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Ochratoxin A in Italian marketed cocoa products

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Abstract

Ochratoxin A (OTA) is a mycotoxin produced by fungi belonging to the *Penicillium* and *Aspergillus* genera. The natural occurrence of OTA in 18 pure cocoa powder samples marketed in Italy and purchased from supermarkets, organic product shops and retail shops was investigated. Nine samples of the eighteen products had no detectable OTA contamination (below 10 ng/kg). Nine positive samples had a toxin concentration ranging between 0.22 and 0.77 μg/kg with a mean of 0.43 μg/kg. All the organic samples analyzed were negative for OTA; of 14 conventional samples analyzed, four samples (28.56%) were above the suggested legal limit (0.5 μg/kg) because they were contaminated with 0.77, 0.53, 0.67 and 0.62 μg/kg, respectively. The optimization of the extraction protocol and comparison of different brands of immunoaffinity column clean-up were carried out. The incidence (50% of examined samples) of the OTA level in Italian cocoa marketed products is an important signal of attention for consumers, because cocoa is widely used in the production of child snack foods and it contributes to OTA daily intake.

Keywords: Cocoa; Ochratoxin A; Immunoaffinity column clean-up

1. Introduction

Nowadays, one of the most widespread problems in advanced technological countries is food quality and safety. Mycotoxin occurrences and related toxic effects in humans and animals represent a major part of food safety (FAO, 1999).

Ochratoxin A (OTA) (Fig. 1) is an important and dangerous secondary metabolite bio-produced by a few moulds belonging to the *Aspergillus ochraceus* and *Penicillium expansum* species (Harwig, Kuiper-Goodman, & Scott, 1983). OTA biosynthesis requires complex fungi–substrate interactions; therefore, its production and accumulation are not easy under normal conditions (Mantle, 2002).

OTA, isolated for the first time by van der Merve, Steyn, and Fourie (1965) from *A. ochraceus*, is chemically a dihydromethyl-isocoumarin moiety linked via the 7-carboxy group, by an amidic bond, to L-β-phenylalanine (Rizzo, Eskola, & Atroshi, 2002).

This chemical structure makes easier its detection by a fluorescence detector and it is possible to achieve a very low limit of quantification and detection by conventional high performance liquid chromatography and fluorescence detector (HPLC–FLD).

In European countries OTA is probably the most ubiquitous mycotoxin, in particular, in Central Europe (Sweden, Germany, Denmark and the UK) and it can be revealed at levels greater than 0.1 μg/kg in more than 90% of human and swine blood samples (Petzinger & Weidenbach, 2002). Main food carriers of OTA are considered beer, cereals, coffee and pork meat (Petzinger & Weidenbach, 2002). Several studies of OTA occurrences in food and beverages have been performed (Blanc, Pittet, Munoz-Box, & Viani, 1998; Gareis & Scheuer, 2000; Hurst & Martin, 1998; Jorgensen, 1998; Pittet, Tornare, Huggett, & Viani, 1996; Rizzo et al., 2002; Skaug, 1999; Solfrizzo, Avvantaggiato, & Visconti, 1998; Thirumala-Devi, Mayo, Reddy, Tangni, Larondelle, & Reddy, 2003; Ueno, Maki, Lin, Furuya, Suguira, & Kawamura, 1998; Zimmerli & Dick, 1995).

There are several scientific reports of OTA carcinogenicity in experimental animals; it is nephrotoxic (Bullerman, 1986; Hald, 1991) hepatoxic, teratogenic

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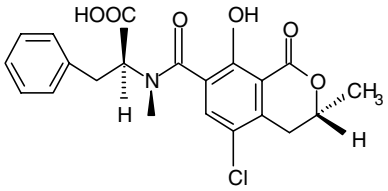

Fig. 1. Chemical structure of ochratoxin A.

and immunosuppressive (Muller, Kielstein, Rosner, Berndt, Heller, & Kohler, 1999; Schlatter, Studer-Rohr, & Rasonyi, 1996) and IARC has classified OTA in group 2B as a possible carcinogenic compound for humans (IARC, 1993).

The mean dietary intake for humans in the European Union was found to be in the range 0.9 (German) to 4.6 ng/kg bw/day (Italy). This level is lower than the provisional tolerable daily intake (PTDI) proposed by the WHO of 5 ng OTA/kg bw/day.

Cereals, beer, pork, coffee are all contributors to OTA daily intake, but cocoa and its related food products have not been considered in detail its use as main ingredient of junk foods, designed and produced for young people, giving a PTDI 16 times higher than the average intake of adults (Petzinger & Weidenbach, 2002).

OTA-contaminated foods are common in Europe and 57% of 6476 food samples were analyzed and found positive for OTA and to be above the detection limit of 0.01 μg/kg (MAFF, 1997; Wolff et al., 2000).

Most of the analyzed samples are cereals, cereals products, breakfast products, oats, maize and flour. Some infant food containing soy products were contaminated by OTA (Petzinger & Weidenbach, 2002); there are no relationships between harvest weather conditions and OTA occurrence. Red wine, beer, tea and coffee are considered to be potentially contaminated by OTA and several surveillance studies were performed, in many countries, on these kinds of beverages.

Cocoa is a very important ingredient in several kinds of foods, such as cakes, biscuits, child-foods, ice-creams and sweets.

In addition, cocoa beans are source of cocoa powder and come from Africa, Central and South America. In all these warm and wet countries, weather and agronomic conditions are favourable for *Penicillium* growth and consequently OTA biosynthesis. In addition, storage and processing conditions of cocoa in these countries are not very safe and mycotoxigenic fungi contamination may be possible at many critical points of the cocoa production chain.

Finally, the cultivation sites are not located near the cocoa transforming sites, so the delivery times are very long and therefore favourable for mould growth.

There are several reported methods for the analysis of OTA in various agricultural products and biological samples. Earlier methods used thin-layer chromatography (TLC) (Pittet & Royer, 2002), while many of the present methods rely on the use of reversed-phase HPLC with an acidic mobile phase and fluorescence detection, since this allows the best sensitivity and selectivity for this assay.

The sample preparation protocols have varied and range from extraction of the material of interest in different solvents followed by a series of liquid–liquid extraction steps, to a direct extraction with more polar materials before an immunoaffinity sample clean-up. However, it is not possible to apply any specific or general protocol, including immunoaffinity columns (used in the analyses of coffee, fresh fruits, or cereals) to cocoa samples.

The main target of this paper was to analyze samples of cocoa powder marketed in Italy for the presence of OTA. This study considers only pure cocoa powder but not cocoa treated with sugars, flavours or other substances. In particular, it compares cocoa products of conventional and organic agriculture, from low technological level countries, whether packed in Europe or not.

A comparison among four extraction protocols and the use of two different immunoaffinity columns was performed to optimize operative conditions for cocoa powder analysis. A similar comparison between two brands of IAC has already been reported but referred only to wine analyses, and it gave a substantial equivalence of both IAC (Castellari, Fabbri, Fabiani, Amati, & Galassi, 2000).

2. Materials and methods

2.1. Materials

Ochratoxin A standard was purchased from Sigma Chemical Company (St. Louis, MO). Water, for the high-performance liquid chromatography (HPLC) mobile phase, was purified in a Milli-Q system (Millipore, Bedford, MA). Phosphate-buffered saline (PBS) was prepared with potassium chloride (0.2 g), potassium dihydrogen phosphate (0.2 g), anhydrous disodium hydrogen phosphate (1.16 g) and sodium chloride (8.0 g) – 900 ml of water for HPLC. The pH was modified at 7.4 and the solution made up to 1 l. All other chemicals and HPLC-grade solvents were purchased from Merck (Darmstadt, Germany).

2.2. Sampling

Eighteen samples (two subsamples for each lot) of pure cocoa powder, no sugar added, were purchased in supermarkets, local stores, and organic product shops in the south of Italy, Naples area. Information about the

Table 1
Brief resume of cocoa powder sampling

Sample	Quantity	Agronomic practice claimed	Comments
Cocoa powder	10	Conventional	Supermarkets
Cocoa powder	2	Conventional	Hard discounts
Cocoa powder	1	Conventional	Retail shop
Cocoa powder	4	Organic	Organic product shops
Cocoa powder	1	Conventional	Automatic chocolate dispenser

origin of the commercial samples (German, Italy, Bolivia, Dominican Republic, generic South America) was on the carton or can labels. All the carton and cans were stored at 4 °C prior to analysis by HPLC. The comparison between different extraction methods was carried out, analyzing a blank sample at the same time.

Table 1 gives a brief description of the collected samples. 22.3% of samples are labelled as organic, 16.7% of samples are produced by international companies, 22.3% of samples are imported from Central and South America and are classified as products of ecological and fair trade markets. Finally, 50% of samples are sold in supermarkets and the other 50% in small shops.

2.3. Preparation of ochratoxin A working solution

Ochratoxin A standard stock solution was prepared by dissolving 1 mg of pure standard in 100 ml of methanol, HPLC grade. Two hundred microlitres of ochratoxin A standard stock solution (10 μg/ml) was transferred to a 100 ml volumetric flask and completely dried under a nitrogen stream at room temperature. It was quickly diluted with pure methanol to obtain a working solution, at 20 μg/l, of a standard working solution and then diluted with methanol. The vials were closed and stored in the dark at 4 °C; the working solution was freshly prepared every week.

2.4. Immunoaffinity columns

Two brands of commercial immunoaffinity clean-up columns, packed and commercialized, Ochraprep® (Rhone Diagnostic Technologies) and OchraTest™ (Vicam Science Technology, MA, USA), were tested and compared. In particular, the behaviour of immunoaffinity columns (IAC) with different OTA extraction protocols from cocoa powder was compared.

2.5. Extraction and clean-up procedures

2.5.1. Method A

According to the Vicam technical note and suggestions for the analysis of coffee powder, 5 g of cocoa powder were extracted with 100 ml of sodium hydrogen carbonate solution (1%). The suspension was homogenized with an Ultraturrax at 13,500 rpm for 1 min, then to 20 ml of filtered suspension, were added 20 ml of PBS solution and 0.01% Tween 20. Ten millilitres of this solution was loaded onto immunoaffinity column and eluted at a flow rate of 1–2 drops/s. The immunoaffinity column was washed, first with 10 ml of PBS 0.01% Tween 20 and 10 ml of pure water and then eluted with 1.5 ml of pure methanol and 1.5 ml of water.

2.5.2. Method B

According to the Rhone Diagnostic Technologies sheet of IAC instructions for the analysis of cocoa powder matrix, 10 g of cocoa powder were re-suspended in 200 ml of sodium hydrogen carbonate solution 1%. The suspension was homogenized in a Waring Blender for 1 min. Twenty millilitre of filtered solution were added to 20 ml of PBS at pH 7.4; all 40 ml of this solution were loaded onto an immunoaffinity column (Rhone Diagnostic Technologies) and eluted at a flow rate of 1–2 drops/s. The column was washed with 20 ml of water and then eluted with 1.5 ml of methanol (acetic acid 2%) and 1.5 ml of pure water.

2.5.3. Method C

According to the official European Ring Test, between 13 different labs of four different countries commissioned by CAOBISCO, 5 g of cocoa powder were extracted with acetonitrile/water (60:40 v/v) plus 400 mg of sodium chloride.

The suspension was homogenized with an Ultraturrax at 13,500 rpm for 2 min and centrifuged at 2.000 g for 20 min and 4 °C (RC 10.10, Jouan S.A., St. Herblain, France). Four millilitres of surnatant were added to 92 ml of PBS buffer at pH 7.4. This solution was loaded onto the immunoaffinity column supplied by Rhone Diagnostic Technologies and eluted at a flow rate at 1–2 drops/s. The column was washed with 20 ml of pure water and then eluted with 1.5 ml of methanol (acetic acid 2%) and 1.5 ml of pure water.

2.5.4. Method D

Ten grammes of cocoa powder were suspended in 200 ml of sodium hydrogen carbonate (1%). The suspension was homogenized in a Waring Blender for 2 min. The solution was centrifuged at 2.000 g for 20 min at 4 °C and divided into four Falcon flasks. 40 ml of filtered surnatant were added to 20 ml of PBS solution. This

solution was loaded onto the immunoaffinity column at a flow rate of 1–2 drops/s. The column was washed with 20 ml of pure water and then eluted with 1.5 ml of methanol (acetic acid 2%) and 1.5 ml of pure water.

All of the above methods reported require 20 μl of sample extract for the analysis by HPLC–FLD.

2.5.5. HPLC equipment

HPLC analyses were performed using LC-10AD pumps and a FLD model RF-10Axl, Shimadzu (Japan), set at an excitation wavelength of 333 nm and emission wavelength of 460 nm. Data acquisition and handling were done by a system control SLC10A with Shimadzu (Japan) software Class VP version 4.3.

A Jupiter (Phenomenex, USA) C_{18} (250×4.6 mm, 5 μm) column was used. HPLC conditions were set up with elution at constant flow of 1 ml/min and $CH_3CN_{(1\%\ \text{acetic acid})}$–$H_2O_{(1\%\ \text{acetic acid})}$ (50:50 v/v) as the starting eluent system. The starting ratio was linearly modified to 100% CH_3–CN in 20 min. After 3 min at a constant ratio, the pumps were taken back to starting conditions in 10 min. Eluent was freshly prepared and filtered (0.22 μm) before use.

All samples were filtered through a 0.22 μm syringe filter (Millipore, Bedford, MA, USA) prior to injection (20 μl) onto the HPLC column.

Mycotoxin identification was performed by comparing retention times and UV spectra of the purified samples with pure OTA standard. A further confirmation was obtained by co-injecting samples together with OTA standard solution.

The average retention time for OTA (12.25 min, RSD 1.5%) was obtained with 10 consecutive injections of the same OTA working solution within the same day. This retention time enhanced the chromatographic resolution of the OTA peak from other matrix interferences. The calculated instrumental detection limit and quantification limit for OTA under these conditions were 0.2 (10 ng/l, S/N 3) and 2 pg (100 ng/l), respectively. In Fig. 2, a chromatographic run of pure OTA standard at concentration of 1 μg/l is shown.

Mycotoxin quantification was carried out by comparing peak areas of the investigated samples to the calibration curve of authentic OTA standards.

In Fig. 3, the calibration curve for OTA, ranging from 0.1 to 100 μg/l, is shown. All chromatographic results were statistically examined, and the relative standard deviation (SD) was calculated.

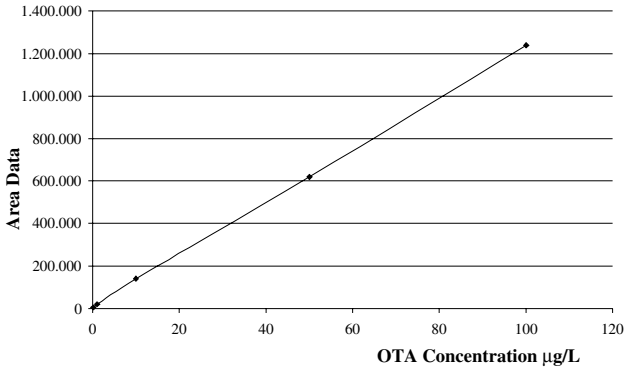

Fig. 3. Calibration Curve for OTA in the range 0.1–100 μg/l.

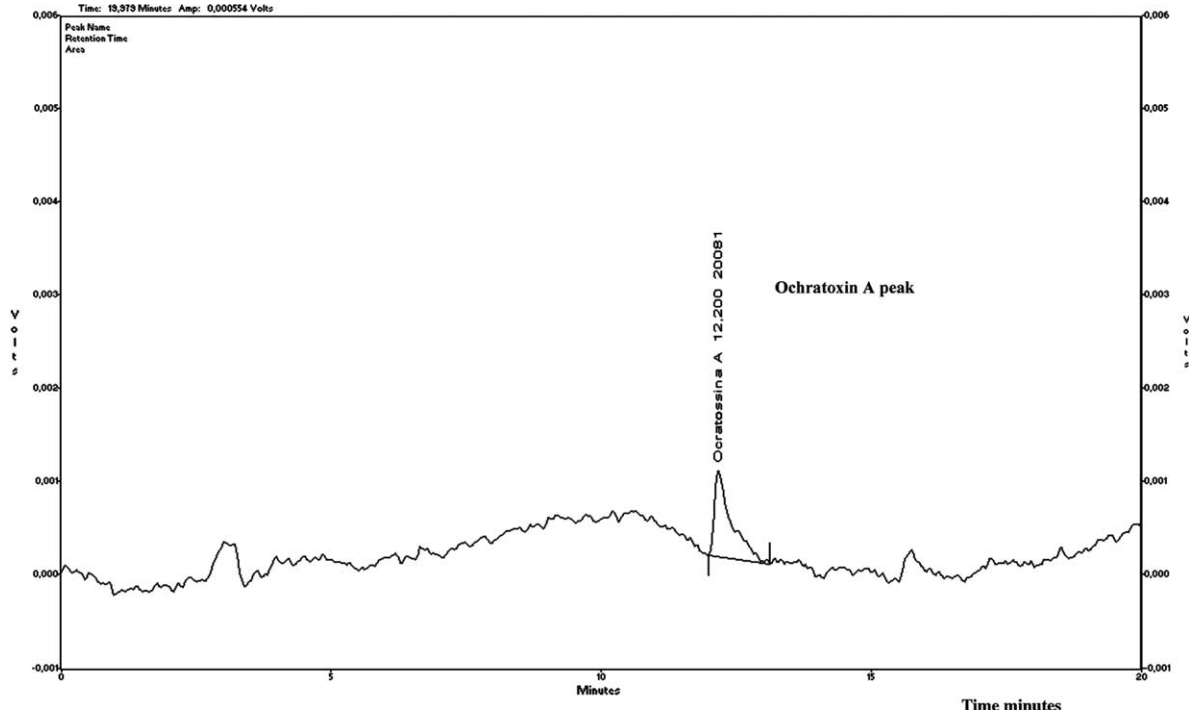

Fig. 2. Chromatographic HPLC run of pure OTA standard at concentration of 1 μg/l.

2.5.6. Recovery experiments

Four extraction protocols were tested to determine recovery efficiency for each method; for this, a blank cocoa powder, previously analyzed with Method B, was spiked at two levels (20 and 2 μg/l) with an OTA working solution. In particular, 10 g of cocoa powder, for Method B and D, were spiked with 10 and 1 ml, respectively, of OTA working solution while 5 g of cocoa powder, for Method A and C, were spiked with 500 μl and 5 ml, respectively, of OTA working solution.

Each test was performed three times, and the value shown is the average of three HPLC measures. All of the residue data shown were corrected for recovery.

3. Results and discussion

In the literature, several methods are used to extract and to analyze OTA from many foods and biological sources, such as coffee, wine, cereals, fruits, blood or plasma.

To detect OTA occurrence in cocoa powder products a fast efficient and sensitive analytical method is needed. In particular, the extraction step is a critical point of all methods, because chromatographic conditions and chemical identification are not related to this kind of food matrix and, consequently, small modifications of the method are needed at these steps.

Cocoa powder is a matrix quite different from fresh fruits, cereals, coffee or wine and its extraction step optimization requires specific study.

A good way to compare different methods and different brands of immunoaffinity columns is to test IACs and extraction conditions, at two spiking OTA levels (2 and 20 μg/kg).

Fig. 2 shows the chromatogram of a standard solution of OTA at a concentration of 1 μg/l (S/N = 5).

The results are shown in Table 2 with the corresponding relative standard deviation (RSD) calculated by Excel Office 2000 Software.

The first consideration is that both IACs are more efficient at the level of 20 μg/kg than at 2 μg/kg, with any method tested.

This result could depend on the immunocapacity of IACs; probably a small amount of OTA is not released from the antibodies, independently of the amount of loaded antigen. Of course, working at low antigen level, this loss becomes proportionally more important, reducing the column performance.

Methods A and C appear to be unsuitable for analyzing cocoa powder because both show low level recoveries with both IAC columns tested; these results depend upon different variables.

First, Method A is suggested by the Vicam Company for coffee matrix and not for cocoa powder and, according to our experience and to the literature reports, Ochratest shows better results than Ochraprep. Second, cocoa is a more fatty matrix than coffee; its post-harvest storage and roasting processes are different and for these reasons Method A is not applicable to cocoa.

Table 2
Resume of recoveries data

IAC tested	Method tested	Level (μg/kg)	Recovery calculated (%)	RSD (%)
Ochraprep	M-A Vicam	2	25.5	2.5
Ochraprep	M-A	20	47.7	1.8
Ochratest	M-A	2	15.2	3.5
Ochratest	M-A	20	56.6	1.6
Ochraprep	M-B Rhone	2	26.4	2.8
Ochraprep	M-B	20	85.9	1.5
Ochratest	M-B	2	17.9	3.4
Ochratest	M-B	20	63.0	2.1
Ochraprep	M-C Ring	2	8.03	2.8
Ochraprep	M-C	20	21.5	3.0
Ochratest	M-C	2	17.9	2.6
Ochratest	M-C	20	37.6	2.9
Ochraprep	M-D Labs	2	20.4	2.3
Ochraprep	M-D	20	83.2	1.9
Ochratest	M-D	2	25.4	2.0
Ochratest	M-D	20	51.1	2.2

Method C has been used by 13 labs during a European Ring Test commissioned by CAOBISCO, on cocoa. The results of this Ring Test show a large standard deviation regarding reproducibility and repeatability between labs; in addition, recovery performances by single labs are not supplied. Method C, in our opinion, has two critical points: first the small quantity of matrix required, 5 g, could not be representative of OTA distribution in cocoa with consequently variable results. Second, this method requires purification of all 96 ml of solution by an IAC column at a flow of 1–2 drops/s, which means 90 min to clean-up for each sample. This volume appears to be very large and OTA may be lost before the elution of IAC with methanol.

Method B appears to be a good starting point to improve the extraction procedure for cocoa powder. This Method shows better recovery with Ochraprep, according to the Rhone Diagnostic technical note, than Ochratest as expected.

Critical points of this Method are the long time needed to perform the analysis and the possible loss of material due to breakdown of filter paper during filtration; however, these two problems can be solved.

Cotton wool or cellulose filters cannot substitute filter paper with the same efficiency, but is possible to obtain a good separation of solids from liquid with a quick centrifugation at 4 °C. The reduction of loss of material is another advantage of this new procedure.

In addition, the centrifugation to saves time and the clear surnatant prepurified by IAC allows an HPLC analysis that is easier to interpret and to quantify.

Method C with this slight modification becomes Method D that ensures a good recovery at both spiking

levels with Ochraprep and an acceptable performance with Ochratest.

Method D has been applied to analyze 18 cocoa powder samples collected in Naples area and the results are shown in Table 3.

Table 3
Natural occurrence data for each cocoa sample analyzed

Sample	Comments	OTA (μg/kg)
1	Supermarket International Trade mark	0.77
2	Supermarket International Trade mark	0.39
3	Supermarket International Trade mark	0.53
4	Supermarket	0.25
5	Supermarket	0.23
6	Supermarket	<L.O.D.
7	Supermarket	<L.O.D.
8	Hard Discount Conventional agriculture	0.67
9	Hard Discount Conventional agriculture	0.42
10	Retail Shop German producer	0.62
11	Supermarket	<L.O.D.
12	Organic shop Conventional agriculture	0.22
13	Organic agriculture South America producer	<L.O.D.
14	Organic agriculture South America producer	<L.O.D.
15	Organic agriculture South America producer	<L.O.D.
16	Organic agriculture	<L.O.D.
17	Organic shop Conventional agriculture South America producer	<L.O.D.
18	Automatic chocolate dispenser	<L.O.D.

The most contaminated cocoa powder samples are numbers 1, 3, 8 and 10; numbers 1 and 3 were produced by international food companies. These large companies use cocoa as an ingredient for biscuits, cakes, sweets and junk food.

On the other hand, the four organic samples, numbers 13, 14 15 and 16, produced in South America and sold in organic shops, are below the limit of detection of 0.5 μg/kg. Cocoa producers of ecological and fair trade markets in the Central and South America, export products of good quality.

Four samples (22%) are over the suggested European Limit (0.5 μg/kg of OTA) for cocoa and two of them are produced by international food companies. In Fig. 4 a positive cocoa sample is shown and in Fig. 5 its co-injection with OTA standard solution confirms OTA occurrence.

Nine samples (50%) are contaminated in the range of 0.22–0.77 μg/kg and these results should provoke Governments to increase the food surveillance for mycotoxins.

These data on the occurrence of OTA are better than those produced by MAFF (1997) in which 20 cocoa samples collected in England in 1998 were all positive for OTA.

In conclusion, the quality of cocoa powder sold in Italian shops can be improved in spite of the fact that 22% of samples were above the OTA legal limit and two of them were being sold in several supermarkets and

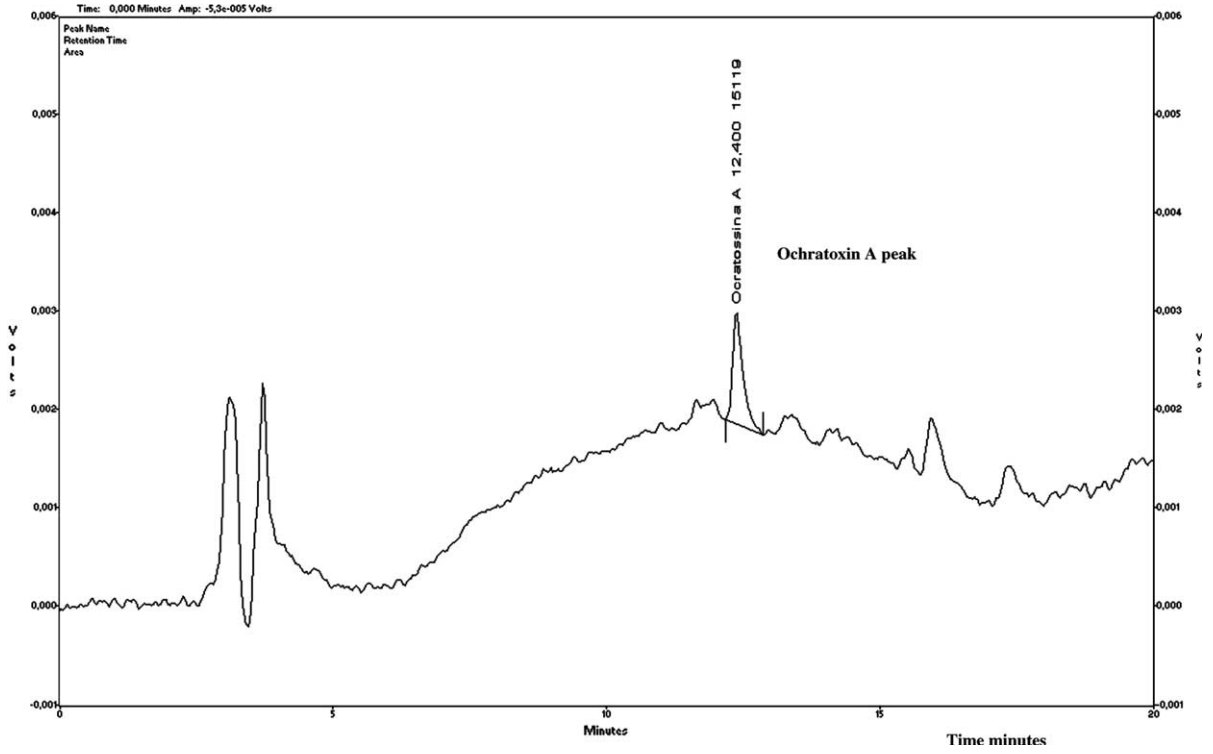

Fig. 4. Chromatographic HPLC run of a positive cocoa powder sample (0.77 μg/kg).

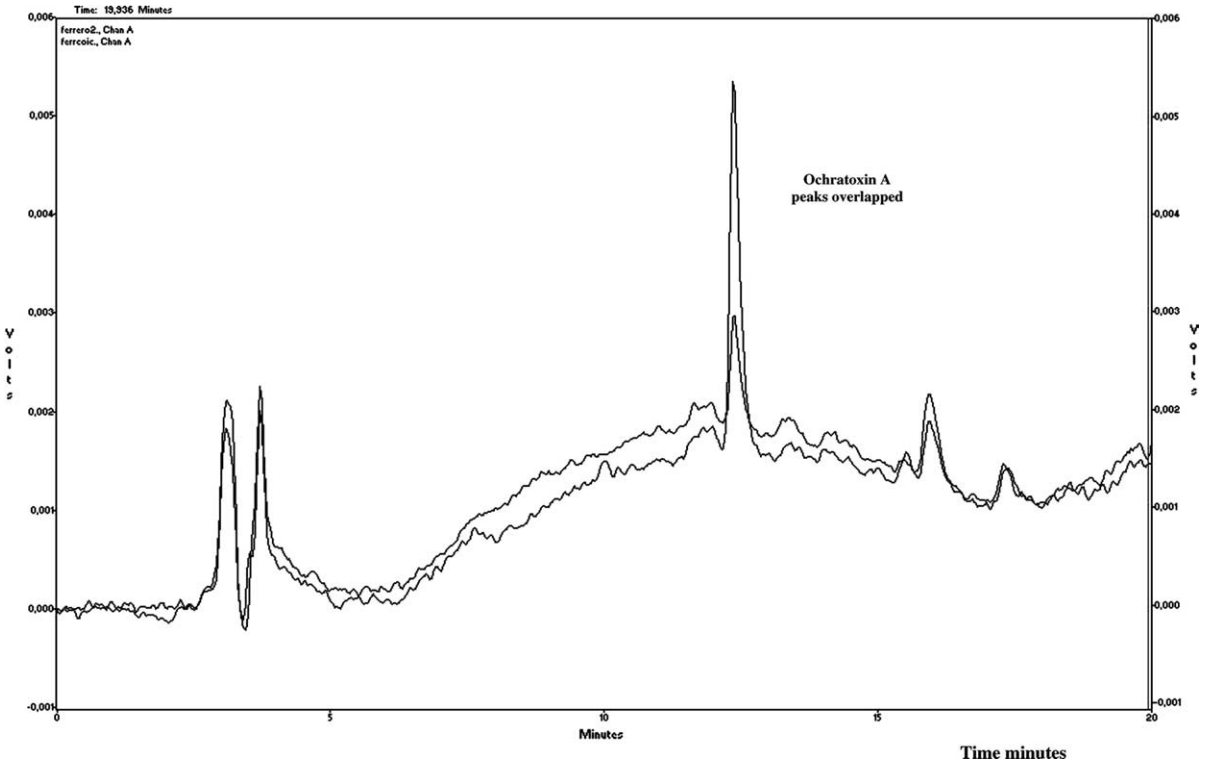

Fig. 5. Chromatographic confirmation of a positive cocoa powder sample (0.77 μg/kg OTA) with co-injenction of standard solution.

retail shops, representing a possible way to increase OTA daily intake, especially for children and young people.

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