was a Jasco model FP-2020 Plus, connected to a Hewlett−Packard 3395 computing integrator.

Chromatographic and Detection Conditions. The optimized mobile phase was a water/acetonitrile/acetic acid mixture (111:87:2, v/v/v). The flow rate was 1 mL min^{-1}, and the temperature was ambient.

Fluorescence excitation and emission wavelengths were 332 nm (4 nm bandwidth) and 460 nm (18 nm bandwidth), respectively.

Solid-Phase Microextraction. Silica fibers (Supelco) with three different coatings, i.e., 85 μm thick polyacrylate (PA) film, 50 μm thick Carbowax/Template Resin (CW/TPR-100) film, and 60 μm thick poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB) film, were employed for comparative studies. The fibers were conditioned as suggested by the producer. A manual SPME device (Supelco) was used to hold the fiber. The extraction was carried out (on beer samples processed as described in the "beer samples" section) under magnetic stirring for 60 min at room temperature. OTA desorption into the SPME−LC interface was performed in the static desorption mode by soaking the fiber in mobile phase for 60 s. Then, the valve was changed to the inject position, and the fiber was exposed for 10 s to the mobile-phase stream. To avoid possible memory effects, the fiber was fully desorbed, before the next extraction, flushing the desorption chamber by syringe injection of 1 mL of the mobile phase.

Beer Samples. A total of 10 pale and strong beer samples (domestic and imported) differing in alcohol content were purchased from a local market and stored at +4 °C. Cool samples were first degassed for 30 min in an ultrasonic bath; then, a beer aliquot was diluted with 0.03% HCl (1:3, v/v), to ensure a final pH ≤3, and an aliquot was filtered through a 0.45 μm Millex-HV-type filter (Millipore). Finally, 1.5 mL of the diluted sample was transferred into a 1.5 mL clear vial (Supelco); the vial was sealed with a hole cap and Teflon-faced silicone septum (Supelco); and the sample was subjected to SPME.

Working solutions were prepared by spiking degassed beer samples with different amounts of OTA standard to obtain the desired concentration levels. Samples were then processed as described above.

The calibration curve was constructed using OTA-free beer samples spiked with variable amounts of the toxin to obtain the following concentration levels: 0.03, 0.06, 0.12, 0.25, 0.5, 1, and 2 ng/mL.

The within-day ($n = 5$) and between-days ($n = 5$ over 5 days) precision (repeatability) were calculated on beer samples spiked at 0.2 ng/mL.

Cleanup by IACs was performed as described by Visconti et al. (10); briefly, degassed beer sample was diluted 1:1 (v/v) with a water solution containing 1% poly(ethylene glycol) (PEG 8000) and 5% $NaHCO_3$. A total of 10 mL of diluted sample was loaded onto a Ochraprep column; the column was washed and finally eluted with 2 mL of MeOH. The methanolic extract was evaporated to dryness, reconstituted with 250 μL of mobile phase, and 20 μL injected.

RESULTS AND DISCUSSION

The first step of the work consisted of the optimization of the SPME conditions for the extraction of OTA from beer samples. A strong dependence of the extraction yield on the pH value was observed (OTA is a weak acid, having pK_a values of ca. 4.4 and 7.5 for the carboxylic and phenolic groups, respectively), indicating that the undissociated form of the analyte is the one preferentially extracted by the polymeric coating of the fiber. The extraction was then carried out at pH 3, where the target compound was present in its undissociated form.

Extraction efficiencies of PA, CW/TPR-100, and PDMS/DVB coatings were evaluated and compared to select the best fiber. The absolute amount of OTA extracted was calculated from peak area values, and a calibration curve was obtained by direct injection of OTA standards. After 1 h of extraction time at ambient temperature (25 ± 1 °C) from a beer sample spiked at 0.2 ng/mL, the absolute amounts extracted were 10, 30, and 35 fmol for PA, PDMS/DVB, and CW/TPR-100, respectively.

The CW/TPR-coated fiber gave the highest recovery; however, co-extraction of matrix components from beer was also

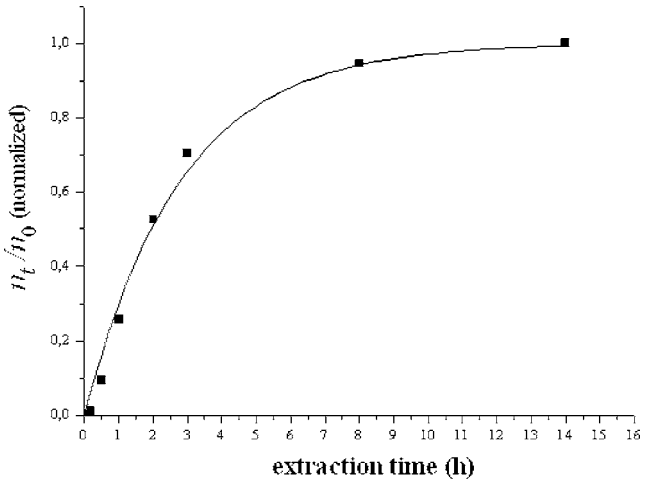

Figure 1. Extraction profiles of OTA from a beer sample spiked at a 0.2 ng/mL level. Fiber, PDMS/DVB; extraction temperature, 25 ± 1 °C.

very efficient (i.e., lower selectivity compared to PDMS/DVB) so that no appreciable improvement could be observed in the signal-to-noise ratio. The PDMS/DVB coating was then chosen for further experiments because it gave the best compromise between extraction efficiency and selectivity.

The amount, n_t, of OTA extracted by the PDMS/DVB fiber at time t, normalized to the equilibrium value n_0, is shown in **Figure 1**; the solid line in **Figure 1** is plotted according to the following equation

$$n_t/n_0 = 1 - \exp(-at) \quad (1)$$

In eq 1, a is a time constant (depending, among other factors, upon the analyte mass-transfer coefficient) and n_0 is given by

$$n_0 = \frac{K_{fs}V_fV_s}{K_{fs}V_f + V_s}C_0 \quad (2)$$

where K_{fs} = fiber-solution partition constant, V_f = volume of the fiber coating, V_s = sample volume, and C_0 = OTA concentration in the sample. From the best fit of eq 1, a value of 0.36 h^{-1} could be estimated for the time constant "a". The average ($n = 3$) value of n_0, estimated from the data at $t = 14$ h, was equal to 110 ± 8 fmol. Considering that the initial amount of OTA in the extraction solution was 186 fmol, this implies that the extraction efficiency of the fiber is ca. 60% under equilibrium condition and ca. 15% after 1 h of extraction (nonequilibrium conditions). Such an apparently low efficiency value is not surprising because SPME is, notoriously, a nonexhaustive extraction method. It is worth noting that the amount of analyte extracted from beer is not significantly different (according to a t test at a 95% confidence level) from that extracted from a standard solution (at the same concentration level); this finding indicates that there is no evidence of matrix effects.

Under equilibrium conditions, the fiber-solution distribution coefficients, K_{fs} (that dictates the maximum preconcentration factor achievable), could be calculated as the ratio between the concentration of the analyte in the fiber coating and in the solution; a log K_{fs} value of 3.7 was estimated.

Equation 1 shows that, provided the "a" term is constant, a linear relationship between n_t and C_0 is observed even under nonequilibrium conditions; then, an extraction time of 60 min was chosen for further experiments.

The temperature above ambient (e.g., 50 °C) produced a ca. 25% response decrease; then, this factor was not investigated further, and all subsequent work was performed at ambient temperature (25 ± 1 °C) in an air-conditioned laboratory environment.

The addition of sodium chloride (up to 200 mg/mL) to beer samples caused a ca. 50% signal enhancement (i.e., improved extraction efficiency, likely because of a salting out effect). However, the adjustment of the ionic strength was not further considered, because it was found to improve co-extraction of beer components, giving rise to more complex chromatograms.

Sample transfer from the fiber to the column is not a crucial step in gas chromatography (GC) because problems arising from slow desorption kinetics from the fiber can be simply counteracted by increasing the injector temperature (and/or the desorption time) and refocusing the injection band on the GC column head. In the case of SPME interfaced to LC, analyte transfer is a highly critical step. The dynamic desorption mode (which could ensure quantitative recoveries) causes a significant increase of both peak width and peak asymmetry (compared to conventional loop injection), deteriorating chromatographic efficiency and resolution. Thus, a static desorption technique was evaluated as a possible alternative. The fiber was soaked in the static mobile phase contained in the desorption chamber (60 μL volume) of the SPME interface for a variable period of time before injection into the LC column. The best conditions were reached after 60 s of static desorption, followed by a 10 s injection time into the mobile-phase stream. This injection mode gave a peak asymmetry factor, measured at 10% peak height, of 1.17, in comparison to 1.14 obtained by direct injection. Under these conditions, the peak shape was not deteriorated and a satisfactory chromatographic efficiency (plate number 36 800 m^{-1}) could be obtained. Obviously, under static injection mode, the complete sample transfer could not be necessarily achieved; indeed, a memory effect was observed, resulting in an estimated carry-over of ca. 12%. Then, before each extraction, the fiber was fully desorbed by the cleaning procedure described in the Experimental Procedures.

Figure 2 reports the SPME−LC−FD chromatograms obtained from (a) an unspiked beer sample and (b and c) a beer sample spiked at 0.03 and 0.25 ng/mL, respectively. As apparent, the optimized mobile phase allowed a good separation from matrix components of OTA that was easily detected at these concentration levels. It should be noted that the separation was performed under simple isocratic elution conditions in less than 16 min. In the 10 different (unspiked) beer samples analyzed, a small peak eluting at the same retention time of OTA was quite invariably observed (see **Figure 2a**). The chromatographic behavior of this peak was identical to that of OTA, even under several gradient elution programs tested. Therefore, it can be reasonably hypothesized that the peak in unspiked beer samples originates from the OTA background level. The difficulty in finding a true blank sample when using the method possessing very low limits of detection (LOD) is not surprising. Consider, for instance, that Nakajima et al. (26), using a method possessing a LOD of 1 pg/mL, have reported an incidence of positive sample higher than 90% during a survey of beers imported in Japan from all over the world. The above hypothesis could be readily verified using a sample cleanup step by IAC (see the Experimental Procedures) that provides a 20-fold preconcentration factor. **Figure 3** clearly shows that the peak at 13.608 min in **Figure 2a** is to be ascribed to a natural OTA contamination (roughly estimated around 9 pg/mL from the peak area

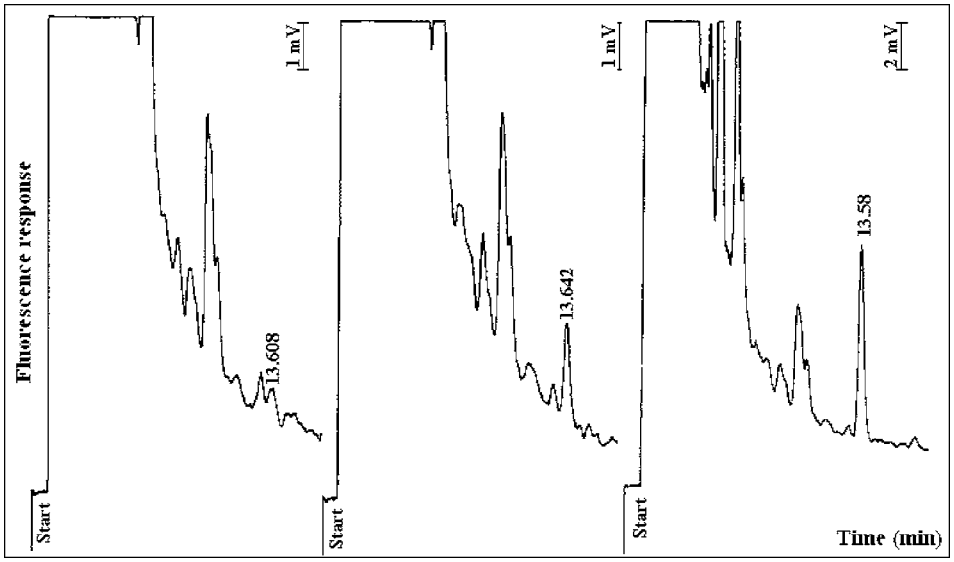

Figure 2. SPME–LC–FD chromatograms relevant to (a) an unspiked beer sample and (b and c) a beer sample spiked with 0.03 and 0.25 ng/mL of OTA, respectively. For chromatographic and detection conditions, see the Experimental Procedures.

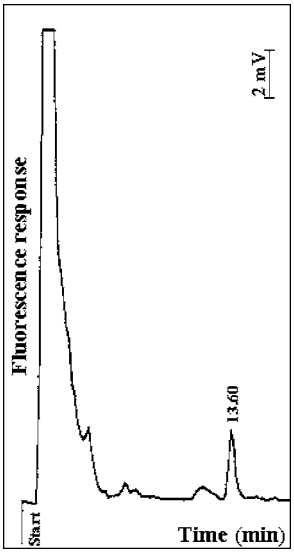

Figure 3. LC–FD chromatogram obtained on the same beer sample of **Figure 2a** after a cleanup step by IAC. For chromatographic and detection conditions, see the Experimental Procedures.

value and a calibration curve obtained by direct injection of OTA standards) of the analyzed beer.

The response of the developed SPME−LC procedure was linear in the ranges of 0.03−2 ng/mL. The unweighted regression line of peak area counts (arbitrary unit) versus [OTA] (ng/mL) was described by the following equation: $y = (0.7_1 \pm 0.3_1) \times 10^5 + (4.45 \pm 0.04) \times 10^6 x$; $R^2 = 0.9996$. The standard error of the regression was $S_{y/x} = 6.9 \times 10^4$.

The limit of quantitation (LOQ), calculated at a signal-to-noise ratio of 10 (noise taken as the standard deviation on the intercept of the unweighted regression line), was 53 pg/mL, well below the Italian guideline value of 0.2 ng/mL (the lowest among those issued in European countries).

The within-day and between-days precision (repeatability) of the method (at 0.2 ng/mL), expressed as RSD, were 4.3 and 5.9%, respectively.

The proposed procedure seems to possess all of the requirements necessary for a routine use to detect noncompliance to guideline (or legal) limits "G" (e.g., 0.2 ng/mL for beer). This implies that the null hypothesis H_0, [OTA] = G, has to be tested (on a statistical basis) against the alternative hypothesis H_1, [OTA] > G. The average peak area value (arbitrary unit, a.u.) for OTA at 0.2 ng/mL is $(8.95 \pm 0.48) \times 10^5$ a.u. ($n = 5$); assuming an equal probability of false positive (α) and false negative (β) of 0.05, the minimum peak area that has to be measured to consider the sample as "noncompliant" is 11.9×10^5 a.u. Using this value to interpolate the calibration curve, a concentration value of 0.25 ng/mL was obtained. When the confidence band on the regression line was taken into account, the standard error on the interpolated value can be obtained and the 95% confidence interval can be calculated as 0.25 ± 0.02 ng/mL. In conclusion, for $\alpha = \beta = 0.05$, only samples containing an OTA concentration higher than 0.27 ng/mL, i.e., "the minimum detectable nonadmissible concentration" (*27*−*29*), should be considered as "noncompliant" using the described procedure. As seen, the intralaboratory precision of the proposed method is such that the minimum detectable nonadmissible concentration is "only" 35% higher than the guideline value. Note that, for a given value of α and β, when the precision of the method becomes lower, the difference between the guideline (legal) limit and the concentration for "noncompliance" declaration becomes higher.

The proposed approach could work as well on other liquid matrixes such as wine; work in this direction is currently in progress.

LITERATURE CITED

(1) Pitt, J. I. Toxigens fungi and mycotoxins. *Br. Med. Bull.* **2000**, *56*, 184−92.

(2) JEFCA (Joint FAO/WHO Expert Committee on Food Additives). Ochratoxin A. In *Safety Evaluation of Certain Mycotoxins in Food*; WHO Food Additives Series 47; FAO Food and Nutrition Paper 74; WHO: Geneva, Switzerland, 2001; p 366.

(3) IARC. *Monograph on the Evaluation of Carcinogenic Risks to Humans, Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins*; International Agency for Research on Cancer: Lyon, France, 1993; Vol. 56, pp 489−521.

(4) Stoev, S. D. The role of ochratoxin A as a possible cause of Balkan endemic nephropathy and its risk evaluation. *Vet. Hum. Toxicol.* **1998**, *40*, 352−360.

(5) Ochratoxin A (WHO Food Additives series 47); 2001, http://www.inchem.org/pages/jecfa.html.

(6) Scientific Cooperation (SCOOP) Task Report 3.2.7. Assessment of dietary intake of ochratoxin A by the population of EU member states; 2002, http://europa.eu.int/comm/food/fs/scoop/index_en.html.

(7) Commission Regulation (EC) 123/2005 of 26 January 2005 amending Regulation (EC) 466/2001 as regards ochratoxin A. *Off. J. Eur. Communities* **2005**, *L 25/3* (Jan 28).

(8) Monaci, L.; Palmisano, F. Determination of ochratoxin A in foods: State-of-the-art and analytical challenges. *Anal. Bioanal. Chem.* **2004**, *378*, 96−103.

(9) Saez, J. M.; Medina, A.; Gimeno-Adelantado, J. V.; Mateo, R.; Jimenez, M. Comparison of different sample treatments for the analysis of ochratoxin A in must, wine and beer by liquid chromatography. *J. Chromatogr., A* **2004**, *1029*, 125−133.

(10) Visconti, A.; Pascale, M.; Centonze, G. Determination of ochratoxin A in domestic and imported beers in Italy by immunoaffinity clean-up and liquid chromatography. *J. Chromatogr., A* **2000**, *888*, 321−326.

(11) Zimmerli, B.; Dick, R. Determination of ochratoxin A at the ppt level in human blood, serum, milk and some foodstuffs by high-performance liquid chromatography with fluorescence detection and immunoaffinity column clean-up: Methodology and Swiss data. *J. Chromatogr., B: Biomed. Sci. Appl.* **1995**, *666*, 85−99.

(12) Medina, A.; Jimenez, M.; Gimeno-Adelantado Valle-Algarra, F. M.; Mateo, R. Determination of ochratoxin A in beer marketed in Spain by liquid chromatography with fluorescence detection using lead hydroxyacetate as a clean-up agent. *J. Chromatogr., A* **2005**, *1083*, 7−13.

(13) Bacaloni, A.; Cavaliere, C.; Faberi, A.; Pastorini, E.; Samperi, R.; Laganà, A. Automated on-line solid-phase extraction-liquid chromatography-electrospray tandem mass spectrometry method for the determination of ochratoxin A in wine and beer. *J. Agric. Food Chem.* **2005**, *53*, 5518−5525.

(14) Arthur, C. L.; Pawliszyn, J. Solid-phase microextraction with thermal desorption using fused silica optical fibers. *Anal. Chem.* **1990**, *62*, 2145−2148.

(15) Arthur, C. L.; Killam, L. M.; Buchholz, K. D.; Pawliszyn, J. Automation and optimization of solid-phase microextraction. *Anal. Chem.* **1992**, *64*, 1960−1966.

(16) Louch, D. S.; Motlagh, S.; Pawliszyn, J. Dynamics of organic compound extraction from water using liquid-coated fused silica fibers. *Anal. Chem.* **1992**, *64*, 1187−1199.

(17) Potter, D. W.; Pawliszyn, J. Detection of substituted benzenes in water at the pg/mL level using solid-phase microextraction and gas chromatography−ion trap mass spectrometry. *J. Chromatogr., A* **1992**, *625*, 247−255.

(18) Zhang, Z.; Pawliszyn, J. Headspace solid-phase microextraction. *Anal. Chem.* **1993**, *65*, 1843−1852.

(19) Potter, D. W.; Pawliszyn, J. Rapid determination of polyaromatic hydrocarbons and polychlorinated biphenyls in water using solid-phase microextraction and GC/MS. *Environ. Sci. Technol.* **1994**, *28*, 298−305.

(20) Kataoka, H.; Lord, H. L.; Pawliszyn, J. Applications of solid-phase microextraction in food analysis. *J. Chromatogr., A* **2000**, *880*, 35−62.

(21) Zambonin, C. G. Coupling solid-phase microextraction to liquid chromatography. A review. *Anal. Bioanal. Chem.* **2003**, *375*, 73−80.

(22) Zambonin, C. G.; Monaci, L.; Aresta, A. Determination of cyclopiazonic acid in cheese samples using solid-phase microextraction and high performance liquid chromatography. *Food Chem.* **2001**, *75*, 249−254.

(23) Zambonin, C. G.; Monaci, L.; Aresta, A. Solid-phase microextraction-high performance liquid chromatography and diode array detection for the determination of mycophenolic acid in cheese. *Food Chem.* **2002**, *78*, 249−254.

(24) Aresta, A.; Cioffi, N.; Palmisano, F.; Zambonin, C. G. Simultaneous determination of acidic mycotoxins in foodstuffs by solid-phase microextraction coupled to high performance liquid chromatography. *J. Agric. Food Chem.* **2003**, *51*, 5232−5237.

(25) Amezqueta, S.; Gonzalez-Penas, E.; Murillo, M.; Lopez de Cerain, A. Validation of a high-performance liquid chromatography analytical method for ochratoxin A quantification in cocoa beans. *Food Addit. Contam.* **2004**, *21*, 1096−1106.

(26) Nakajima, M.; Tsoubouchi, H.; Miyabe, M. A survey of ochratoxin A and aflatoxins in domestic and imported beers in Japan by immunoaffinity and liquid chromatography. *J. AOAC Int.* **1999**, *82*, 897−902.

(27) Desimoni, E.; Mannino, S. Uncertainty of measurement and legislative limits. *Accredit. Qual. Assur.* **1998**, *3*, 335−337.

(28) Desimoni, E.; Mannino, S.; Brunetti, B. On the assessment of compliance with legal limits. Part 1: Signal and concentration domains. *Accredit. Qual. Assur.* **2001**, *6*, 452−458.

(29) Christensen, J. M.; Holst, E.; Olsen, E.; Wilrich, P. T. Rules for stating when a limiting value is exceeded. *Accredit. Qual. Assur.* **2002**, *7*, 28−35.

Received for review October 27, 2005. Revised manuscript received December 28, 2005. Accepted January 17, 2006. Financial support from the University of Bari is gratefully acknowledged.

JF052666O