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Aflatoxins and Ochratoxin A in Red Paprika for Retail Sale in Spain: Occurrence and Evaluation of a Simultaneous Analytical Method

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Aflatoxins are the only mycotoxins with legal limits for spices in the European Union. A further limit for ochratoxin A is expected to be adopted soon. Thus, rapid simultaneous methods for quantifying the five mycotoxins are sought. Liquid extraction, immunoaffinity column cleanup, and HPLC-FD with a wavelength program were optimized for the analysis of the five mycotoxins in paprika, a complex fatty matrix. The limits of detection ranged from 0.3 to 0.6 μg/kg. Repeatability (RSDr) ranged from 7.9 to 13.4%, and recoveries were between 61.4 and 77.8%, in both cases at the lower spike level. Aflatoxins, when found, were far below the two legal limits of 5 μg/kg for aflatoxin B_1 and 10 μg/kg for total aflatoxins. Ochratoxin A was more frequently found, with a mean of 11.8 μg/kg, and in a more varied range (SD = 18.9 μg/kg). When an automation of the precolumn derivatization step was attempted, the procedure proved to be unfeasible, but experience derived from this trial and from the general employment of this reaction enables some comments on the possibilities and limitations of this procedure and on research for an alternative one to be made.

KEYWORDS: Mycotoxins; aflatoxins; ochratoxin A; Spanish paprika; immunoaffinity column; simultaneous food analysis

INTRODUCTION

A few varieties of pepper fruit (*Capsicum annuum*) are extensively employed as spices after drying and grinding. In most Spanish-speaking countries this is called "pimentón" and normally refers to the red varieties, although the name commonly used worldwide is paprika, which includes even yellow color varieties. Traditionally, the most important producing countries in the world have been China, Mexico, and Turkey (*1*) and Spain and Hungary within the European Union.

Contamination of red pepper spice by aflatoxins and ochratoxin A may occur at any stage of production, from preharvest to drying and storage. The European Commission (EC) set a legal limit for the first time in 2002, although only for aflatoxins. Several attempts to establish a maximum level for OTA have failed so that the last Commission Regulation (*2*), despite setting limits for many other foodstuffs, postponed a figure for spices. The lack of enough information regarding its usual content in spices, and particularly in paprika (*3*), together with incomplete data of dietary exposure for infants and children (*4*), might be the reasons for this delay. However, maximum levels of 10–20 μg/kg are agreed for the commercial transactions within the international spice trade (*3*). A legal limit of 10 μg/kg is currently being considered for approval by the EC (*5*).

In a monitoring of spices marketed in Portugal, aflatoxins were found in 8 of 12 samples of paprika, with aflatoxin B_1 ranging from 1 to 20 μg/kg (*6*). In Turkey, in a study of 90 samples of three different types of red pepper, aflatoxins were found in 12, with amounts ranging from 1.1 to 97.5 μg/kg (*7*). In October 2004 the discovery of levels above the EC limits in Hungarian paprika led to its withdrawal from the market. Subsequently, 70 ground red pepper samples, most of them from commercial outlets of Hungary, were analyzed for both aflatoxins and ochratoxin A, with 17 samples containing aflatoxin B_1, 7 of which ranged from 6.1 to 15.7 μg/kg, and 32 contained ochratoxin A, 8 of them with amounts from 10.6 to 66.2 μg/kg (*8*). Both authors and health authorities attributed this to the mixing of domestic batches with imports from tropical countries. Of 55 samples of different spices purchased in popular markets of Morocco, all 14 samples of red paprika were contaminated (average = 2.88 μg/kg aflatoxin B_1), these spices being those found with the highest level (*9*). With regard to contents of ochratoxin A in paprika, scientific references are limited. A recent review on foodstuffs (*10*) states that paprika was found among the most contaminated products, together with nutmeg, chilli, coriander and pepper. An interesting and comprehensive

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study of its occurrence in red paprika commercialized in Murcia (Spain) but grown and processed in four different places (Murcia itself, Zimbabwe, Brazil, and Peru) has been published recently (*3*). The product from Peru was reported as the most contaminated, but great differences were noticeable between the four regions of origin within this country with average levels ranging from 7.4 to 101.5 μg/kg. Samples from Zimbabwe and Brazil had very low and similar average levels (3–4 μg/kg), whereas the local production showed levels of <1 μg/kg.

As a result of the current high costs of manufacturing paprika, an increasing import market is developing from overseas countries, namely Peru, Brazil, Morocco, South Africa, Nigeria, Zimbabwe, India and Pakistan (*1, 3*). Developing countries often have climatic conditions that enable fungal proliferation and, furthermore, production of mycotoxins. In addition, they often lack the technology and infrastructure necessary for adequate manufacturing practices and routine food monitoring. The increasing need for multimycotoxin analysis has promoted the research on simultaneous methods with the employment of adequate extraction and cleanup procedures. With this objective an important role is being played by the immunoaffinity columns (IAC). Three years ago, an original simultaneous method was published for aflatoxins and ochratoxin A in bee pollen (*11*). On this basis, a modification has been carried out for red paprika and a subsequent application to samples obtained from local retail outlets and distributors.

MATERIAL AND METHODS

Chemicals and Reagents. HPLC-grade solvents and pro-analysis grade chemicals were from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) was supplied by Riedel-de Haën (Sigma-Aldrich laborchemikalien, Seelze, Germany). Phosphate-buffered saline (PBS) (pH 7.4, 100 mM sodium phosphate, 9% NaCl) was from Fluka (Madrid, Spain). HPLC-grade water was obtained with a Waters Milli-Q system.

Individual standard stock solutions of aflatoxins B_1, B_2, G_1, and G_2 in benzene/acetonitrile (98:2) and ochratoxin A in benzene/acetic acid (99:1) were purchased from Supelco Co. (Bellefonte, PA). Commercial solutions of aflatoxins (3 μg/mL) and ochratoxin A (50 μg/mL) were diluted in acetonitrile to obtain individual stock solutions (1000 μg/L) of each mycotoxin. A multicomponent solution of 100 μg/L was prepared by dilution in PBS, the benzene layer being evaporated with the help of a nitrogen stream, and eventually made up to volume with PBS (pH 7.4, 100 mM sodium phosphate, 9% NaCl). Appropriate dilutions of this were made with PBS to obtain volumes of 20 mL, which are the volumes of both standards and extracted/enriched samples to be passed trough the IAC. These standards and working solutions were stored at −20 °C when not in use.

Extraction Procedure. Amounts of 4 g of paprika powder were placed in a centrifuge tube, 20.0 mL of methanol/water (80:20) were added, and the mixtures were sonicated with a Microson XL 2007 (Misonix Inc., New York) for 15 s to assist extraction. All were then centrifuged for 15 min (temperature range = 0–20 °C) at 10000 rpm (11190*g*), and 10.0 mL of the supernatants was collected and transferred into 50 mL volumetric flasks and diluted to volume with PBS.

Immunoaffiniy Column Cleanup. An aliquot of 20 mL of each aqueous extract (16% methanol), equivalent to 0.8 g of paprika powder, was filtered through a 0.45 μm Millex-HV PVDF filter of 25 mm i.d. (Millipore, Billerica, MA) onto an AflaOchra cartridge from Vicam (Watertown, MA), at a flow rate of 2 drops/s. The cartridge was rinsed with 15 mL of reagent water and dried by passing air through it for 2−3 s with a syringe. Mycotoxins were then eluted with 2 mL of methanol into a conical bottom flask (Supelco, Madrid, Spain), at a flow rate of 1 drop/s. The colorless methanolic extract was concentrated to dryness in a heater block at 45 °C, assisted with a gentle stream of nitrogen.

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

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

Chromatographic conditions were as follows: mobile phase flow rate, 1 mL/min, from 0 to 12.3 min, 0.7 mL/min for 5 min, and then 1 mL/min until stop time; injection volume, 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 as follows: 100% A isocratically for 12 min, 100% of B in A for 0.3 min, and 100% B isocratically for 8.7 min. Detection was carried out using a wavelength program with, respectively, excitation and emission wavelengths of 360 and 440 nm until time 17 min, for aflatoxins, and then of 332 and 478 nm, respectively, for ochratoxin A.

The chromatographic system was calibrated for quantitation of mycotoxins with standard working solutions previously passed through an IAC, evaporated to dryness, addition of TFA and redissolved in water:acetonitrile, as indicated above for the samples. The quantitative analysis was carried out by peak area measurement using the external standard method. The calibration curves and linear regression equations were obtained for the four aflatoxins and ochratoxin A (5.0, 10.0, 20.0, 30.0, 40.0, and 50.0 μg/L). The limit of detection (LOD) was calculated in accordance with the method of Glaser et al. (*12*), so that seven samples of paprika powder were added of a mycotoxins multicomponent solution with concentrations from 2- to 5-fold the estimated LOD. Precision was tested, at two levels (1 and 5 μg/kg), with three samples spiked with multicomponent standards of mycotoxins submitted to the same cleanup and derivatization process.

LC-MS Analysis. An HPLC-MS system, from Waters Alliance 2795, coupled to a Waters ZQ4000 quadrupole mass spectrometer (Waters, Milford, MA), was used. Chromatographic conditions were the same as above and mass spectrometer working conditions were optimized with a standard of ochratoxin A. The mass spectrometer was operated in the negative electrospray ionization (ESI−) mode. Capillary voltage was 3.0 kV, and nitrogen was used as spray gas. Source and desolvation temperatures were set at 80 and 250 °C, respectively, with a flow of 411 L/h for the latter. The target ions were *m*/*z* 404.1 and 402.1 in SIR mode. The cone voltage was 40 V, cone flow was 60 L/h, and cone temperature was 20 °C.

Samples. To test for robustness of the method and occurrence of both groups of mycotoxins, 21 samples of red paprika powder were purchased from the local retail outlets. Selection was made so as to be representative of the different qualities and brands on sale in Spain. This included selection of both sweet and hot varieties. Apart from two Protected Designation of Origin for Pimentón, most of this commodity is commercialized in Spain by a few big packagers, but with various trademarks. Consequently, the samples were taken over a somewhat extended period (years 2004 through 2007) so as to get the most representative results. Packs were from 500 g to the more frequent 75 g, but some of the samples were in bulk. In all cases they were labeled as being of domestic origin.

RESULTS AND DISCUSSION

Extraction and Cleanup. In a formerly developed method for the same analytes in bee-pollen (*11*), the extractant mixture was acetonitrile/water (60:40). A study from Stroka et al. (*13*) on extractants applied to dry matrices, such as paprika, animal feeds and infant formula, concluded that acetonitrile based extractants could give rise to absorption of unpredictable water amounts by the sample, thus yielding erroneously high results. They concluded that MeOH/water mixtures prevented this. In fact, in a subsequent collaborative study they employed this

Table 1. pH Values of the Supernatant MeOH/Water Extract and of the Water- or PBS-Diluted Extracts

	pH		
sample	extract	H_2O diluted	PBS diluted
1	5.76	4.88	6.85
2	5.68	4.83	6.82
3	5.83	5.02	6.87
4	5.87	5.07	6.87
mean	5.79	4.95	6.85

mixture (*14*). Thus, a shift to MeOH/water (80:20) was decided on for our method. Sonication also revealed itself as an excellent tool for shaking, and 15 s were seen to be enough for completion of extraction. As recommended by Vicam, for other matrices, and followed by other authors, for bee-pollen (*11*) and for chilli powder (*15*), dilution of the supernatant extract was made with water, but very low and unpredictable recoveries were observed for ochratoxin A, particularly for the higher spike levels. Some authors state that pH value is crucial at this point. Recoveries of aflatoxins, especially aflatoxin G_1, decrease when the pH of the solution to be passed for cleanup is below pH 4 or above pH 8 (*16*). Extraction of ochratoxin A is lower for neutral than for acidic media, the optimum reported being pH 1.5 (*17*), but the optimum pH for cleanup is near neutral. Paprika is quite an acidic matrix. Measurements of pH were made for the supernatant extracts and for the water and PBS diluted solutions of four different samples (**Table 1**). In order to find out if differences in pH values were significant the Student's *t* test for paired values was applied, the level of significance being $p = 6.32 \times 10^{-6}$, thus meaning that the solvents H_2O and PBS provide significantly different pH values. As shown in **Table 1**, the extraction was being performed at a correct pH for both kinds of mycotoxins, but not the cleanup after dilution with water. Probably, a weakened binding affinity of the ochratoxin A antibodies was produced at such an acidic pH. Thus, dilution before IAC passage was subsequently made with PBS.

The red and clear supernatant extract became turbid when dilution was made either with water or PBS. This colloidal-like solution remained stable and passage through the IAC was thought to involve the risk of physical obstruction of the binding sites. Furthermore, colloid-like particles remaining in the IAC eluate might interfere with the subsequent TFA derivatization, a critical reaction as will be discussed next. Filtration through 0.45 μm pore filters again gave a clear red-orange solution, and no fouling occurred when filters of 25 mm i.d. were employed. No further filtration was needed and no guard column was used before LC separation.

Derivatization with TFA. Precolumn derivatization with TFA to enhance fluorescence emission of aflatoxins B_1 and G_1 is an option when no postcolumn device is available. As is well-known, an acid catalytic addition of water across the double bond of the furan ring occurs, yielding derivatives B_{2a} and G_{2a} The reaction is delicate and can vary greatly with external conditions and operators. To reach an acceptable reproducibility, it needs the precise addition of very small volumes of reagents and a very precise reaction time after addition of TFA to the dried residue (30 s) and after addition of the acetonitrile:water solvent (another 30 s). Furthermore, TFA is a fuming reagent, harmful if inhaled and corrosive to skin. An automatic derivatization should overcome all these drawbacks and this was attempted by an appropriate programming of the automatic injector. Eluated analytes from the IAC were recovered in the autosampler vials, evaporated to dryness and put in the carousel

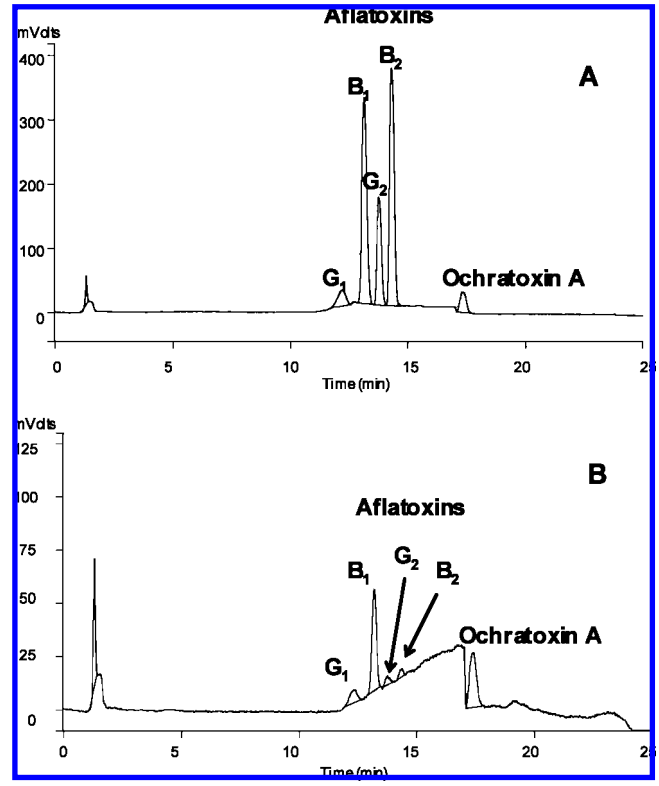

Figure 1. HPLC chromatograms corresponding to a fortified paprika powder at 5 μg/kg of aflatoxins and ochratoxin A (**A**) and to a real sample (**B**).

of the LC autoinjector device. Programming of the autoinjector arm was made so as to reproduce exact reaction conditions. Nevertheless, the procedure proved unviable. From our experience, a vigorous shaking (vortexing) of reagents with the dried residue is critical, as well as the use of pure and recently opened (anhydrous) TFA. This may be common knowledge for many researchers or analysts, but is rarely stated and, thus, is often a source of mistakes for unskilled analysts. In addition, no septum from manufacturers was found to be resistant enough to the TFA attack when passing the needle of the autoinjector, which resulted in a contamination of the samples owing to a massive bleeding from the septa.

LC Analysis and Validation of Method. Figure 1 shows chromatograms of a real and a fortified sample. Separation of aflatoxins and ochratoxin A is readily accomplished. Occurrence of false positives has been reported for ochratoxin A in certain cases whose cause not yet been determined (*18, 19*), which makes a confirmation advisable, either with a derivatization to the ochratoxin A methyl ester or with a mass spectrometer. Despite the more frequently reported positive mode, a better signal was provided by our mass spectrometer when it was operated in the ESI− mode (**Figure 2**).

Recoveries at the upper spike level are very high for aflatoxins B, but much lower for aflatoxins G and ochratoxin A (**Table 2**). At the lower spike level recoveries are lower, especially for aflatoxin G_2 and ochratoxin A. For this range of concentrations, the Commission Regulation (EC) 401/2006 recommends values from 70 to 110%. At the upper spike level, recovery values are within this range; at the lower spike level, values are also within this range except for aflatoxin G_2 and ochratoxin A. With the formerly published method for bee pollen (*11*), recoveries were of the same order for the four aflatoxins (90 and 80% at the upper and lower spike levels, respectively). The LOD values now, as compared with the former method, are similar for

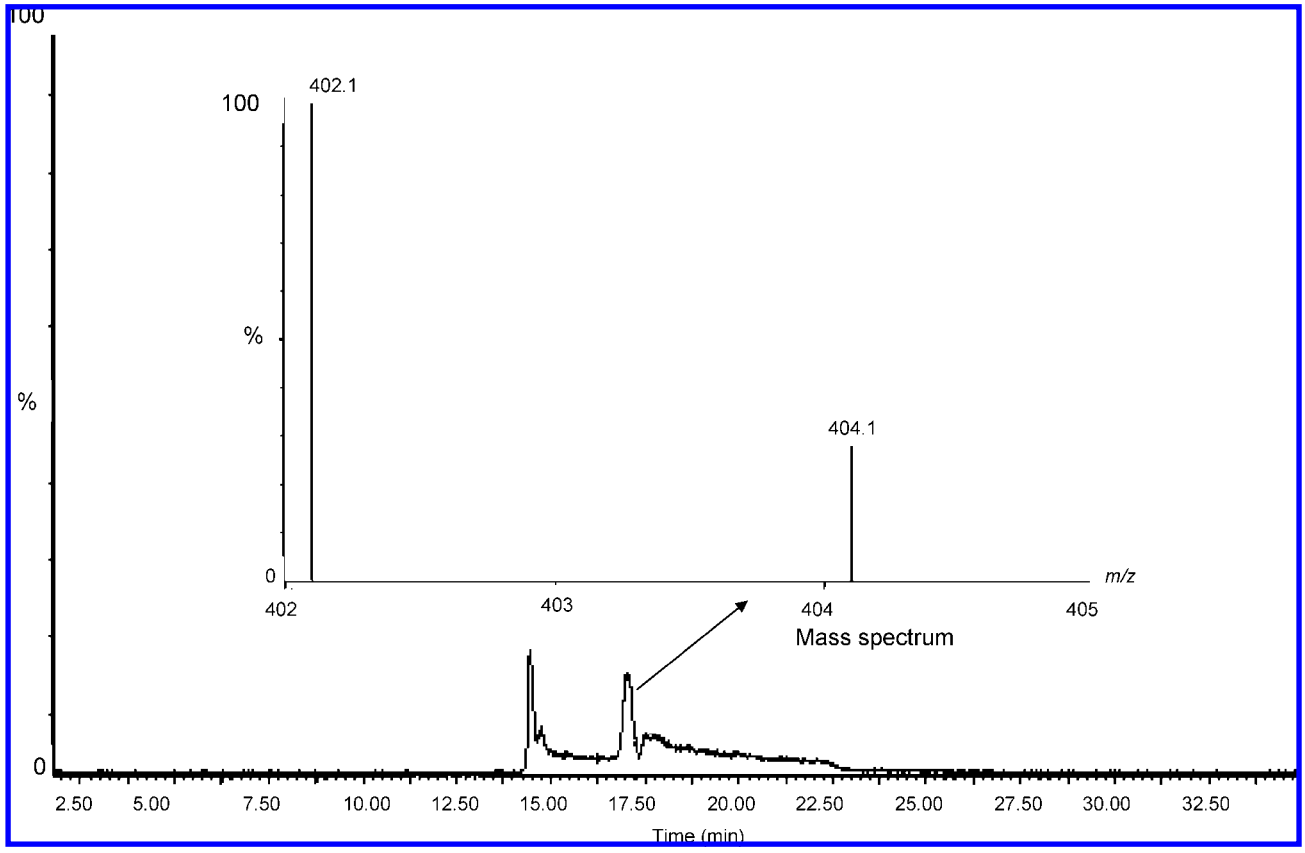

Figure 2. HPLC-MS chromatogram and mass spectrum of ochratoxin A from a real sample.

Table 2. Validation of Method for Analysis of Aflatoxins and Ochratoxin A

		aflatoxin				
	paprika spike level (μg/kg)	B_1	B_2	G_1	G_2	ochratoxin A
limit of detection (μg/kg)		0.3	0.3	0.6	0.4	0.4
repeatability,[a] RSDr (%)	1.0	8.9	7.9	13.4	10.6	8.9
	5.0	9.3	5.1	11.0	5.8	9.9
recovery[a] (%)	1.0	77.8 ± 0.1	73.6 ± 0.1	71.5 ± 0.1	61.4 ± 0.1	63.9 ± 0.1
	5.0	99.7 ± 0.5	97.8 ± 0.3	71.7 ± 0.4	70.7 ± 0.2	75.7 ± 0.4

[a] Recovery, mean ± SD; repeatability, (SD/mean) × 100.

aflatoxin B_1 and ochratoxin A, but somewhat higher for the other three. The repeatability for the new method is similar for both spike levels, but worse than for those obtained for the former of bee pollen. The EC Regulation employs the Horwitz equation, and the results for this are of 45.3 and 12.6%, respectively, for each spike level, which are far above those attained in our method (**Table 2**). Consequently, all five mycotoxins largely comply with it.

A simultaneous method for aflatoxins, ochratoxin A, and zearalenone in rye, rice, and pig feed (which employs extraction with acetonitrile/water, TFA derivatization, and AflaOchraZea IAC cleanup) yielded very dissimilar recoveries (*20*), but the lowest was also for aflatoxin G_2 (50–65%). However, lower LOD values were reported for this method, probably because of the different matrices and method of calculation.

A recent method (*21*) for botanical roots (extraction with MeOH/BS and AflaOchra cleanup) has compared the three derivatization techniques (TFA, Kobra cell/and UV). Results for aflatoxins were comparable in all three, although the RSD was slightly higher when the precolumn technique was applied. The recoveries for aflatoxins were in the range of 60–70% and those for ochratoxin A, 50–60%, depending on the particular kind of root. More recently, Sobolev (*22*), with a Florisil cleanup and a postcolumn UV cell, reported recoveries of 68–93% for aflatoxins.

As mentioned before, Stroka et al. (*14*) carried out a collaborative study for aflatoxins with HPLC-FD in dried figs and paprika, with a postcolumn Kobra cell. Recoveries at spike levels of 2.4 and 9.6 μg/kg ranged from 71 to 92%, respectively, for total aflatoxins, and at spike levels of 1.0 and 4.0 μg/kg of aflatoxin B_1 recoveries were of 82–101%. RSD values for repetitivity were 4.6–23.3% for total and 3.1–20.0% for aflatoxin B_1. Aflatoxins were determined with matrix solid-phase dispersion in chilli powder (*15*) and different spices (*23*). Recoveries of 88–95% for each of two spike levels are double than ours, RSD below 6% and LOD of 0.10–0.25 μg/kg were reported for the former, with a Kobra cell. Finally, Almela et al. (*3*) determined ochratoxin A with OchraTest IAC, HPLC-MS and reported recoveries of 73–83% at spike levels of 1–5 μg/kg, respectively.

Analysis of Samples. Positive contamination was considered for those samples with amounts above the LOD. As shown in

Table 3. Mycotoxins (Micrograms per Kilogram) in Samples of Ground Red Paprika

	aflatoxin				
sample	B_1	B_2	G_1	G_2	ochratoxin A
1	1.0	–[a]	–	0.8	2.4
2	–	–	–	0.8	1.3
3	1.4	0.6	–	0.7	–
4	3.8	0.7	–	–	73.8
5	1.5	0.6	0.9	–	0.7
6	0.8	–	–	0.6	5.1
7	0.9	–	–	–	–
8	0.7	–	–	0.5	–
9	0.9	–	–	–	–
10	–	–	–	–	–
11	1.1	0.6	–	0.6	7.0
12	0.8	–	–	–	3.0
13	1.4	0.6	1.1	0.6	6.6
14	0.8	0.6	–	0.5	2.7
15	0.7	–	–	–	–
16	0.7	–	–	–	–
17	0.7	–	–	–	11.6
18	1.8	0.6	–	–	6.4
19	0.8	–	–	–	13.1
20	0.7	–	–	–	24.6
21	0.7	–	–	–	7.6
mean	1.1	0.6	1.0	0.6	11.9
max	3.8	0.7	1.1	0.8	73.8
min	0.7	0.6	0.9	0.5	0.7
SD	0.7	0.1	0.2	0.1	18.9
90th percentile	1.6	0.7	1.1	0.8	21.2
no. positive samples	19	7	2	8	14
% of total samples	90	33	10	38	67

[a] –, not detected.

Table 3, 19 samples (90% of total) were contaminated with aflatoxin B_1, but at levels far below the legal limit of 5 μg/L. Aflatoxins B_2, G_1, and G_2 were quantitated in seven, two, and eight samples, respectively, at even much lower levels than the former aflatoxin B_1. This is in agreement with results from other authors (*5, 6, 9*) for spices: aflatoxin B_1 is always the most abundant of the four and, hence, a good indicator of aflatoxin contamination. None of the 21 samples reached the legal limit of 10 μg/L for the sum of the four aflatoxins, the maximum being one sample with 4.5 μg/L.

With regard to ochratoxin A, 15 samples were found to be contaminated by HPLC-FD, but 1 was not confirmed by HPLC-MS, which amounts to a contamination of 67% of total samples. As seen in **Table 3**, amounts of ochratoxin A were found in a most varied range (SD value = 18.9 μg/L).

The amounts for aflatoxins were within the calibration range in all the cases, whereas for ochratoxin A one of the samples had to be reinjected after dilution with water/acetonitrile (90:10).

A few years ago aflatoxin contents were regulated for spices in the European Union. At an early date a first legal limit for ochratoxin A is expected to be added to the last European Union regulation. To our knowledge, no completely simultaneous analytical method for aflatoxins and ochratoxin A has yet been reported for paprika. There is a tendency to extrapolate the use of an analytical method, officially validated for a particular matrix, to the same mycotoxins in other commodities (*16, 17, 21*). This applies especially to the IAC cleanup step for many analysts. It should be taken into account that the behavior of each matrix might be different; thus, IAC should be tested for every mycotoxin or group of mycotoxins and for every matrix.

The behavior of an IAC filled with aflatoxins and ochratoxin A antibodies has been studied for the analysis of paprika. A classical precolumn derivatization for aflatoxins has been applied, and advantages and limitations of this reaction have also been studied. The method has proved to have performance very similar to that of other currently official ones, but in addition it enables the simultaneous determination of five mycotoxins in a single run. Because automation of the derivatization step has not been feasible, a further study should aim at applying it to one of the postcolumn derivatization devices, namely, those employing the bromination or UV activation. Upon examination of its molecular structure, no a priori modification would be forecast to affect ochratoxin A derived from this treatment.

With regard to samples, aflatoxins, when found, never reached either of the two legal limits. However, ochratoxin A was more frequently found and over a more varied range.

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