JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Simultaneous Immunoaffinity Column Cleanup and HPLC Analysis of Aflatoxins and Ochratoxin A in Spanish Bee Pollen

RAFAEL J. GARCIA-VILLANOVA,*,† CARLOS CORDÓN,† ANA M. GONZÁLEZ PARAMÁS,† P. APARICIO,† AND M. EUGENIA GARCIA ROSALES‡

Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Farmacia, Universidad de Salamanca, Campus "Miguel de Unamuno", E-37007 Salamanca, Spain, and Centro de Estudios Tecnológicos, Industrial y de Servicios, No. 40, Tabasco, Mexico

Bee pollen is a major substrate for mycotoxins growth when no prompt and adequate drying is performed by the beekeeper after collection by bees. Regulatory limits for aflatoxins and ochratoxin A are currently in force in the European Union for a rising list of foodstuffs, but not for this. An immunoaffinity column cleanup process has been applied prior to the analysis of aflatoxins B_1, B_2, G_1, and G_2 and ochratoxin A (OTA). Optimization of the HPLC conditions has envolved both a gradient elution and a wavelength program for the separation and fluorimetric quantitation of all five mycotoxins at their maximum excitation and emission values of wavelength in a single run. The higher limit of detection (μg/kg) was 0.49 for OTA and 0.20 for aflatoxin B_1. Repeatability (RSDr) at the lower limit tested ranged from 9.85% for OTA to 6.23% for aflatoxin G_2, and recoveries also at the lower spiked level were 73% for OTA and 81% for aflatoxin B_1. None of the 20 samples assayed showed quantifiable values for the five mycotoxins.

KEYWORDS: Mycotoxins; aflatoxins; ochratoxin A; spanish bee pollen; immunoaffinity column; simultaneous food analysis

INTRODUCTION

Aflatoxins are a family of mycotoxins produced mainly by two *Aspergillus* species particularly abundant in areas of the world with hot, humid climates. *Aspergillus flavus*, which is ubiquitous, produces B aflatoxins. *A. parasiticus*, which produces both B and G aflatoxins, has more limited distribution. All of them, including the M_1, are considered to play a role in liver cancer etiology (carcinogenic to humans, Group 1, IARC) (*1*), and, besides that, aflatoxin B_1 is immunosuppressive (*2*).

The next most studied mycotoxin is ochratoxin A, hereafter OTA. It belongs to a group of five produced by *Penicillium verrucosum* and by several species of *Aspergillus*, most notably *A. ochraceus*. In temperate climates, *P. verrucosum* is the main source of its contamination in foods, while *Aspergillus* species predominate in warmer countries, notably in tropical regions (*2*). OTA is a potent nephrotoxic and is believed to be the cause of the Balkan endemic nephropathy. Still there is no adequate evidence for its renal carcinogenicity (possibly carcinogenic to humans, Group 2B, IARC) (*3*).

Both groups of mycotoxins can occur in a wide range of raw food commodities, including cereals, nuts and nut products, dried fruit, coffee, and spices. Aflatoxins have been also reported in cocoa, oil seeds, dried peas, and beans; OTA has been investigated in figs, wines (*4*, *5*), beers (*6*), pigs' kidneys (*7*, *8*), and more recently in both green (*9*) and roasted coffee (*10*). Legal limits have been set for human food and animal feed in many countries. Three years ago, a Commission Regulation (*11*) established for all of the European Union maximum permissible limits for the four aflatoxins for a number of foodstuffs. Two subsequent amendments (*12*, *13*) were made including limits for OTA (from 3−10 μg/kg) and enlarging application to other items.

Thin-layer chromatography (TLC) has been used for identification and quantitation of aflatoxins during the past three decades and of OTA for more than 20 years, because it is easy, fast, and inexpensive (*15*). HPLC with fluorescence detection (FD) is nowadays the method of choice because of the available short and high-resolution columns and of the sensitivity of fluorescence detectors, together with its potential for automation (*15*, *16*). Extraction is normally performed in acetonitrile−water, methanol−water, or even chloroform. An effective cleanup of the raw extract is required for purification of the analytes. To do so, more convenient techniques have now replaced the liquid−liquid separation with the employment of either solid-phase silica alone or of multifunctional columns. However, most of the past decade and recent articles on the analysis of aflatoxins and OTA deal with immunoaffinity columns (IAC) cleanup.

* Author to whom correspondence should be addressed (telephone +34-923-294537; fax +34-923-294515; e-mail rgvill@usal.es).
† Universidad de Salamanca.
‡ Centro de Estudios Tecnológicos.

10.1021/jf048882z CCC: $27.50
Published on Web 10/28/2004

Thus, the four B and G aflatoxins have been isolated for analysis in corn and peanuts (*17*); peanut butter (*18*); tahini (a sesame butter) (*19*); peanuts, fig, maize gluten, soya, and copra (*20*); peanut butter, paprika powder, and pistachio and fig pastes (*21*); spices (*22*); medicinal herbs and plant extracts (*23*); baby foods (*24*); and animal feeds (*25*). Immunoaffinity cleanup for analysis of OTA is reported for rye and trout feed (*26*), wheat (*27*), barley (*28*), baby foods (*29*), dried vine fruit (*30*), wines (*4*, *5*, *31*), beer (*6*, *31*), and soluble (*32*) green (*9*) and roasted (*33*, *34*) coffee. Some of these works deal with interlaboratory comparison. Precolumn derivatization is usually performed to enhance fluorescence emission of aflatoxins B_1 and B_2; a postcolumn bromination is less common because of the need of a reaction chamber (the Kobra cell) but is more efficient. The more recent studies report higher recoveries than the older ones, so that detection limits below 1 μg/kg and an analytical precision of $\pm$30% are currently achievable for all five mycotoxins in most foods.

Few simultaneous methods for analysis of mycotoxins can be found in the literature. The first published was a TLC of aflatoxin B_1 and OTA in black olives, using lead acetate as a cleanup solution and hexane for defatting (*35*). Two simultaneous HPLC determinations of citrinin and OTA in wheat and barley (*36*) and in cheese (*37*) were then reported. Simultaneous analysis of aflatoxins, OTA, and zearalenone in grains was performed with a complicated but somewhat effective C18 column on-line sample cleanup device (*38*) and, quite recently, with an IAC cleanup (*39*). OTA and zearalenone were determined with HPLC-FD in maize using β-cyclodextrin as a mobile phase additive (*40*) and more recently in soil with the help of a C8 column for cleanup (*41*). Based on a correlation with fungal volatil metabolites, an attempt at analyzing OTA and deoxynivalenol with electronic nose was made in barley (*42*). Finally, an interesting application of the solid-phase microextraction technique for the simultaneous determination of OTA and cyclopiazonic, mycophenolic, and tenuazonic acids in cornflakes by HPLC-DAD has been recently reported (*43*).

The increasing need for multimycotoxin analysis makes necessary simultaneous determination following adequate extraction and cleanup procedures. Bee-collected pollen is a major substrate for mycotoxins growth when no prompt and adequate drying is performed by the beekeeper after collection by bees. No data for mycotoxins in pollen are found in scientific literature, with the exception of a screening ELISA test reported in Spanish bee pollen for the sum of aflatoxins (*44*). The annual bee pollen production in Spain is estimated to be over 1000 tons. Most of it is for the export market where it fetches the highest price because of its esteemed floral composition, near 80% from *Cistus ladanifer*. IAC for the simultaneous cleanup of samples for analysis of aflatoxins and OTA are now commercially available, so that we have applied one of them to Spanish bee pollen samples for the analysis of aflatoxins B_1, B_2, G_1, and G_2 and OTA. An optimization of the HPLC conditions has been made for the separation and FD quantitation of all five mycotoxins at their maximum excitation and emission values of wavelength in a single run.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. HPLC-grade solvents and pro-analysis grade chemicals were delivered from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) was supplied by Riedel-de Haën (Sigma-Aldrich laborchemikalien, Seelze, Germany). HPLC-grade water was obtained with a Waters-MilliQ system.

Individual standard stock solutions of aflatoxins B_1, B_2, G_1, and G_2 and OTA, in benzene−acetonitrile (98:2), were purchased from Supelco Co. (Bellefonte, PA). The stock solutions of aflatoxins (3 μg/mL) and OTA (50 μg/mL) were mixed and diluted in water:acetonitrile (90:10) to obtain solutions of mixed mycotoxins. These standard and working solutions were stored at −20 °C when not in use.

Samples. Twenty samples of bee-collected pollen were assayed for their mycotoxin contents. Samples were either supplied by Spanish beekeepers or bought in a herbalist's shop between 1998 and 2000 and were stored frozen until analysis. No questionnaire on the harvesting, processing, or storing conditions was elaborated. Moisture ranged from 4.1% to 8.4% (average, 5.9%). Selection was made so as to be representative of the Spanish bee pollen produced either for domestic or for export markets. The palynological composition was as follows: 72% *Cistaceae* (of which, 92.7% *Cistus ladanifer*); 4% *Boraginaceae*, *Papilonaceae*, *Asteraceae*, and *Fagaceae*, each; 1% *Rosaceae* and *Ericaceae*, each; and 10% of other botanical families. Sensory characters (color, smell, and taste) were those of the commonly known bee pollen.

Extraction Procedure. Frozen samples (100 g) were ground to obtain a homogeneous powder. An aliquot (50 g) of it was placed in a beaker, 100.0 mL of acetonitrile:water (60:40) was added, and the mixture was sonicated (Microson XL2007, Misonix Inc., NY) to assist extraction. The sample was then homogenized for 15 min with a Polytron PT 10-35 (Kinematica, Luzern, Switzerland) and centrifuged for 10 min at 4000 rpm, and the supernatant was collected and filtered through filter paper. A 10.0 mL portion of the filtrate was transferred into a 50.0 mL volumetric flask and diluted to volume with water.

Immunoaffinity Column Cleanup. An aliquot of 10.0 mL of the aqueous extract (equivalent to 1.0 g of pollen) was filtered through a 13 mm fiber filter from Pall Corp. (Ann Arbor, MI) and was transferred to an AflaOchra cartridge column, from Vicam (Watertown, MA), at a flow rate of 2 drops/s. The cartridge was rinsed with 10 mL of water. Mycotoxins were then eluted with 2 mL of methanol, at a flow rate of 1 drop/s. The yellow drops that eluted early were discarded, and the colorless methanolic extract was concentrated to dryness with a gentle stream of nitrogen.

Precolumn Derivatization. The residue was redissolved with 50 μL of TFA and mixed for 30 s. Next, 200 μL of water:acetonitrile (90:10) was added, mixed for a further 30 s, and, finally, transferred to an autosampler vial.

LC Analysis. Mycotoxins were quantitated with a Varian chromatographic system (Walnut Creek, CA) which consisted of 9012Q pump, 9100 autoinjector, and 9075 fluorescence detector. Separation of aflatoxins and OTA was performed in a Waters Nova-Pack (Milford, MA) reverse phase C18 column, 4 μm particle size, 150 × 3.9 mm i.d. A specific Nova-Pack guard column was placed between the autoinjector and column. All of the chromatographic information was reprocessed in a Star Workstation (ver. 4.5) supplied by Varian.

Chromatographic conditions were as follows: mobile phase flow rate 1 mL/min between time 0−12.3 min, 0.7 mL/min for 5 min, and then 1 mL/min until stop time; injection volume of 50 μL; and solvents, A, water:acetonitrile:methanol (83:8.5:8.5), and B, water:acetonitrile:acetic acid (49.5:49.5:1). The elution program was 100% A isocratically for 9 min, 100% of B in A for 3.30 min, and 100% B isocratically for 9 min. Detection was carried out using a wavelength program with, respectively, excitation and emission wavelengths of 360 and 440 nm until time 18, for aflatoxins, and then of 390 and 477 nm, respectively, for OTA.

The chromatographic system was calibrated for quantitation of mycotoxins with standard working solutions previously passed through an immunoaffinity cartridge, evaporated to dryness, TFA added, and redissolved in water:acetonitrile, as indicated above for the samples.

RESULTS AND DISCUSSION

Extraction and Cleanup. The extraction procedure was optimized to facilitate the complete removal of the analytes. The addition of extractants to the ground sample of pollen followed by vigorous stirring gave rather poor extraction yields. A sonication and subsequent homogenization stage resulted in a better performance when the time for each step was appropriate.

Table 1. Validation of Method

	mycotoxin				
	B_1	B_2	G_1	G_2	OTA
limit of detection (μg/kg pollen)	0.20	0.13	0.10	0.13	0.49
repeatability RSDr (%)					
0.625 μg/kg pollen	7.64	8.45	7.97	6.23	9.85
2.5 μg/kg pollen	3.42	5.69	2.35	2.23	7.11
% recoveries in spiked pollen					
0.625 μg/kg pollen	81	84	79	80	73
2.5 μg/kg pollen	93	91	90	92	81

Concerning the extractant, different protocols recommended by Vicam were tested. Acetonitrile:water (60:40) showed better extraction yields than the combination of methanol:water (80:20) and NaCl. The extract was eluted through the IAC and rinsed with water to rid it of possible interferents at the subsequent chromatographic analysis. The volume of water was adjusted to reach a clear eluate. All aflatoxins B_1, B_2, G_1, and G_2 and OTA were subsequently removed from the IAC by passing methanol with a volume adjusted to reach the highest yield. The early drops from this methanolic eluate showed a yellow color, most probably because of carotenoid species. This fraction was assayed for a potential content of the analytes searched for in a spiked pollen sample. A flat chromatogram was obtained, indicating that the analytes had not eluted yet. Consequently, it was decided to discard the early yellow fraction with an aim to protect the chromatographic circuit from unwanted material, yet perhaps in an elevated concentration.

LC Analysis. After nitrogen stream drying, TFA was added to enhance the fluorescence emission of aflatoxins B_1 and G_1. An acid catalytic addition of water across the double bond of the furan ring occurred, yielding derivatives B_{2a} and G_{2a}. Calibration graphs were obtained for the four aflatoxins (0.1, 0.5, 1.0, 5.0, 10.0, and 20.0 μg/L each) and for OTA (0.5, 1.0, 5.0, 10.0, and 20.0 μg/L). Regression coefficients were all above 0.995. The limit of detection was calculated according to Glaser et al. (*45*), so that seven samples of ground bee pollen were added to a mycotoxins multicomponent solution with concentrations from 2- to 5-fold the estimated detection limit. Precision was tested, at two levels, with powdered samples ($n = 7$) spiked with multicomponent standards of mycotoxins submitted to the same cleanup and derivatization process (**Table 1**).

A comparison of the method performance can be made with a recent and authoritative collaborative study (*22*) for aflatoxins performed also on various dry matrixes: pistachio and fig pastes and paprika powder. The authors also employed IAC cleanup, but a different extractant and a postcolumn bromination for derivatization. Recoveries of 71−92% were reported for the four aflatoxins spiked at levels of 2.4−9.6 μg/kg, quite in line with our results (**Table 1**) for the higher spiked level (2.5 μg/kg) and even for the lower one (0.625 μg/kg). For aflatoxin B_1, they reported 82−102% at spiked levels of 1.0−4.0 μg/kg, also in line with our results. Concerning the repeatability (%RSDr), they reported values ranging from 4.6% to 23.3% for the four aflatoxins and from 3.1% to 20.0% for aflatoxin B_1.

Regarding the determination of OTA, the IAC method of Solfrizzo (*27*), also employed on dry matrixes such as wheat, oats, rye, and trout feed, reports quite comparable results. Another dry matrix, barley, has been used for a collaborative study (*29*). The authors also employed acetronitrile:water as extractant and report a wide range for the figures of recovery, at a spiked level of 4 μg/kg, as well as for the repeatability.

Consequently, the method described herein does not vary significantly from other existing IAC methods concerning the figures for validation of the method. Its main feature lies in its ability for the simultaneous analysis of the five mycotoxins most searched for and, particularly, with a European regulatory limit for an increasing number of foodstuffs.

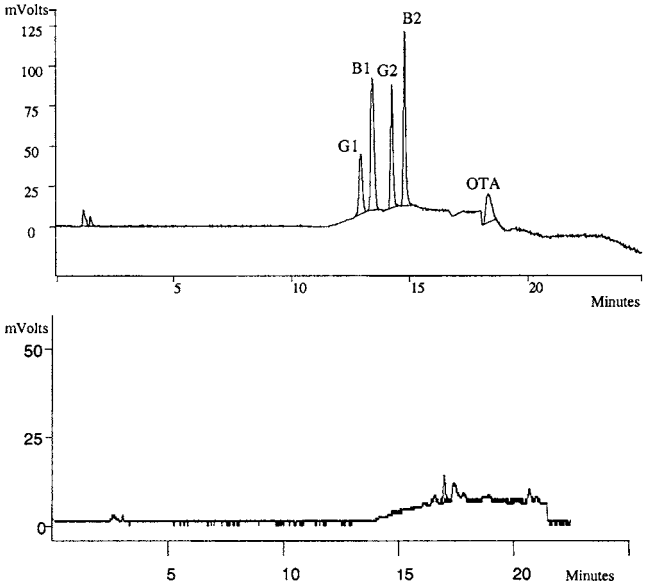

Figure 1. HPLC chromatograms corresponding to a fortified bee pollen at 2.5 μg/kg (top) and to a real sample (bottom).

Analysis of Samples. None of the 20 samples showed quantifiable values for the five mycotoxins assayed (**Figure 1**). The levels, if present, should be very far from those currently in force for other regulated (*12*, *13*) foodstuffs, ranging from 2 to 8 μg/kg for B_1 and from 4 to 15 μg/kg for the sum of all four aflatoxins, and from 3 to 10 μg/kg for OTA. The occurrence of false positives has been reported for OTA in certain cases not yet determined (*10*, *26*), which makes advisable a confirmation either with a derivatization to the OTA methyl ester or with mass spectrometry. In our case, there was no need for this because no positive value was obtained in the samples analyzed.

The only previous reference for Spanish bee pollen is that of Serra and Escolà (*44*), which employed a screening ELISA test for the sum of four aflatoxins; a high variation in replicates has recently been reported for analysis in tahini (*20*). They found values below 5 μg/kg (the limit of detection) in all 20 samples assayed. To avoid biotic hazards (i.e., contamination with pathogenic moulds and bacteria, either from the natural habitat or from human handling), they recommended two practices: first, to collect the pollen no later than 48 h after the pollen trap was installed and, second, to reduce its water content to a water activity value (a_w) under 0.60 no later than 24 h after collection from the trap. The results from our study and a survey on the beekeeper's sector reveal that these practices have been generalized during the past decade, so that a drying process is usually applied for 2 h, in a 1−2 cm bed, in a 40 °C air stream. Of the 20 samples, only one surpassed 7% of humidity, which is the recommended limit.

Although no regulation is currently in force for mycotoxins in bee pollen in the E.U., commercial transactions usually include an analysis of aflatoxins. Official control of both types of mycotoxins herein studied should be considered for a further Commission Regulation concerning Quality Standards of bee pollen.

Regarding the analytical method itself, performance is adequate from a regulatory point of view. During the last 5 years, more and more foodstuffs have been added to a list for control of aflatoxins and (quite recently) OTA. The burden of

regulatory limits makes it necessary to count on ease, performance, and, even more, simultaneous analytical methods, which in our case has been accomplished.

ACKNOWLEDGMENT

We thank Dr. J. Sánchez (Departamento de Botánica) for his assistance in the pollen sample selection and for general recommendations, and G. H. Jenkins for his revision of the English version of the manuscript.

LITERATURE CITED

(1) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene*; International Agency for Research on Cancer: Geneva, 2002; Vol. 82, pp 171−174.

(2) Report of the Third Joint FAO/WHO/UNEP International Conference on Mycotoxins, Tunis (Tunisia), March 3−6, 1999, pp 1−14.

(3) *IARC Monographs 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 489−490.

(4) Lopez de Ceraín, A.; González-Peñas, E.; Jiménez, A. M.; Bello, J. Contribution to the study of ochratoxin A in Spanish wines. *Food Addit. Contam.* **2002**, *19*, 1058−1064.

(5) Shephard, G. S.; Fabiani, A.; Stockenström, S.; Mshicileli, N.; Sewram, V. Quantitation of ochratoxin A in South African wines. *J. Agric. Food Chem.* **2003**, *51*, 1102−1106.

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

(7) Sharman, M.; Norwich, U. K.; MacDonald, S.; Gilbert, J. Automated liquid chromatographic determination of ochratoxin A in corn, barley and kidney: collaborative study. *J. Chromatogr., A* **1992**, *603*, 285−289.

(8) Clarke, J. R.; Marquardt, R. R.; Frohlich, A. A.; Pitura, R. J. Quantification of ochratoxin A in swine kidney by enzyme-linked immunosorbent assay using a simplified sample preparation procedure. *J. Food Prot.* **1994**, *57*, 991−995.

(9) Paulino de Moraes, M. H.; Luchese, R. H. Ochratoxin A on green coffee: Influence of harvest and drying processing procedures. *J. Agric. Food Chem.* **2003**, *51*, 5824−5828.

(10) Sibanda, L.; Saeger, S. D.; Van Peteghem, C. Optimization of solid-phase cleanup prior to liquid chromatographic analysis of ochratoxin A in roasted coffee. *J. Chromatogr., A* **2002**, *959*, 327−330.

(11) Commission Regulation (EC) No. 466/2001, Official Journal of the European Communities, L77, 16/3/2001, 1.

(12) Commission Regulation (EC) No. 472/2002, Official Journal of the European Communities, L75, 16/3/2002, 18.

(13) Commission Regulation (EC) No. 683/2004, Official Journal of the European Communities, L106, 15/4/2004, 3.

(14) Lin, L.; Zhang, J.; Wang, P.; Wang, Y.; Chen, J. Thin-layer chromatography of mycotoxins and comparison with other chromatographic methods. *J. Chromatogr., A* **1998**, *815*, 3−20.

(15) Holcomb, M.; Wilson, D. M.; Truckess, M. W.; Thompson, H. C. Determination of aflatoxins in food products by chromatography. *J. Chromatogr., A* **1992**, *624*, 341−352.

(16) Valenta, H. Chromatographic methods for the determination of ochratoxin A in animal and human tissues and fluids. *J. Chromatogr., A* **1998**, *815*, 75−92.

(17) Urano, T.; Trucksess, M. W.; Page, S. W. Automated affinity liquid chromatography system for on-line isolation, separation and quantitation of aflatoxins in methanol−water extracts of corn or peanuts. *J. Agric. Food Chem.* **1993**, *41*, 1982−1985.

(18) Patey, A. L.; Sharman, M.; Gilbert, J. Determination of aflatoxin B_1 levels in peanut butter using an immunoaffinity column cleanup procedure: interlaboratory study. *Food Addit. Contam.* **1990**, *7*, 515−520.

(19) Nilufer, D.; Boyacioglu, D. Comparative study of three different methods for the determination of aflatoxins in tahini. *J. Agric. Food Chem.* **2002**, *50*, 3375−3379.

(20) Barmark, A. L.; Larsson, K. Immunoaffinity column cleanup/liquid chromatographic determination of aflatoxins: an interlaboratory study. *J. AOAC Int.* **1994**, *77*, 46−53.

(21) Stroka, J. S.; Anklan, E.; Jörissen, U.; Gilbert, J. Immunoaffinity column cleanup with liquid chromatography using postcolumn bromination for determination of aflatoxins in peanut butter, pistachio paste, fig paste and paprika powder: collaborative study. *J. AOAC Int.* **2000**, *83*, 320−340.

(22) Garner, R. C.; Whattam, M. W.; Taylor, P. J. L.; Stow, M. W. Analysis of United Kingdom purchased spices for aflatoxins using an immunoaffinity column cleanup procedure followed by high-performance liquid chromatography analysis and postcolumn derivatisation with pyridinium bromide perbromide. *J. Chromatogr., A* **1993**, *648*, 485−490.

(23) Reif, K.; Metzger, W. Determination of aflatoxins in medicinal herbs and plant extracts. *J. Chromatogr., A* **1995**, *692*, 131−136.

(24) Stroka, J. S.; Anklan, E.; Jörissen, U.; Gilbert, J. Determination of aflatoxin B_1 in baby food (infant formula) by immunoaffinity column cleanup liquid chromatography with postcolumn bromination: collaborative study. *J. AOAC Int.* **2001**, *84*, 1116−1123.

(25) Holcomb, M.; Thompson, H. C. Analysis of aflatoxins (B_1, B_2, G_1 and G_2) in rodent feed by HPLC using postcolumn derivatization and fluorescence detection. *J. Agric. Food Chem.* **1991**, *39*, 137−140.

(26) Solfrizzo, M.; Avantaggiato, G.; Visconti, A. Use of various cleanup procedures for the analysis of ochratoxin A in cereals. *J. Chromatogr., A* **1998**, *815*, 67−73.

(27) Scudamore, K. A.; MacDonald, S. J. A collaborative study of an HPLC method for the determination of ochratoxin A in wheat using immunoaffinity column cleanup. *Food Addit. Contam.* **1998**, *15*, 401−410.

(28) Entwisle, A. C.; Williams, A. C.; Mann, P. J.; Slack, P. T.; Gilbert, J. Liquid chromatographic method with immunoaffinity column cleanup for determination of ochratoxin A in barley: collaborative study. *J. AOAC Int.* **2000**, *83*, 1377−1383.

(29) Burdaspal, P.; Legarda, T. M.; Gilbert, J. Determination of ochratoxin A in baby food by immunoaffinity column cleanup with liquid chromatography: interlaboratory study. *J. AOAC Int.* **2001**, *84*, 1445−1452.

(30) MacDonald, S.; Wilson, P.; Barnes, K.; Damant, A.; Masse, R.; Mortby, E.; Shepherd, M. J. Ochratoxin A in dried vine ruit: method development and survey. *Food Addit. Contam.* **1999**, *16*, 253−260.

(31) Visconti, A.; Pascale, M.; Centonze, G. Determination of ochratoxin A in wine and beer by immunoaffinity column cleanup and liquid chromatographic analysis with fluorometric detection: collaborative study. *J. AOAC Int.* **2001**, *84*, 1818−1827.

(32) Pittet, A.; Tornare, D.; Huggett, A.; Viani, R. Liquid chromatographic determination of ochratoxin A in pure and adulterated soluble coffee using an immunoaffinity column cleanup procedure. *J. Agric. Food Chem.* **1996**, *44*, 3564−3569.

(33) Entwisle, A. C.; Williams, A. C.; Mann, P. J.; Russell, J.; Slack, P. T.; Gilbert, J. Combined phenyl silane and immunoaffinity column cleanup with liquid chromatography for determination of ochratoxin A in roasted coffee: collaborative study. *J. AOAC Int.* **2001**, *84*, 444−450.

(34) Santos, E. A.; Vargas, E. A. Immunoaffinity column cleanup and thin-layer chromatography for determination of ochratoxin A in green coffee. *Food Addit. Contam.* **2002**, *19*, 447−458.

(35) Le Tutour, B.; Tantaoui-Elaraki, A.; Aboussalim, A. Simultaneous thin layer chromatographic determination of aflatoxin B_1 and ochratoxin a in black olives. *J.-Assoc. Off. Anal. Chem.* **1984**, *67*, 611−612.

(36) Lepom, P. Simultaneous determination of mycotoxins citrinin and ochratoxin A in wheat and barley by high-performance liquid chromatography. *J. Chromatogr., A* **1986**, *355*, 335−339.

(37) Vazquez, B. I.; Fente, C.; Franco, C.; Cepeda, A.; Prognon, P.; Mahuzier, G. Simultaneous high-performance liquid determination of ochratoxin and citrinin in cheese by time-resolved luminiscence using terbium. *J. Chromatogr., A* **1996**, *727*, 185−193.

(38) Chamkasem, N.; Cobb, W. Y.; Latimer, G. W.; Salinas, C.; Clement, B. A. Liquid chromatographic determination of aflatoxins, ochratoxin A and zearalenone in grains, oilseeds an danimal feeds by postcolumn derivatization and on-line sample cleanup. *J.-Assoc. Off. Anal. Chem.* **1989**, *72*, 336−341.

(39) 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.

(40) Seidel, V.; Poglits, E.; Schiller, K.; Lindner, W. Simultaneous determination of zearalenone in maize by reversed-phase high-performance liquid chromatography with fluorescence detection and β-cyclodextrin as mobile phase additive. *J. Chromatogr., A* **1993**, *635*, 227−235.

(41) Mortensen, G. K.; Strobel, B. W.; Hansen, H. C. Determination of zearalenone and ochratoxin A in soil. *Anal. Bioanal. Chem.* **2003**, *376*, 98−101.

(42) Olsson, J.; Borjesson, T.; Lundstedt, T.; Shnurer, J. Setection and quantification of ochratoxin A and deoxynivalenol in barley grains by GC-MS and electronic nose. *J. Chromatogr., A* **2002**, *72*, 203−214.

(43) Aresta, A.; Cioffi, N.; Palmisano, F.; Zambonin, C. G. Simultaneous determination of ochratoxin A, cyclopiazonic, mycophenolic and tenuazonic acids in cornflakes by solid-phase microextraction coupled to high-performance liquid chromatography. *J. Agric. Food Chem.* **2003**, *51*, 5232−5237.

(44) Serra Bonvehí, J.; Escolà Jordà, R. Nutrient composition and microbiological quality of honeybee-collected pollen in Spain. *J. Agric. Food Chem.* **1997**, *45*, 725−732.

(45) Glaser, J. A.; Foerst, D. L.; Mckee, G. D.; Quave, S. A.; Budde, W. L. Trace analysis for wastewater. *Environ. Sci. Technol.* **1981**, *15*, 1426−1430.

Received for review July 7, 2004. Revised manuscript received September 13, 2004. Accepted September 14, 2004. This work has been made within the framework of the "Programa Nacional de Recursos y Tecnologías Agroalimentarias" of the INIA and financed by the Spanish Ministerio de Ciencia y Tecnología.

JF048882Z