JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Accelerated Solvent Extraction of Ochratoxin A from Rice Samples

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In this paper, accelerated solvent extraction (ASE) for the analysis of ochratoxin A (OTA) is applied for the first time. Optimization of the method is given for the extraction of OTA from rice samples. Several parameters such as type of solvent, temperature, pressure, static time, and cell size were investigated thoroughly to find the optimal extraction conditions. The optimum ASE operating conditions were methanol as extraction solvent, 1500 psi, 40 °C, 5 min of static time, 50% flush volume, 60 s of purge, 1 cycle, and 11 mL cell size. The total extraction time was ∼15 min. OTA was determined by liquid chromatography coupled with a fluorescence detector and confirmed by methyl ester derivatization. The analytical performance of the method was monitored with quality control parameters. Finally, the optimized method was used to evaluate 12 rice samples, 1 of which was positive with an OTA content of 4.17 ng/g. The proposed method offers the possibility of a fast and simple process to obtain a quantitative extraction of OTA.

KEYWORDS: Ochratoxin A; accelerated solvent extraction; rice

INTRODUCTION

Ochratoxin A (OTA), 7-carboxyl-5-chloro-8-hydroxyl-3,4-dihydro-3*R*-methylisocoumarin-7-L-*β*-phenyl-alanine, is a colorless crystalline compound that belongs to a group of closely related derivatives of isocoumarin linked to L-phenylalanine and classified as pentaketides (*1*). OTA is a secondary metabolite produced by the genera *Penicillium* (e.g. *P. verrucosum*) and *Aspergillus* (e.g. *A. ochraceus*); it has been shown to be teratogenic and immunosuppressive and has been implicated in Balkan nephropathy in humans. The International Agency for Research on Cancer (IARC) lists OTA as possibly carcinogenic to humans (group 2B) (*2*). A level of 5 ng/g of OTA in cereals has been established in the European Union as maximum permitted limit (*3*).

Natural occurrence of OTA has been reported from temperate subtropical and tropical climates in several foods including rice (*4*, *5*). Rice is one of the most important crops in the world; in 2002/2003, 408 661 000 metric tons were consumed (*6*). The United Nations has launched a major international drive to increase the production of rice, the staple food for more than half of the world's population.

OTA, due to the co-presence of phenolic and carboxylic groups, has an acid nature ($pK_a = 4.4$). Therefore, OTA is soluble in organic solvents, such as chloroform, methanol, and acetonitrile in acid medium and also in diluted aqueous sodium bicarbonate (*7*). OTA analysis is a multiple-step process usually based on extraction, cleanup, and determination. Detection methods for OTA and mycotoxins in general are based on thin-layer chromatography, enzyme-linked immunosorbent assay (ELISA), or mainly liquid chromatography (LC) (*8*). Common extraction methodologies are based on the solubility of OTA in organic solvents. Frequently used cleanup procedures are liquid−liquid extraction or solid-phase extraction (*9*).

Accelerated solvent extraction (ASE) is a methodology that uses solvents at relatively high pressure and temperatures at or above the boiling point. The experimental parameters of the extraction are temperature, pressure, static time, cell size, and solvent used (*10*−*13*). The ASE technique consists, briefly, in enclosing a solid sample in a cell and sealing it. The cell is filled with an extraction solvent. Under static conditions (static time), the fluid is held in the cell under elevated temperature and pressure for short periods of time (5−10 min); this step is also denominated a cycle. This permits contact between the solvent and the sample for efficient extraction. Fresh solvent is flushed through the cell, and compressed gas is used to purge the sample extract from the cell into a collection vessel. A fast extraction is possible with ASE due to the use of high temperatures and pressures to keep the solvent in liquid state. This enhances efficiency compared to extractions at room temperature and atmospheric pressure (*14*).

The extraction process in ASE has three subsequent steps. First, the analytes must diffuse from the core of the matrix to the surface, and, next, they are transferred from the surface into the extraction fluid. Finally, the analytes are eluted. The ASE rate is limited by the slowest of these three steps. In ASE, mass transfer from the core into the solvent is controlled by molecular diffusion. The respective diffusion coefficient is determined by

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10.1021/jf051560q CCC: $30.25
Published on Web 10/26/2005

the structure of solutes, extraction temperature, and type of extraction solvent, which provides the opportunity to use a wide variety of solvents, even those not effective in conventional extraction methods (*15*).

The objective of this study is to optimize the ASE of OTA from rice and to make use of the proposed method in naturally contaminated rice samples.

MATERIALS AND METHODS

Chemicals. OTA crystalline material was purchased from Sigma (St. Louis, MO). A stock standard solution of OTA at 500 μg/mL in methanol was prepared and kept wrapped in aluminum foil at −20 °C, because OTA gradually breaks down under UV light. OTA working solutions were prepared by dilution in the same solvent and stored in glass-stoppered tubes at −20 °C.

High-performance liquid chromatography (HPLC) grade methanol, toluene, dichloromethane, acetonitrile, ethyl acetate, and acetic acid were supplied by Merck (Darmstadt, Germany). Formic acid was obtained from Scharlau (Barcelona, Spain). Deionized water (0.125 μS) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA).

Sampling. Rice (*Oryza sativa*) samples were collected from different rice packers and supermarkets. All samples were stored in sealed plastic bags and kept at 25 °C in a dark and dry place. The samples were divided with a subsample divider. A 200 g subsample was milled and collected in a plastic bag and stored under the same conditions until analysis.

Sample Fortification Procedure. The recovery (percentage of standard added to sample that is recovered after extraction and cleanup) of the extraction method was determined by sample fortification. Five grams of milled rice was fortified 1 h before extraction with a solution of OTA in methanol at 0.05 μg/mL. The OTA fortification solution (0.05 μg/mL) was prepared in methanol and used for quantification of the analyte recovered after extraction. Samples were fortified with 0.5 mL of this solution in order to have 5 ng of OTA/g of rice, which is the maximum permitted limit in cereals.

ASE. An automated Dionex ASE 200 system (Dionex Co., Sunnyville, CA) was used for OTA extractions. Stainless steel extraction cells were sealed at one end with circular cellulose filters of 1.98 cm diameter (Dionex Co.) when organic solvents were to be used and with 1.98 cm diameter glass microfiber filters for water or methanol/water/ammonia (70:29:1 v/v) as extraction solvent. Five grams of milled rice was poured into an 11 mL extraction cell, fortified with the OTA fortification solution, and closed. Several extraction solvents were tried, for example, methanol, toluene, dichloromethane, acetonitrile, ethyl acetate, water, methanol/water (70:30 v/v), methanol/water/ammonia (32%) (70:29:1 v/v), and methanol/acetic acid (99:1 v/v).

At the end of each extraction a total extract volume of ∼12 mL was evaporated to dryness using a Büchi Rotavapor R-200. After redissolving in methanol, the extract was poured into an assay tube, evaporated at 55 °C with a gentle stream of nitrogen, and taken to a final volume of 0.5 mL at room temperature. This final solution was stored at 4 °C until injection at room temperature on the liquid chromatograph−fluorescence detector (LC-FLD) for analysis.

LC-FLD Determination. A Shimadzu (Kyoto, Japan) SCL-6A liquid chromatography system equipped with two LC-6A pumps, a Rheodyne model 7125 injector (20 μL loop), and an SRF-535 fluorescence detector were used. A LC Phenomenex column Luna C_{18} (5 μm) (150 × 4.6 mm i.d.) was employed with a mobile phase consisting of methanol/0.1 M formic acid (70:30 v/v) at a flow rate of 0.7 mL/min according to the method of Blesa et al. (*5*). Detection of OTA was carried out using 334 and 464 nm as wavelengths for excitation and emission, respectively.

Confirmation Procedure. The identity of OTA was confirmed by methyl ester formation according to the method of Zimmerli and Dick (1995) (*16*). Briefly, this technique consists of adding 2.5 mL of methanol and 0.1 mL of concentrated hydrochloric acid to 200 μL of OTA residue. The vial is closed and kept overnight at room temperature. The reaction mixture is evaporated to dryness and the residue redissolved in mobile phase. Then 20 μL is analyzed using LC-FLD. The reaction yields ∼90% (*5*).

Table 1. Mean Recoveries of OTA Obtained after Extraction from a Spiked Rice Sample[a]

solvent conditions	extraction recovery (%) ± RSD (%)
methanol	77.2 ± 5.0
methanol/water (70:30)	59.1 ± 6.2
methanol/water/ammonia (70:29:1)	42.5 ± 5.9
methanol/acetic acid (99:1)	44.2 ± 5.7
toluene	59.2 ± 8.0
water	16.3 ± 7.1
dichloromethane	29.2 ± 10.3
ethyl acetate	16.4 ± 6.7
acetonitrile	26.1 ± 11.2

[a] Spiking at 5 ng of OTA/g of rice. Extractions were performed in triplicate. ASE operating conditions: 1000 psi, 40 °C, 1 cycle, 5 min static time, 11 mL cells, 60 s purge time.

RESULTS AND DISCUSSION

Achieving maximum efficiency is probably the greatest concern in ASE method development. Because several parameters influence the extraction efficiency, a general discussion of these parameters is presented.

Solvent Selection. ASE can be performed with almost any solvent or mixture of solvents (except strong concentrated bases and acids). The selection of a suitable extraction solvent is the first challenge in ASE method development (*17*). Several solvents and combinations of solvents [methanol, methanol/water (70:30), methanol/water/ammonia (70:29:1), methanol/acetic acid (99:1), toluene, water, dichloromethane, ethyl acetate, acetonitrile] were tried for the extraction of OTA from rice. ASE conditions for these trials were as follows: 1000 psi, 40 °C, 1 cycle, 5 min static time, and 11 mL cells. It was decided to work with these solvents for three reasons. Primarily, some of them are used for the extraction of OTA by several authors obtaining good recoveries (*18*−*21*). Secondarily, OTA, as an acid ($pK_a = 4.4$), is moderately soluble in organic solvents in acid medium. Finally, in ASE it is possible to use solvents that are not effective in conventional methods because the solubilizing power is increased by high temperature and pressure (*15*). Results of this first trial are shown in **Table 1**.

These results show that under these conditions methanol was the solvent with best recoveries of OTA. However, it was decided to perform various experiments with all solvents, changing the temperature (40, 60, and 80 °C) and pressure (500, 1000, 1500, 2000, and 2500 psi). According to Gan et al. (*22*), along with time, these are the most important variables that can affect the extraction efficiency by ASE.

After this was done, we selected the conditions of highest recoveries of OTA with every solvent. For each solvent it was possible to obtain better recoveries of OTA by using a pressure and temperature higher than the conditions tried at first (40 °C and 1000 psi). Methanol is still the solvent with the best recovery values (**Table 2**). Therefore, it was chosen as the solvent for ASE of OTA from rice followed by its detection by LC. The use of methanol can be pointed out as a general advantage for extracting because in this way the use of halogenated solvents is avoided, the extraction procedure is facilitated because no mixtures have to be prepared, and methanol is a low-cost solvent.

Effect of Temperature and Pressure. The use of high temperatures during the extraction process affects the properties

Table 2. ASE Operating Conditions of Best Recoveries of OTA for Each Solvent[a]

solvent	ASE conditions: pressure (psi)	ASE conditions: temp (°C)	% of recovery
methanol	1500	40	95.0
methanol/water (70:30)	500	40	70.5
methanol/water/ammonia (70:29:1)	500	40	72.8
methanol/acetic acid (99:1)	1000	60	64.0
toluene	1500	60	74.8
water	1500	60	39.3
dichloromethane	1500	60	51.3
ethyl acetate	1000	40	21.1
acetonitrile	1000	40	34.1

[a] Spiking at 5 ng of OTA/g of rice. ASE operating conditions: 11 mL cells, 1 cycle, 5 min static time, 60 s purge time.

of a solvent. It increases diffusion rates and the capacity to solubilize analytes. Interactions between analytes and matrix components are weakened, and there is a decrease in viscosity and surface tension. With the use of high pressure in the extraction process, the solvent is kept in a liquid state when temperatures at or above the boiling point are being used. It also improves the extraction efficiency by forcing the solvent into areas that would not normally be contacted using atmospheric conditions (*8*, *14*).

A temperature of 40 °C was chosen for ASE of OTA from rice because it gave recoveries of >90%. As the temperature was increased above 40 °C, the extraction efficiency decreased. As was mentioned before, high temperatures will increase solubility and mass transfer, but selectivity also decreases (*8*); therefore, matrix components are coextracted. Considering that rice has an elevated content of starch and other components highly soluble in alcohol at high temperatures, it is feasible that under these extracting conditions their solubility in methanol is higher than the solubility of OTA, causing saturation of the solvent and a low content of OTA in solution.

ASEs of OTA from rice samples with methanol were carried out using different pressures (500, 1000, 1500, 2000, and 2500 psi). The highest recovery of OTA was obtained at 1500 psi. A decrease in ASE recoveries of OTA was observed when pressures >1500 psi were utilized (2000 and 2500 psi) at 40 °C, even when several authors (*8*, *12*, *14*) have not found a relationship between pressure and recovery. In this case higher pressure along with temperature improves a better extraction of matrix components than of OTA.

Effect of Cell Size and Number of Cycles. During the optimization of the method, two different cell sizes were tried, that is, 11 and 22 mL. For both sizes the same quantity of rice (5 g) and the conditions previously established were used.

There were no differences between recoveries of OTA by using either the 11 or 22 mL cell size. However, with the 11 mL cells a lower quantity of solvent is used, which is in general an environmental and economic advantage.

To investigate the effects of extraction time on extraction efficiency, time was varied by augmenting the number of extraction cycles (one, two, and three cycles). When multiple cycles are used, the application of static time (5 min in this case) and the solvent flush step are repeated. These would improve the penetration of solvent into the sample interstices and the contact of the solvent with the analyte. In this study, we did not observe an enhancement in recoveries of OTA by increasing the time used in extraction; thus, a 5 min cycle was set for the extraction of OTA from rice.

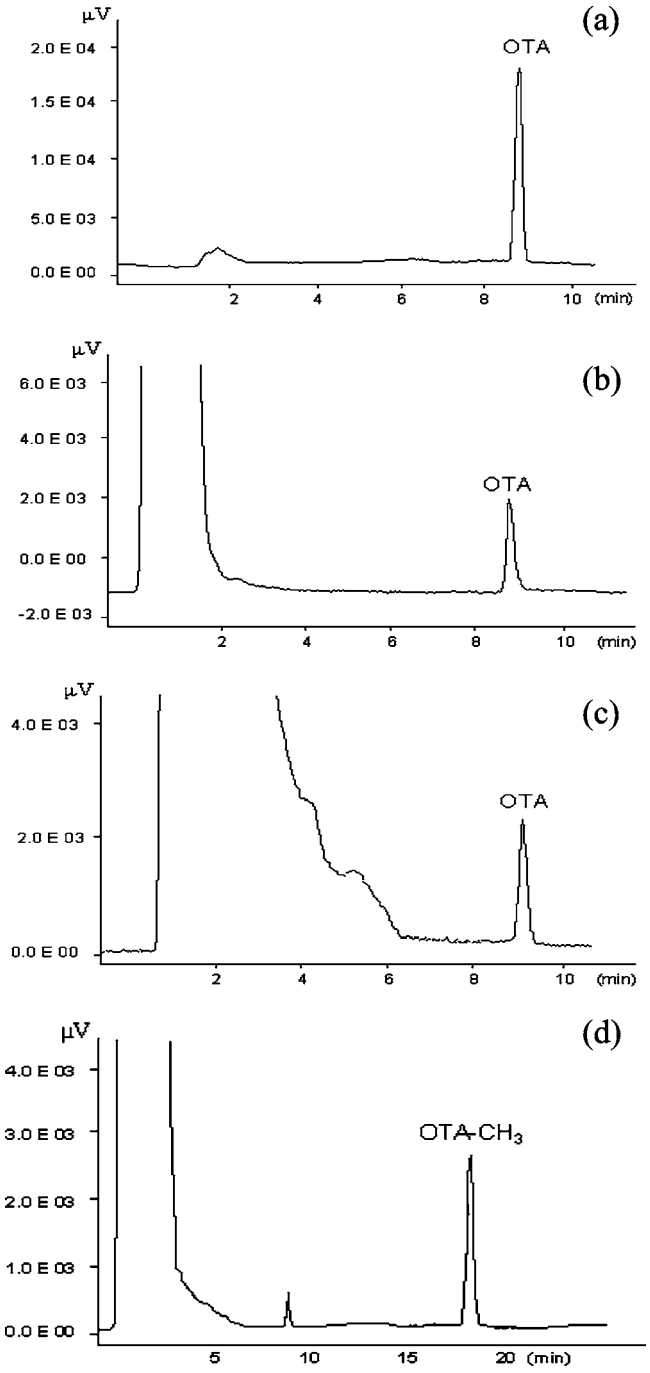

Figure 1. LC-FLD chromatograms: (**a**) fortification solution, 0.05 μg/mL of OTA; (**b**) rice sample spiked at 5 ng/g of OTA; (**c**) positive rice sample with an OTA content of 4.17 ng/g; (**d**) methylated rice sample spiked at 5 ng/g of OTA extract.

Analytical Performance. The mean recovery of fortified rice samples ($n = 8$) at a level of 5 ng/g of OTA was 94.0%, with a relative standard deviation of 2.5%. These values at a fortification level of 15 ng/g remain almost the same. At this fortification level, the mean recovery of the method is 90.7%, with a relative standard deviation of 2.8%. The values obtained for recovery and relative standard deviation of the optimized method are in agreement with Commission Directive 2002/26/EC for methods of analysis of OTA in foodstuffs (*23*). These results indicate good recovery and reproducibility of the method optimized in this work. Methodologies such as solid-phase extraction and liquid−liquid extraction yield 60−120% (*24*), considering that the proposed method of ASE of OTA from

rice with LC-FLD offers also several advantages; for example, it is an automated procedure that allows up to 24 programmed extractions to be performed, the quantity of solvents used is lessened, and it does not require a large amount of sample. Therefore, it is a good alternative that allows the analysis to be performed with good precision and accuracy.

Several parameters were taken into consideration to establish quality control of the method, such as limit of detection and limit of quantification, which were calculated by applying the 3σ criterion (*25*), and inter- and intraday variations. The limits of detection and quantification were 0.01 and 0.03 ng/g, respectively. The analytical work was conducted on three different days in triplicate to detect any day-to-day effects. Certification exercises on several mycotoxins indicate the possibility that the variation (inter- and intraday) values for duplicate recovery experiments do not exceed 15% (*26*). At a fortification level of 5 ng/g of OTA, an interday variation of 5.5% and an intraday variation of 4.7% were obtained. According to these results it can be said that the analytical work conducted fulfills both requirements.

Application of the Optimized Method to Real Rice Samples. Twelve rice samples were purchased and treated as described under Materials and Methods. The optimized ASE method was applied for the analysis of OTA in these samples. One of these samples contained an OTA level of 4.17 ng/g. According to Commission Regulation (EC) No. 472/2002, this level is below that established as the maximum permitted level of OTA in cereals.

The chromatograms corresponding to the optimized ASE of OTA in a spiked rice sample and a naturally contaminated rice sample are shown in **Figure 1** as are the chromatograms of the OTA fortification solution and of the methylated extract. There were no interfering peaks near the retention time of OTA, allowing the quantitation of the analyte. Retention times for OTA and methylated OTA (OTA-CH_3) were 8.53 and 18.2 min, respectively.

LITERATURE CITED

(1) Steyn, P. S. Ochratoxins and related dihydro-isocoumarins. In *Mycotoxins: Production, Isolation, Separation, and Purification*; Betina, V., Ed.; Elsevier Science: New York, 1984; pp 183−216.

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

(3) Commission Reglament (CE) 472/2002, of 12 March 2002. *Off. J. Eur. Communities* **2002**, *L 075*, 0018−0020.

(4) Karin, A.; Gunnar, J.; Karl, H. Ochratoxin A in rice cultivars after inoculation of *Penicillium verrucosum*. *Nat. Toxins* **1998**, *6*, 73−84.

(5) Blesa, J.; Berrada, H., Soriano, J. M.; Moltó, J. C.; Mañes, J. Rapid determination of ochratoxin A in cereals and cereal products by liquid chromatography. *J. Chromatogr. A* **2004**, *1046*, 127−131.

(6) U.S. Department of Agriculture (USDA), Foreign Agricultural Service (FAS), Aug 2002.

(7) Miraglia, M.; De Santis, B.; Grossi, S.; Brera, C. Mycotoxins. In *Handbook of Food Analysis*; Nollet, M. L., Ed.; Hogeschool Gent: Ghent, Belgium, 2004; pp 85−100.

(8) Giergielewicz-Mozajska, H.; Da Browski, L.; Namiesnik, J. Accelerated solvent extraction (ASE) in the analysis of environmental solid samples—some aspects of theory and practice. *Crit. Rev. Anal. Chem.* **2001**, *31*, 149−165.

(9) Scott, P. M.; Trucksess, M. W. Application of immunoaffinity columns to mycotoxin analysis. *J. AOAC Int.* **1997**, *80*, 941−949.

(10) Adou, K.; Bontoyan, W. R.; Sweeney, P. J. Multiresidue method for the analysis of pesticide residues in fruits and vegetables by accelerated solvent extraction and capillary gas chromatography. *J. Agric. Food Chem.* **2001**, *49*, 4153−4160.

(11) Gentili, A.; Perret, D.; Marchese, S.; Sergi, M.; Olmi, C.; Curini, R. Accelerated solvent extraction and confirmatory analysis of sulfonamide residues in raw meat and infant foods by liquid chromatography electrospray tandem mass spectrometry. *J. Agric. Food Chem.* **2004**, *52*, 4614−4624.

(12) Heemken, O. P.; Theobald, N.; Wenclawiak, B. W. Comparison of ASE and SFE with Soxhlet, sonication and methanolic saponification extractions for the determination of organic micropollutants in marine particulate matter. *Anal. Chem.* **1997**, *69*, 2171−2180.

(13) Alonso-Salces, R. M.; Korta, E.; Barranco, A.; Berrueta, L. A.; Gallo, B.; Vicente, F. Determination of polyphenolic profiles of basque cider apple varieties using accelerated solvent extraction. *J. Agric. Food Chem.* **2001**, *49*, 3761−3767.

(14) Richter, B. E.; Jones, B. A.; Ezzel, J. L.; Porter, N. L.; Avdalovic, N.; Pohl, C. Accelerated solvent extraction: a technique for sample preparation. *Anal. Chem.* **1996**, *68*, 1033−1039.

(15) Lou, X. W.; Janssen, H. G.; Cramers, C. A. Parameters affecting the accelerated solvent extraction of polymeric samples. *Anal. Chem.* **1997**, *69*, 1598−1603.

(16) 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 enhanced fluorescence detection and immunoaffinity column cleanup: methodology and Swiss data. *J. Chromatogr. B* **1995**, *666*, 85−99.

(17) Dionex Corp. *ASE 200, Accelerated Solvent Extractor Operator's Manual*; Document 031149, revision 03; Sunnyvale, 1997.

(18) Scudamore, K. A.; Hetmanski, M. T.; Nawaz, S.; Naylor, J. Rainbird. Determination of mycotoxins in pet foods sold for domestic pets and wild birds using linked-column immunoassay clean-up and HPLC S. *Food Addit. Contam.* **1997**, *14*, 175−186.

(19) Göbel, R.; Lusky, K. Simultaneous determination of aflatoxins, ochratoxin A and zearalenone in grains by new immunoaffinity column/liquid chromatography. *J. AOAC Int.* **2004**, *87*, 411−416.

(20) Richard, J. L.; Plattner, R. D.; May, J.; Liska, S. L. The occurrence of ochratoxin A in dust collected from a problem household. *Mycopathologia* **1999**, *146*, 99−103.

(21) Filali, A.; Ouammi, L.; Betbeder, M.; Baudrimont, I.; Soulaymani, R.; Benayada, A.; Creppy, E. E. Ochratoxin A in beverages from Morocco: a preliminary survey. *Food Addit. Contam.* **2001**, *18*, 565−568.

(22) Gan, J.; Papiernik, S. K.; Koskinen, W. C.; Yates, S. D. Evaluation of accelerated solvent extraction (ASE) for analysis of pesticide residues in soil. *Environ. Sci. Technol.* **1999**, *33*, 3249−3253.

(23) Commission Directive 2002/26/CE of 13 March 2002. *Off. J. Eur. Communities* **2002**, **L 075**, 0038−0043.

(24) Wood, G. M.; Patel, A.; Entwisle, A. C.; Boenke, A. Ochratoxin A in wheat: a second intercomparison of procedures. *Food Addit. Contam.* **1996**, *13*, 519−539.

(25) Wennrich, L.; Popp, P.; Moder, M. Determination of chlorophenols in soils using accelerated solvent extraction combined with solid-phase microextraction. *Anal. Chem.* **2000**, *72*, 546−551.

(26) Hald, B.; Wood, G. M.; Boenke, A.; Schurer, B.; Finglas, P. Ochratoxin-A in wheat—an intercomparison of procedures. *Food Addit. Contam.* **1993**, *2*, 185−207.

Received for review June 30, 2005. Revised manuscript received September 13, 2005. Accepted September 26, 2005. This work has been supported by the Spanish Ministry of Education and Science (AGL-2003-01407). L.G. thanks the CONACyT of Mexico (Consejo Nacional de Ciencia y Tecnología) for a grant. C.J. thanks the Spanish Ministry of Education and Science for a grant.

JF051560Q