Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 108 (2008) 272–276
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical Methods

Simple liquid chromatography assay for analyzing ochratoxin A in bovine milk

L. González-Osnaya, J.M. Soriano *, J.C. Moltó, J. Mañes

Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain

Received 2 October 2006; received in revised form 25 June 2007; accepted 3 October 2007

Abstract

Ochratoxin A (OTA) is a mycotoxin with teratogenic and carcinogenic properties. Animal intake of feedstuffs contaminated with OTA may cause that some residues may be found in bovine milk, therefore, its analysis requires a highly sensitive, simple and precise technique.

This method is based on a liquid–liquid extraction with methanol, followed by filtration and extract concentration. Liquid chromatography coupled to fluorescence detection was used for OTA analysis. In this way, several impurities are filtered off and OTA is quantified with a mean recovery of $93.0 \pm 7.4\%$ and a limit of detection of $0.01\ \mathrm{ng\,mL^{-1}}$. Therefore, this methodology allows a simple quantitative extraction of OTA from bovine milk.
© 2007 Published by Elsevier Ltd.

Keywords: Ochratoxin A; Milk; Liquid chromatography

1. Introduction

Ochratoxin A (OTA) is a secondary fungi metabolite mainly produced by *Penicillium verrucosum*, *Aspergillus ochraceus* and *Aspergillus carbonarius* (Eskola, Parikka, & Rizzo, 2001; Van der Merwe, Steyn, Fourie, Scott, & Theron, 1965). These fungi are natural opportunist biodeterioration agents of carbohydrate-rich agricultural commodities worldwide from latitudes ranging from cool to tropical temperate; therefore, this mycotoxin can occur in a large variety of commodities, such as cereals, dried fruits, coffee, beer, wine, and, because of a carry-over effect, in milk, blood, liver, kidney, and poultry meat from animals fed with contaminated feed (Mantle, 2002; Visconti, Pascale, & Centonze, 1999). Because of the persistence of OTA in the food chain, exposure to the compound is a potential human health hazard given that OTA has been experimentally shown to be teratogenic, a potent renal carcinogen, immunosuppressive, an enzyme inhibitor and has been implicated in Balkan endemic nephropathy, a disease characterized by progressive renal fibrosis in humans; it also has been associated with increased incidence of tumors of the upper urinary tract (KuiperGoodman, 1996; Vrabcheva et al., 2004). OTA is listed as a possible human carcinogen by the International Agency for Research on Cancer (IARC) based on sufficient evidence in experimental animals and inadequate human evidence (IARC, 1993).

Contaminated feed is the main source for mycotoxins infestation of farm animals. Oral intake of fungal metabolites with feed results in a negative impact on all relevant parameters of animal production. Moreover, under experimental conditions mycotoxins and/or their metabolites can be traced in milk (Gareis & Wolff, 2000). After consumption of OTA contaminated food and feed, the mycotoxin is distributed into different tissues; in cows the bioavailability of ochratoxin A is low, since the microflora in the rumen of cows hydrolyzes OTA to the less toxic metabolite ochratoxin α (Hult, Teiling, & Gatenbeck, 1976; Kiessling, Pettersson, Sandholm, & Olsen, 1984); therefore, OTA concentration in bovine milk is expected to be very low; however this low concentration show that

* Corresponding author. Tel.: +34 963543056; fax: +34 963544954.
E-mail address: jose.soriano@uv.es (J.M. Soriano).

0308-8146/$ - see front matter © 2007 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2007.10.013

cattle feed is frequently highly contaminated with OTA considering that the excretion rate may vary according with OTA intake (Guerre, Bailly, Benard, & Burgar, 2000). Direct contamination of milk with OTA can occur by growth of toxigenic *Aspergillus* and *Penicillium* species in raw milk if hygienic quality of milk handling is poor; however nowadays this is very unlikely since large-scale producers have implemented food safety measures such as good manufacturing practices (GMPs) and the hazard analysis and critical control point (HACCP) system.

OTA analysis is a multiple step process usually based on extraction, cleanup and determination. Detection methods for OTA and mycotoxins in general are mainly based on thin-layer chromatography, enzyme-linked immunosorbent assay (ELISA) or mainly liquid chromatography (LC) (Miraglia, De Santis, Grossi, & Brera, 2004). Chromatographic methods generally require multiple steps prior to detection, including extraction, extensive sample clean-up, pre-concentration, and sometimes derivatization of the analyte. So far, enzyme-linked immunosorbent assay (ELISA) methods are the most common immunoassay techniques used in OTA analysis due to the capability for parallel analysis of multiple samples. Although detection limits of LC and ELISA are comparable, the latter technique suffers from false positive results (because of cross-reactions) and, more importantly, from false negative results, therefore confirmatory test based on liquid or gas chromatography are required; such sample treatment not only makes the analysis time-consuming and costly but also requires trained personnel (Monaci, Tantillo, & Palmisano, 2004). Common extraction methodologies are based on the solubility of OTA in organic solvents, such as chloroform, methanol, acetonitrile in acid medium and diluted aqueous sodium bicarbonate (Steyn, 1984). These extraction procedures are liquid–liquid extraction or solid-phase extraction (Scott & Trucksess, 1997). Most methodologies use for clean-up, home-made silica gel cartridges or commercial available cartridges; other methodologies utilize immunoaffinity columns (Ferrufino-Guardia, Tangni, Larondelle, & Ponchaut, 2000; Skaug, Helland, Solvoll, & Saugstad, 2001). The aim of this work was to combine a simple extraction methodology with appropriate liquid chromatography conditions in order to have an easier and friendlier to the environment technique to determine OTA in milk at trace levels.

2. Materials and methods

2.1. Chemicals

OTA crystalline material was purchased from Sigma (St. Louis, MO, USA). A stock standard solution of OTA at 500 μg mL^{-1} in methanol was prepared and kept wrapped in aluminum foil at −20 °C, due to OTA gradually breaks down under UV light. OTA working solutions were prepared by diluting in the same solvent and stored in glass-stopped tubes at −20 °C.

High performance liquid chromatography (HPLC) grade acetonitrile, methanol and acetic acid were supplied by Merck (Darmstadt, Germany). Deionised water (0.125 μS) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Sampling

Milk samples were collected from different supermarkets in Valencia (Spain). All samples were stored in sealed plastic cups and kept at −20 °C until extraction and analysis by liquid chromatography.

2.3. Extraction and clean-up

The recovery (percentage of standard added to sample that is recovered after extraction and clean-up) of the extraction method was determined by sample fortification. Samples ($n = 5$) were fortified at a level of 0.02, 0.06 and 0.1 ng of OTA mL^{-1} of milk. OTA fortification solution was prepared in methanol and used for quantification of the analyte recovered after extraction.

A volume of 2 mL of methanol was added to a 2 mL portion of fortified milk in an assay tube. This solution was vortexed for one minute and then it was filtered through a nylon acrodisk (0.45 μm) under vacuum. The extract was then evaporated to dryness with N_2 at 45 °C. Once cooled, it was reconstituted in 100 μL of methanol and briefly sonicated before injection.

2.4. LC-FLD Determination

A Shimadzu (Kyoto, Japan) SCL-6A liquid chromatography system equipped with two LC-6A pumps, a Rheodyne Model 7125 injector (20 μL loop) and an RF-10AXL fluorescence detector were used. Chromatographic conditions are based upon previous work made by González-Osnaya, Soriano, Moltó, and Mañes (2006), these are as follows: a LC Phenomenex column Luna 5 μm C18 100 A (150 × 4.60 mm i.d.) with a mobile phase consisting of acetonitrile:water:acetic acid (50:49:1 v/v/v) at a flow rate of 0.4 mL min^{-1}. Detection of OTA was carried out using 334 and 464 nm as wavelengths for excitation and emission, respectively.

2.5. Confirmation procedure

In case of finding a naturally contaminated sample, the identity of OTA was to be confirmed by methyl ester formation according to Zimmerli and Dick (1995). Briefly, this technique consists in adding 2.5 mL of methanol and 0.1 mL of concentrated hydrochloric acid to 200 μL of OTA residue. The vial is closed and kept overnight at room temperature. The reaction mixture is evaporated to dryness and the residue re-dissolved in mobile phase (see LC-FLD determination). Then 20 μL are analyzed using liquid chromatography coupled on fluores-

cence detection with the same conditions as in OTA analysis.

3. Results and discussion

3.1. Extraction and clean-up

As outlined in the introduction, analytical methods for OTA determination in milk involve an extraction step with several organic solvents, often halogenated, and the presence of a strong acid. Previous studies on analysis of OTA made by our group (González-Osnaya et al., 2006; Juan, González, Soriano, Moltó, & Mañes, 2005), has demonstrated that the use of methanol as extraction solvent for OTA offers high recoveries, lower cost and also a more environmental friendly technique.

The extraction methodology proposed is short and easily performed. When this procedure was applied to some of the samples, the filtration step was difficult as the filter clogged, therefore, an extra step had to be incorporated for further clean-up; centrifugation at 2880*g*, 16 °C during 20 min highly improved filtration.

The use of an alcohol for the extraction leads to aggregation of the casein micelles by dehydration, therefore structures of average sizes as large as 9 μm are precipitated (Agboola & Dalgleish, 1996; Huppertz, Fox, & Kelly, 2004); with the separation of these particles, by filtration and/or centrifugation, cleaner extracts are obtained. In the cases of whole and low fat milk, after centrifugation upper cream layers were completely discarded. This latter clean-up step did not compromise recovery of the analyte.

The experimental results obtained with this protocol show that the use of cartridges or other more expensive clean-up options as immunoaffinity columns may be avoided. Some impurities may remain in the extract, but as it will be discussed further, they do not interfere with the analysis.

3.2. Chromatography

Most methods for the analysis of OTA are based on LC-FLD with and acidic aqueous mobile phase mixed either with methanol or acetonitrile; because of the chemical stability of silica gel-based columns, the acidic system is favored (Tafuri, Ferracane, & Ritieni, 2004; Ueno et al., 1998; Zimmerli & Dick, 1995). In this way, the chromatography of OTA is not awkward and the retention time is not very sensible to slight changes in the composition of the eluent. Under the chromatographic conditions studied, OTA has a retention time of 22.8 min which allows not having any interfering peaks near the peak of OTA. The day-to-day variation of the retention time for OTA was 1.4%. As it can be seen in Fig. 1a and b, at the time OTA is retained, all impurities that may remain in the extract are long gone; therefore OTA may be accurately quantified. At the end of each day of analysis, mobile phase was run by the entire chromatographic system during two hours for cleansing. Due to the absence of a certified standard reference material, the accuracy (trueness) of the results was not determined.

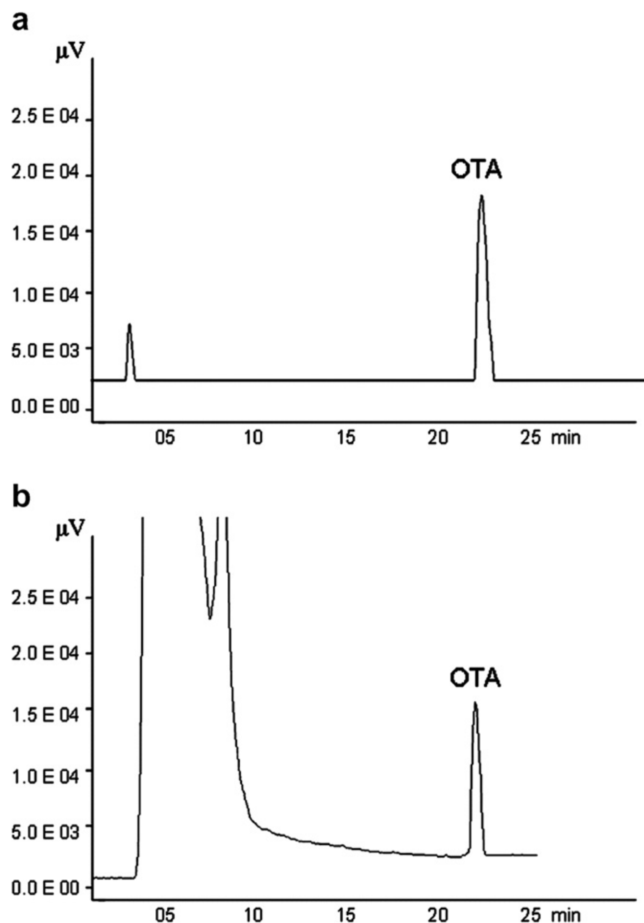

Fig. 1. LC-FLD chromatograms: (a) fortification solution 1.2 ng mL^{-1} of OTA and (b) milk sample spiked at 0.06 ng mL^{-1} of OTA.

3.3. Method performance parameters

With the described system a detection limit (3σ criterion) (Wennrich, Popp, & Moder, 2000) of 0.01 ng mL^{-1} was achieved. This detection value is in agreement with the rest of the proposed methodologies for the extraction of OTA from milk.

The mean recovery of OTA from bovine milk at different concentration values may be seen in Table 1. The analytical work was conducted on three different days in triplicate to detect any day-to-day effects. Certification exercises on several mycotoxins indicate the possibility that

Table 1
Mean recovery of OTA from bovine milk

OTA fortification level (ng mL^{-1})	Recovery ± RSD[a] (%)
0.02	91.6±4.0
0.06	90.8±6.9
0.1	93.0±7.4

[a] Relative Standard Deviation.

the variation (inter- and intraday) values for duplicate recovery experiments do not exceed 15% (Hald, Wood, Boenke, Schurer, & Finglas, 1993). At a fortification level of 0.1 $ng\,mL^{-1}$ of OTA, an interday variation of 6.2% and an intraday variation of 4.7% were obtained. The value obtained for relative standard deviation of the method developed is in agreement with the Commission Directive 2002/26/EC for methods of analysis of OTA in foodstuffs (EC, 2002). According to these results it can be concluded that the analytical work conducted fulfills these requirements.

3.4. Application to samples

The method developed has been used on bovine milk purchased in different stores and supermarkets ($n = 61$). These samples included whole ($n = 16$), low fat ($n = 20$) and fat-free milk ($n = 12$), also imitation or substitute milk was included ($n = 13$); these last samples comprised soy, almond, oat, wheat and rice beverages; which are labeled and commercialized as soy milk, rice milk, etc.

None of the samples contained OTA above the detection level. However, small amounts of OTA in milk are of importance for consumers of large quantities of milk, due to low concentrations of OTA can contribute to a significant portion of the total intake of the toxin. In Norway and Sweden, OTA was detected in 12% and 14% of the samples, respectively (range 10–58 $ng\,L^{-1}$) (Breitholtzemanuelsson, Olsen, Oskarsson, Palminger, & Hult, 1993; Skaug, 1999). In Germany, OTA was not detected in any of the 121 samples analyzed (Valenta & Goll, 1996). No OTA contamination is desirable, a trace may be acceptable but given what is so far known about the adverse properties of OTA; screening test should ensure reliable and cost effective food control as a preventive strategy.

4. Conclusion

The methodology developed in this work allows having good OTA recovery and reproducibility, it does not require a large quantity of solvent and for its simplicity and rapidness it is a good alternative that allows performing analysis of OTA in milk with good precision and accuracy at relatively lower cost.

Acknowledgments

This work has been supported by the Spanish Ministry of Education and Science (AGL-2006-04438). L. González-Osnaya thanks the grant given by CONACyT of México (Consejo Nacional de Ciencia y Tecnología).

References

Agboola, S. O., & Dalgleish, D. G. (1996). Enzymatic hydrolysis of milk proteins used for emulsion formation. 1. Kinetics of protein breakdown and storage stability of the emulsions. *Journal of Agricultural and Food Chemistry, 44*(11), 3631–3636.

Breitholtzemanuelsson, A., Olsen, M., Oskarsson, A., Palminger, I., & Hult, K. (1993). Ochratoxin-A in cows milk and in human-milk with corresponding human blood-samples. *Journal of AOAC International, 76*(4), 842–846.

EC (2002). Commission Directive 2002/26/CE of 13 March 2002. Official Journal of the European Communities. No. L 075 of 16/03/2002, pp. 0038–0043.

Eskola, M., Parikka, P., & Rizzo, A. (2001). Trichothecenes, ochratoxin A and zearalenone contamination and *Fusarium* infection in Finnish cereal samples in 1998. *Food Additives and Contaminants, 18*(8), 707–718.

Ferrufino-Guardia, E. V., Tangni, E. K., Larondelle, Y., & Ponchaut, S. (2000). Transfer of ochratoxin A during lactation: Exposure of suckling via the milk of rabbit does fed a naturally contaminated feed. *Food Additives and Contaminants, 17*(2), 167–175.

Gareis, M., & Wolff, J. (2000). Relevance of mycotoxin contaminated feed for farming animals and carry over of mycotoxins in food of animal origin. *Mycoses, 43*, 79–83.

González-Osnaya, L., Soriano, J. M., Moltó, J. C., & Mañes, J. (2006). Extraction and analysis of ochratoxin A in bread using pressurised liquid extraction and liquid chromatography. *Journal of Chromatography A, 1113*(1–2), 32–36.

Guerre, P., Bailly, J. D., Benard, G., & Burgar, V. (2000). Milk excretion of the mycotoxins: Which risks for the consumer. *Revue de Medecine Veterinaire, 151*(1), 7–22.

Hald, B., Wood, G. M., Boenke, A., Schurer, B., & Finglas, P. (1993). Ochratoxin-A in wheat—An intercomparison of procedures. *Food Additives and Contaminants, 10*(2), 185–207.

Hult, K., Teiling, A., & Gatenbeck, S. (1976). Degradation of ochratoxin-A by a ruminant. *Applied and Environmental Microbiology, 32*(3), 443–444.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004). Properties of casein micelles in high pressure-treated bovine milk. *Food Chemistry, 87*(1), 103–110.

IARC (1993). Some naturally occurring substances: items and constituents, heterocyclic aromatic amines and mycotoxins. *Monographs on the evaluation of carcinogenic risks to humans*. Lyon, France: International Agency for Research on Cancer.

Juan, C., González, L., Soriano, J. M., Moltó, J. C., & Mañes, J. (2005). Accelerated solvent extraction of ochratoxin A from rice samples. *Journal of Agricultural and Food Chemistry, 53*(24), 9348–9351.

Kiessling, K. H., Pettersson, H., Sandholm, K., & Olsen, M. (1984). Metabolism of aflatoxin, ochratoxin, zearalenone, and 3 trichothecenes by intact rumen fluid, rumen protozoa, and rumen bacteria. *Applied and Environmental Microbiology, 47*(5), 1070–1073.

KuiperGoodman, T. (1996). Risk assessment of ochratoxin A: An update. *Food Additives and Contaminants, 13*, 53–57.

Mantle, P. G. (2002). Risk assessment and the importance of ochratoxins. *International Biodeterioration & Biodegradation, 50*(3–4), 143–146.

Miraglia, M., De Santis, B., Grossi, S., & Brera, C. (2004). Mycotoxins. In M. L. Nollet (Ed.), *Handbook of food analysis* (pp. 85–93). Ghent, Belgium: Spring.

Monaci, L., Tantillo, G., & Palmisano, F. (2004). Determination of ochratoxin A in pig tissues by liquid-liquid extraction and clean-up and high-performance liquid chromatography. *Analytical and Bioanalytical Chemistry, 378*(7), 1777–1782.

Scott, P. M., & Trucksess, M. W. (1997). Application of immunoaffinity columns to mycotoxin analysis. *Journal of AOAC International, 80*(5), 941–949.

Skaug, M. A. (1999). Analysis of Norwegian milk and infant formulas for ochratoxin A. *Food Additives and Contaminants, 16*(2), 75–78.

Skaug, M. A., Helland, I., Solvoll, K., & Saugstad, O. (2001). Presence of ochratoxin A in human milk in relation to dietary intake. *Food Additives and Contaminants, 18*(4), 321–327.

Steyn, P. S. (1984). Ochratoxins and related dihydro-isocoumarins. In V. Betina (Ed.), *Mycotoxins: Production, Isolation, Separation, and Purification* (pp. 183–190). New York: Elsevier Science.

Tafuri, A., Ferracane, R., & Ritieni, A. (2004). Ochratoxin A in Italian marketed cocoa products. *Food Chemistry, 88*(4), 487–494.

Ueno, Y., Maki, S., Lin, J., Furuya, M., Sugiura, Y., & Kawamura, O. (1998). A 4-year study of plasma ochratoxin A in a selected population in Tokyo by immunoassay and immunoaffinity column-linked HPLC. *Food and Chemical Toxicology, 36*(5), 445–449.

Valenta, H., & Goll, M. (1996). Determination of ochratoxin A in regional samples of cow's milk from Germany. *Food Additives and Contaminants, 13*(6), 669–676.

Van der Merwe, K., Steyn, P., Fourie, L., Scott, D., & Theron, J. (1965). Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh.K. *Nature*, 1112–1113.

Visconti, A., Pascale, M., & Centonze, G. (1999). Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography. *Journal of Chromatography A, 864*(1), 89–101.

Vrabcheva, T., Petkova-Bocharova, T., Grosso, F., Nikolov, I., Chernozemsky, N., Castegnaro, M., et al. (2004). Analysis of ochratoxin A in foods consumed by inhabitants from an area with Balkan Endemic Nephropathy: A 1 month follow-up study. *Journal of Agricultural and Food Chemistry, 52*(8), 2404–2410.

Wennrich, L., Popp, P., & Moder, M. (2000). Determination of chlorophenols in soils using accelerated solvent extraction combined with solid-phase microextraction. *Analytical Chemistry, 72*(3), 546–551.

Zimmerli, B., & Dick, R. (1995). Determination of ochratoxin-A at the ppt level in human blood, serum, milk and some foodstuffs by high-performance liquid-chromatography with enhanced fluorescence detection and immunoaffinity column cleanup – methodology and Swiss data. *Journal of Chromatography B: Biomedical Applications, 666*(1), 85–99.