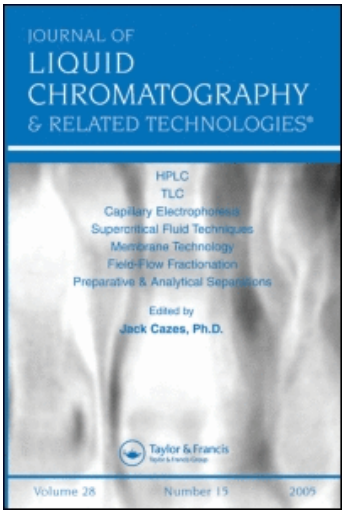

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 02 March 2003

To cite this Article Brera, Carlo , Grossi, Silvana , de Santis, Barbara and Miraglia, Marina(2003) 'High Performance Liquid Chromatographic Method for the Determination of Ochratoxin A in Cocoa Powder', Journal of Liquid Chromatography & Related Technologies, 26: 4, 585 — 598
To link to this Article: DOI: 10.1081/JLC-120017906
URL: http://dx.doi.org/10.1081/JLC-120017906

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JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 26, No. 4, pp. 585–598, 2003

High Performance Liquid Chromatographic Method for the Determination of Ochratoxin A in Cocoa Powder

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ABSTRACT

Ochratoxin A (OTA) is a potent nephrotoxin that can affect human health through their diet. Cereals and coffee represent, by far, the main contributors to OTA exposure, but recently other food commodities like wine, raisins, and cocoa products have been found to be additional sources of intake for consumers. Ochratoxin A contamination in cocoa derived products presents an emerging risk for consumer. Recently, both monitoring programs and research have been aimed at ascertaining the status of contamination worldwide, and critical control points in cocoa processing. At the moment, all studies confirmed that cocoa can result in contamination quite frequently, and at levels worthy of interest. This paper describes a study to carry out a method of analysis for the determination

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DOI: 10.1081/JLC-120017906
1082-6076 (Print); 1520-572X (Online)

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of OTA in cocoa derived products. Performance characteristics of the method, as within-day and between-day repeatability, reproducibility, and accuracy are described, showing reliable results adequately matching the criteria suggested by the European Committee for Standardization (CEN) for the analysis of mycotoxins. The advantages resulting from this method are mainly addressed to the saving of time of analysis, and the possibility to detect ng/kg levels of ochratoxin A without interfering peaks, representing, therefore, a useful tool for the control of foodstuffs, in accordance with the upcoming communitary legislation.

Key Words: Cocoa; Ochratoxin A; Mycotoxins.

INTRODUCTION

Recently, safety issues related to exposure in the diet to xenobiotics for the consumers, are receiving more and more critical attention. This is because of a higher, than in the past, awareness of consumers to the quality of his own dietary habit. So far, mycotoxins do not represent a perceivable risk by the consumer, despite their very high carcinogenic potency, and in some cases, genotoxicity. Among mycotoxins, ochratoxin A (OTA) represents one of the most widespread and hazardous substances. In fact, it is well recognised that the occurrence of OTA in food can seriously impair human health.[1] Its potential correlation with targeted pathologies, such as Balkan Endemic Nephropathy (BEN), has been strongly forwarded by researchers in some regions of Eastern Europe.[2,3] It has also often been found to be at high levels in human serum and breast milk.[4–7]

In 1998 the European Commission's Scientific Committee for Food recommended that it would be prudent to reduce exposure to OTA as much as possible, e.g. to below 5 ng/kg bw/day.[8]

Ochratoxin A can be found in many food products of plant origin, and in several animal origin food products, with the exclusion of fish.[9] Various sources of contamination are significant for OTA, with cereals acting as a major contributor, followed by wine, beer, spices, coffee, and cocoa. Ochratoxin A contamination in cocoa could result in an emerging risk for the consumer, especially in consideration of the particularly high risk groups of the population consuming cocoa derived products, such as children. Unfortunately, due to many possible sources of uncertainties or lack of information (reliability of sampling procedures and of analytical data, influence of technological procedures, consumption data), the main food sources of OTA intake for humans are still in debate. However, from a recent survey, performed within SCOOP-EU task 3.2.7, aimed at the evaluation of OTA

exposure by the EU consumer, cocoa products can contribute to the exposure in a minimal way (4% of the total contribution), even if it is at a level that is significant when related to critical groups of consumers, such as children.[10]

Cocoa can act as a substrate for mould growth in the favourable moist climatic conditions of countries where it is cultivated. Moreover, other developing sites for mould attack are fermentation and drying, or when beans get wet during transport and storage conditions.

The most relevant species producing OTA in cocoa are *Aspergillus carbonarius* and *Penicillium viridicatum*.

From the literature, there is no direct correlation between ochratoxin A and the visible mould on the beans, with no particular origin more susceptible than others. Generally, lower levels in cocoa butter, than in the non-fat fraction (powder/cake), have been found.

As far as ochratoxin A occurrence in cocoa products, different results were obtained. Contamination levels within 100–500 μg/kg were reported[11] in 18 out of 56 cocoa beans samples and in 16 out of 19 samples of roasted cocoa samples, while no OTA was detected in all 15 analysed roasted cocoa beans samples, at a limit of detection of 0.1 μg/kg.[12] More recently, 100% of positive results in 40 samples of cocoa powder and 34 samples of ready-mixed cocoa drinks, falling in the range 0.09–1.800 μg/kg and 0.005–0.054 μg/kg, respectively, and 91% of positive samples of 56 samples of chocolate drinks within the range < 0.01–0.630 μg/kg, were found.[13]

Further research is needed, but it has been estimated that it is possible that up to 20% of cocoa bean production could be excluded from consumption, depending on the permitted levels to be set by the European Commission. In this respect, the European Commission invited each member state to provide, by December 2003, as much data and information as possible, addressed to the identification of the critical control points in all food production chains potentially susceptible to OTA contamination, to the evaluation of the actual status of OTA contamination in finished products, and to the development of appropriate methodology for extraction, measurement, and determination of OTA levels in cocoa derived products.

Toward this aim, a large number of surveys are expected to be performed with the need of handling large amounts of data related to OTA analysis in cocoa samples. In addition, the involvement of the cocoa industry in handling a method to be used for the implementation of preventive activities based upon HACCP principles should be considered.

Even if some analytical methods are available,[14–16] they are very old in some cases and not in accordance with the most modern concepts of good laboratory practices and quality assurance principles, or not suitable for control activities, or not in compliance with some international new analytically trends, such as the ban of halogenated solvents.

The aim of this study has been, therefore, devoted to carry out a fast and sensitive HPLC method of analysis for OTA determination in cocoa powder samples, in order to handle a methodology to be performed in compliance with the forthcoming legal limits probably fixed at communitary levels.

EXPERIMENTAL

Reagents and Apparatus

During the analysis, unless otherwise stated, only reagents of recognised analytical grade and only distilled water or water of grade 1, according to EN ISO 3696, have been used. All solvents were HPLC grade.

Ochratoxin A Calibrant Solutions

Stock solutions: Ochratoxin A primary stock solution was purchased from Sigma at a initial concentration of 50 μg/mL in toluene : acetic acid, (98 : 2; V : V). The concentration of this stock solution has been checked using a UV spectrophotometer, and stored at $4^{\circ}C \pm 0.1$. One hundred microliter of primary stock solution were pipetted into a glass vial and diluted to 5 mL with 4900 μL of toluene/acetic acid mixture, at a concentration of 1 μg/mL. One hundred microliter of the 1 μg/mL ochratoxin A solution were pipetted into a 10 mL volumetric flask. After solvent evaporation under a stream of nitrogen, the reference standard was redissolved in 10 mL methanol : deionised water 70 : 30 to give a final concentration of 10 ng/mL.

Calibration Curve

Starting from 10 ng/mL ochratoxin A reference standard solution, appropriate aliquots were drawn to build up a five-level calibration curve, at mass concentrations of working standard solutions of 0.025, 0.1, 0.25, 0.5, and 1.0 ng/mL, respectively. Equal volumes (200 μL) of working standard solutions were injected. Ochratoxin A mass in nanograms, corresponding to the fluorescence of the samples, was calculated by the linear regression equation of the obtained calibration curve.

Phosphate Buffered Saline (PBS) (pH 7.4)

Phosphate buffered saline can be prepared from potassium chloride (0.20 g), potassium dihydrogen phosphate (0.20 g), anhydrous disodium hydro-

gen phosphate (1.16 g), and sodium chloride (8.00 g), and added to 900 mL purified water. After dissolution, pH must be adjusted to 7.4 (with 0.1 mol/L HCl or 0.1 mol/L NaOH, as appropriate) and the solution taken to 1.0 L.

HPLC Equipment

The HPLC system consisted of an isocratic Gilson 321 pump regulated at 1.0–1.2 mL/min flow rate (Gilson, Middleton, WI 53562-0027), equipped with an injection system of 200 µL injection loop (Rheodyne 7125), a fluorescence detector fitted with flow cell and set at 333 nm (excitation) and 460 nm (emission) (Jasco FP-1520, JASCO Corporation, Tokyo, Japan). Data were processed with a Unipoint–Gilson chromatographic data handling software. The analytical HPLC column used was a C18 Phenomenex Kromasil reverse phase (250 × 4.6 mm), packed with 5 µm particle size and maintained under controlled temperature with a column oven regulated at 40°C ± 1°C. A mobile phase of acetonitrile : water : acetic acid [50 : 49 : 1 (V : V : V)] flowing at 1.0–1.2 mL/min was used.

Principle of the Method

Samples have been extracted with aqueous solutions of sodium hydrogen carbonate under controlled conditions of pH, diluted with a solution of PBS, filtered, and cleaned up by immunoaffinity columns (IAC) containing antibodies specific to ochratoxin A. Ochratoxin A is eluted with methanol, separated by reversed-phase HPLC, and quantified by fluorescence.

Procedure

Extraction

Two extraction procedures can be used: (a) Weigh, to the nearest 0.1 g, a test portion of 5 g into a high speed blender. Add 100 mL of aqueous solution of sodium hydrogen carbonate (0.1%) and polyethylene glycol (0.3%). Stopper the jar and blend samples at high speed for 2 min; or (b) Weigh, to the nearest 0.1 g, a test portion of 5 g into a 300 mL Erlenmeyer flask. Add 100 mL of aqueous solution of sodium hydrogen carbonate (0.1%) and polyethylene glycol (0.3%). Stopper the flask and shake samples at medium speed for 30–45 min in wrist action shaker.

Filter the extract through folded filter paper. Draw 20 mL of filtrate and dilute with 20 mL of PBS. Mix thoroughly. Apply the diluted sample to the IAC, as described underneath. If the sample should be slightly cloudy, re-filter the extract through glass microfiber filters.

Clean-Up

Conditioning of Immunoaffinity Columns

Immunoaffinity columns (Ochraprep Rhone-Diagnostics) should be kept at room temperature prior to conditioning. For conditioning, apply 5 mL of PBS on the top of the column and let them pass at a speed of 2–3 mL/min through the column, avoiding letting the column run dry.

Connect the IAC to the vacuum manifold or equivalent, and attach the reservoir to the IAC.

Pipette 30 mL (equivalent to 0.75 g of sample) of the diluted extract into the reservoir, and pass through the IAC at a flow rate of about 1–2 drops/second by gravity. Flow rate should not exceed 5 mL/min. The IAC must not run dry. Wash the IAC with 10 mL PBS, and then with 10 mL de-ionised water at a flow rate of about 1–2 drops/second. Dry the column by either applying a light vacuum for 5–10 s or passing air through the IAC by means of a syringe for 10 s. Remove the IAC from the vacuum manifold and place it over a silanised vial.

Elute OTA in a two steps procedure: (1) Apply 0.50 mL of methanol on the column and let it pass through by gravity. (2) Wait for 1 min and apply a second portion of 0.75 mL methanol. Collect the applied elution solvent by passing air through, as much as possible, after most has passed through by gravity. Add 0.50 mL of de-ionised water, mix vigorously by Vortex, and store the sample test solution at 4°C until HPLC analysis.

HPLC Analysis

Inject 200 μL, out of 1.75 mL eluate (equivalent to 0.086 g of sample), into the chromatographic apparatus by full loop injection system, at least three times for each injection.

The limit of detection of the method calculated as 3 signal-to-noise ratio, was 0.06 μg/kg. As far as quantitative analysis was concerned, if the ochratoxin A signal of the naturally contaminated samples fell outside the calibration graph, the amount of sample injected was adjusted by concentrating or diluting the sample test solution.

RESULTS AND DISCUSSION

Precision

In order to test the performance characteristics of the method, three naturally contaminated cocoa powder batches (low, medium, and high level) were analysed in two different days for within-day and between-day repeatability evaluation. The obtained results are shown in Table 1. Each contaminated sample was analysed ten times within the two days. All the average levels and standard deviations satisfactorily fitted each other.

As far as reproducibility (R) is concerned, the same set of analyses has been repeated after a month, changing the operator and the instrumentation. The obtained results ($N = 20$ for each level of contamination) are described in Table 2. All reproducibility values were satisfactory in terms of accordance with the international standards related to the carry out of an analytical method, resulting in much lower corresponding values, as calculated from Horwitz equation, from which the corresponding values at found concentration levels were 61.60%, 49.52%, and 35.13% vs. 23.07%, 18.18%, and 17.54% for low, medium, and high levels of contamination, respectively. In addition, in all of the three levels of contamination, the ratio r/R, that has to fall within 0.5 and 0.7 as a result of a method with good precision, fully met this condition. Moreover, the results showed good performance, as also shown if compared with Horrat values (Table 3). In addition, all values satisfactorily fell within the performance characteristics for ochratoxin A analysis, suggested by European Committee for Standardization (CEN),[17] that are $\leq 40\%$ as RSD_r at levels of concentration of less than 1 μg/kg and $\leq 20\%$ as RSD_r at levels of concentration within 1–10 μg/kg.

Accuracy

Accuracy was evaluated by recovery factor calculations and summarised in Table 4.

Three different spiking levels were added to blank samples, i.e., low (0.15 μg/kg), medium (0.50 μg/kg), and high (2.0 μg/kg) levels of contamination. The spiking levels were chosen within the range of concentration levels currently under discussion, as maximum tolerable limits by the European Commission for cocoa derived products.

All the spiked samples were left in a fume cupboard overnight to allow the evaporation of the solvent, and successively analysed along the analytical cycle, including unknown samples.

The obtained values fell within 78–96%, showing a good method performance and in accordance with recovery values suggested by CEN for the same levels of concentration.

Table 1. Within and between-day Repeatability (r) and Relative Standard Deviations (RSD) at low, medium, and high level of contamination.

		Low level		
1st Day	Average (μg/kg)[a] = 0.12	s_r[b] = ±0.02	Within-day repeatability (r_{w1})[c] = 0.06	$RSD_{r1}\%$[d] = 16.65
2nd Day	Average (μg/kg) = 0.11	s_r = ±0.02	Within-day repeatability (r_{w2}) = 0.06	$RSD_{r2}\%$ = 18.18
1st Day + 2nd Day	Average (μg/kg) = 0.11	s_r = ±0.02	Between-day repeatability (r_b) = 0.06	$RSD_{r1+r2}\%$ = 18.18
		Medium level		
1st Day	Average (μg/kg) = 0.52	s_r = ±0.06	Within-day repeatability (r_{w1}) = 0.17	$RSD_{r1}\%$ = 11.53
2nd Day	Average (μg/kg) = 0.52	s_r = ±0.07	Within-day repeatability (r_{w2}) = 0.20	$RSD_{r2}\%$ = 13.46
1st Day + 2nd Day	Average (μg/kg) = 0.52	s_r = ±0.07	Between-day repeatability (r_b) = 0.20	$RSD_{r1+r2}\%$ = 13.46
		High level		
1st Day	Average (μg/kg) = 1.67	s_r = ±0.23	Within-day repeatability (r_{w1}) = 0.65	$RSD_{r1}\%$ = 13.77
2nd Day	Average (μg/kg) = 1.60	s_r = ±0.19	Within-day repeatability (r_{w2}) = 0.53	$RSD_{r2}\%$ = 11.87
1st Day + 2nd Day	Average (μg/kg) = 1.63	s_r = ±0.20	Between-day repeatability (r_b) = 0.57	$RSD_{r1+r2}\%$ = 12.26

[a]Number of replicates = 10.
[b]s_r defined as the standard deviation, calculated from results generated under repeatability conditions.
[c]$r_{w1,w2,b}$ defined as the value below which the absolute difference between two single test results obtained under repeatability conditions (i.e. same sample, same operator, same apparatus, same laboratory, and short interval of time) may be expected to lie within a specific probability (typically 95%) and calculated as $r = 2{,}8 \times s_r$.
[d]RSD_r calculated from results generated under repeatability conditions $[(s_r/x)-100]$.

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Table 2. Reproducibility values calculated on the same set of samples in different conditions and time.

Level	Average (μg/kg)[a]	s_R[b]	R[c]	r_b	r_b	RSD_R%[d]	RSD_{RH}%[e]
Low	0.13	±0.03	0.085	0.06	0.70	23.07	61.60
Medium	0.55	±0.10	0.28	0.20	0.71	18.18	49.52
High	1.71	±0.30	0.85	0.57	0.67	17.54	35.13

[a]Number of replicates = 20 for each level of contamination.
[b]s_R defined as the standard deviation, calculated from results under reproducibility conditions.
[c]R defined as the value below which the absolute difference between single test results obtained under reproducibility conditions (i.e. on identical material obtained by operators in different laboratories, using the standardised test method) may be expected to lie within a certain probability (typically 95%) and calculated as $R = 2{,}8 \times s_R$.
[d]RSD_R as calculated from results generated under reproducibility conditions $[(s_R/x)-100]$.
[e]RSD_{RH} as calculated from Horwitz's formula.

Table 3. Horrat values[a] for the three different levels.

Level	RSD_R% observed	RSD_{RH}% predicted	Horrat value[a]
Low	23.07	61.60	0.37
Medium level	18.18	49.52	0.36
High	17.54	35.13	0.50

[a]Horrat value: RSD_R (observed)/RSD_{RH} (predicted). The RSD_{RH} predicted was derived by the Horwitz equation.

Table 4. Average recovery factors (%), for OTA spiked samples at different levels.

Added level (μg/kg)	Number of replicates	Average found level (μg/kg)	Average recovery ± SD (%)
0.15	5	0.12	82 ± 4
0.50	5	0.43	86 ± 2
2.0	5	1.88	94 ± 2

Optimisation of the Method

Typical chromatograms of OTA standard and OTA naturally contaminated cocoa beans are shown in Figs. 1 and 2.

In order to obtain the best accuracy throughout the whole analytical chain, three different extraction solvent were used: (a) aqueous solution of sodium hydrogen carbonate (0.1%) and polyethylene glycol (PEG) 8000 (0.3%), adjusting pH to 7.8 ± 0.1 before the extraction, (b) with PEG, without adjusting pH, and (c) without PEG, and adjusting pH at 7.8 ± 0.1 before the extraction step.

Chromatograms of the obtained results following the three procedures are shown in Fig. 3. It could be concluded, that the most efficient procedure was obtained by adding PEG to the sample and by adjusting pH at 7.8 ± 0.1 with hydrochloric acid 0.1 mol/L before the extraction [Fig. 3(a)]. In the other case,

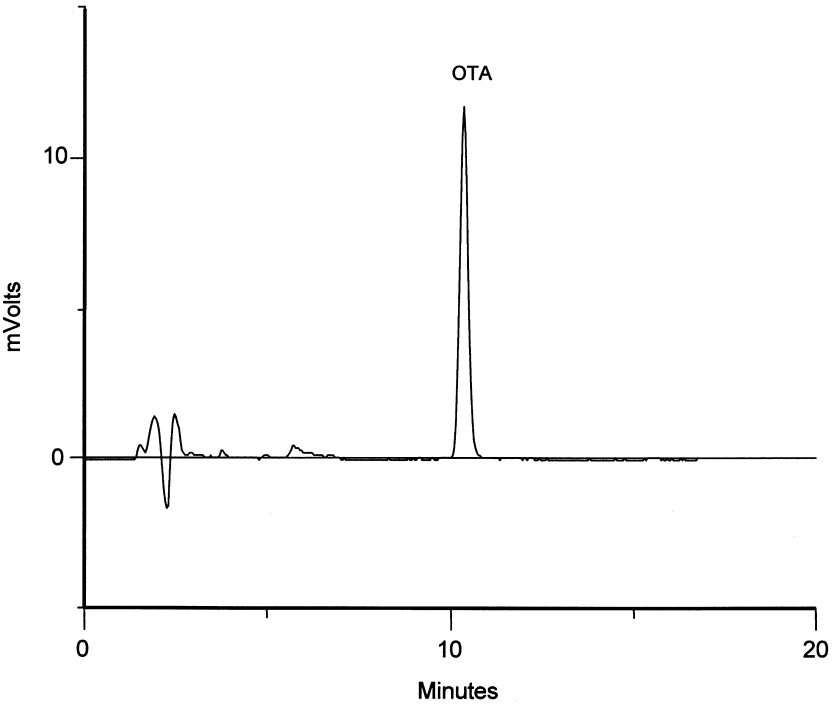

Figure 1. Chromatogram of 0.5-ng injection of ochratoxin A standard. Column: Phenomenex, Kromasil (250×4.6 mm, 5 µm); mobile phase: acetonitrile : water : - acetic acid [50 : 49 : 1 (V : V : V)]; flow-rate: 1.2 mL/min; detection: fluorescence at 330 nm excitation and 460 nm emission.

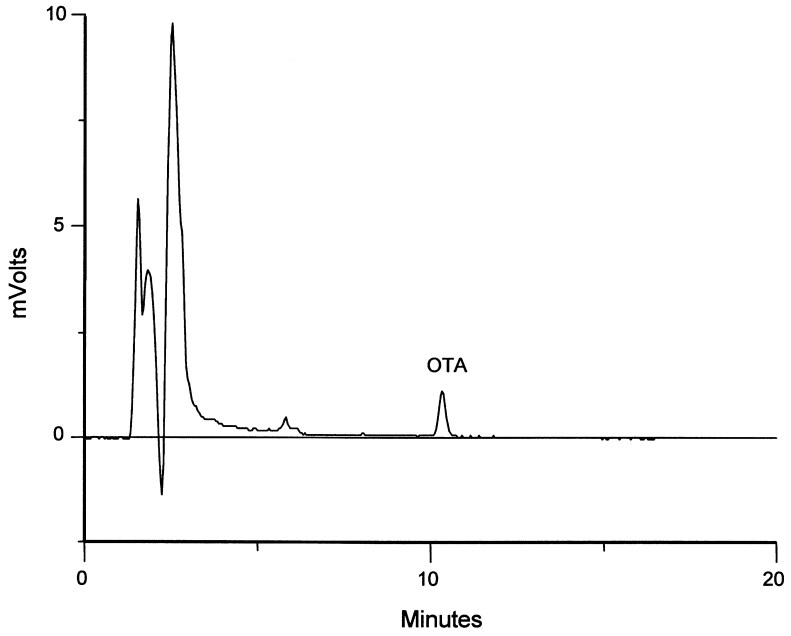

Figure 2. Chromatogram of naturally contaminated cocoa bean (low level). Phenomenex, Kromasil (250 × 4.6 mm, 5 μm); mobile phase: acetonitrile : water : acetic acid [50 : 49 : 1 (V : V : V)]; flow-rate: 1.2 mL/min; detection: fluorescence at 330 nm excitation and 460 nm emission.

it should be considered, that due to the extraction step, pH increased about 0.5 pH units as a result of the co-extraction of alkalinising substance from cocoa powder, and if pH conditions are not under control, the pH registered value was as high as about 9.0 ± 0.1. This condition is probably the result of incompatibility with the following cleanup step by IACs, where the linkage between antibodies sites and ochratoxin is better enhanced at moderately alkaline conditions, [Fig. 3(b)]. Finally, by excluding PEG from the extraction solvent, even if at controlled pH, a decrease of the output occurred, probably due to the lower strength of the extraction solvent [Figure 3(c)].

A mixture of methanol–sodium hydrogen carbonate (0.1%), (20 : 80 v : v) was also used in order to check a stronger extraction power of ochratoxin A, but serious difficulties in filtration steps were encountered. With this extraction solvent, a centrifugation step should be added for obtaining the extract to be successively cleaned up by IAC; for this reason, this extraction mixture was abandoned, since it is too time consuming.

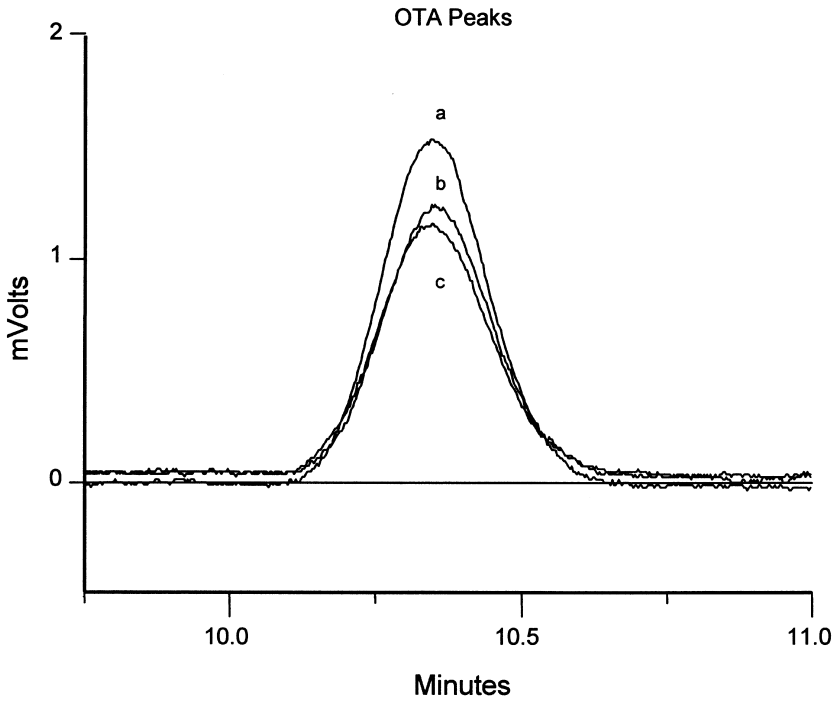

Figure 3. Chromatograms of OTA extraction efficiency ion accordance with three different procedures, (a) with PEG added to the sample and by adjusting pH at 7.8 ± 0.1 with hydrochloric acid 0.1 mol/L, before the extraction; (b) with PEG added to the sample without pH adjustment; (c) without PEG and with pH adjustment at 7.8 ± 0.1 with hydrochloric acid 0.1 mol/L, before the extraction.

Furthermore, in order to speed up the analytical procedure without any loss of ochratoxin A, the eluate from the IAC was diluted with an appropriate amount of water before the injection onto HPLC, instead of taking the samples to dryness with nitrogen and re-dissolving it with the mobile phase. This procedure assured faster time of analysis, no additional handling of the sample by the operator, and increased recovery factors.

Additionally, chromatographic column length can be reduced at 150 mm, and flow rates can be increased at 1.2 mL/min, having as a final result, a reduction of the analysis time. Total loop injection was preferred to the partial loop procedure in consideration of the best accuracy and repeatability of retention times and of peak areas.

CONCLUSIONS

The method, tested for performance characteristics assessment, showed good results and reliability when compared to the reference values for mycotoxins. Furthermore, the method proved to be robust, easy-to-use, not time consuming, sensitive, and suitable for control and research purposes.

For this reason, this method is suitable to meet the need for a fast and reliable method for OTA determination in cocoa powder, also, in view of the proximal maximum tolerable limits under discussion by European Commission.

Therefore, the method is predominantly addressed both to public laboratories involved in official control activity, and to private laboratories dealing with the HACCP principles implementation, and it can also be used for monitoring studies aimed at the evaluation of the status of contamination of commercial products.

REFERENCES

1. Kuiper-Goodman, T. Food safety: Mycotoxins and phycotoxins in perspective. In *Mycotoxins and Phycotoxins–Developments in Chemistry, Toxicology and Food Safety*; Miraglia, M., van Egmond, H.P., Brera, C., Gilbert, J., Eds.; Alaken, Inc.: Fort Collins, Colorado, 1998; 25–48.
2. Radovanovic, Z. Epidemiological characteristics of Balkan endemic nephropathy. In *Eastern Regions of Yugoslavia in Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours*; Castegnaro, M., Plestina, R., Dirheimer, G., Chernozemsky, I.N., Bartsch, H., Eds.; IARC: International Agency for Research on Cancer: Lyon, 1991; 11–20.
3. Petkova-Bocharova, P.; Castegnaro, M. Ochratoxin A in human blood in relation to Balkan endemic nephropathy and renal tumours in Bulgaria. In *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours*; Castegnaro, M., Plestina, R., Dirheimer, G., Chernozemsky, I.N., Bartsch, H., Eds.; IARC: International Agency for Research on Cancer: Lyon, 1991; 135–137.
4. Micco, C.; Miraglia, M.; Brera, C.; Corneli, S.; Ambruzzi, A. Evaluation of ochratoxin A level in human milk in Italy. Food Addit. and Contam. 1995, **12** (3), 351–354.
5. Miraglia, M.; Brera, C.; Corneli, S.; De Dominicis, R. Ochratoxin A in Italy: status of knowledge and perspectives. In *Human Ochratoxicosis and its Pathologies*; Creppy, E.E., Castegnaro, M., Dirheimer, G., Eds.; John Libbey Eurotext: Montrouge, France, 1993; Vol. 231, 129–139.

6. Miraglia, M.; De Dominicis, A.; Brera, C.; Corneli, S.; Cava, E.; Menghetti, E.; Miraglia, E. Ochratoxin A levels in human milk and related food samples: An exposure assessment. Nat. Toxins **1995** (3), 436–444.
7. Palli, D.; Miraglia, M.; Saieva, C.; Masala, G.; Cava, E.; Colatosti, M.; Corsi, A.M.; Russo, A.; Brera, C. Serum levels of ochratoxin A in healthy adults in Tuscany: Correlation with individual characteristics and between repeat measurements. Cancer Epidem. Biomar. **1999** (3), 265–269.
8. European Commission for Food, DGXXIV: SCF, Working Group on Contaminants Opinion on ochratoxin A CS/CNTM/MYC/14, 28 September 1998.
9. Krogh, P. Ochratoxins in food. In *Mycotoxins in Food*; Krogh, P., Ed.; Food Science and Technology, 1987; 97–121.
10. European Commission for Food SCOOP Task 3.2.7., Exposure assessment Task 3.2.7 Assessment of dietary intake of Ochratoxin A by the population of EU Member States, *in press.*
11. van Egmond, H.P; Speijers, G.J.A. Survey of data on the incidence and levels of ochratoxin A in food and animal feed worldwide. J. Natl. Toxins **1994**, 125–144.
12. Majerus, P.; Cutka, I.; Dreyer A.; El-Dessouki, S.; Eyrich, W.; Reusch, H.; Schurer, B.; Waiblinger, H.U. Zur Belastungssituation von ochratoxin A in lebensmitteln pflanzlichen. Ursprungs, Deut. Lebensm.-Rundsch. **1993**, *89*, 112–114.
13. Engel, G. Ochratoxin A in sweets, oil seeds, and dairy products, Arch. Lebensmittelhyg. **2000**, *51*, 98–101.
14. Sharman, M.; MacDonald, M.; Gilbert, J. Automated liquid chromatographic determination of Ochratoxin A in cereals and animal products using immunoaffinity column clean-up. J. Chromatogr. **1992**, *603*, 285–288.
15. Hurst, W.J.; Martin, Jr., R.A.; High performance liquid chromatography determination of ochratoxin A in artificially spiked cocoa beans. J. Chromatogr. **1983**, *265*, 353–356.
16. Hurst, W.J.; Martin, Jr., R.A. High-performance liquid chromatographic determination of ochratoxin A in artificially contaminated cocoa beans using automated sample clean-up. J. Chromatogr. A **1998**, *810*, 89–94.
17. European Committee for Standardization (CEN), Food analysis, Biotoxins. Criteria of analytical methods of mycotoxins, 1999; CR 13505.

Received September 10, 2002
Accepted October 15, 2002
Manuscript 5962