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# Detection and quantification of Ochratoxin A in milk produced in organic farms

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## Research highlights

Detection of Ochratoxin A with HPLC method. ► Safety of organic production. ► Mycotoxins in dairy products.

## Abstract

Dairy cows (as all other ruminants) possess physiological systems for mycotoxins' detoxification. However, in organic farming practices the detoxification could be impaired because of possible higher contamination of feedstuff, changes in rumen pH and other factors. So the organic milk could be at risk in this respect. The results of an investigation on the presence of Ochratoxin A (OTA) in 63 samples of organic and 20 samples of conventional milk are reported in this paper. The quantification was carried out by means of the HPLC method described by Sørensen and Elbæk (2005). The method has been modified in the purification step so as to shorten the time of analysis and lower the cost of the assay.

Three organic out of 63 "organic" samples resulted positive for OTA, with amounts ranging from 0.07 to 0.11 ppb.

Keywords: Milk; Ochratoxin A; Liquid chromatography; Organic farming; Mycotoxins.

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## 1. Introduction

Ochratoxin A (OTA;  $C_{20}H_{18}ClNO_6$ , molecular weight = 403.82 g/mol) is a mycotoxin produced by different species of *Aspergillus* (*A. ochraceus*, *A. melleus*, *A. sulphureus*, *Aspergillus* section *Nigri*, *A. carbonarius*, *A. awamori*) and *Penicillium* (*P. verrucosum*, *P. crysogenum* and *P. nordicum*) (Bayman & Baker, 2006; Magan, 2006; Zheng et al., 2005; Bayman and Baker, 2006; Magan, 2006, Zheng et al., 2005).

Its hepatotoxic, nephrotoxic and teratogenic effects are well documented (Boudra & Morgavi, 2006). OTA has been classified by the International Agency of Research in Cancer (IARC) as a carcinogenic of 2B class (Muscarella, Palermo, Rotunno, Quaranta, & D'Antini, 2004) and it seems also involved in the Balkan Endemic Nephropathy (BEN) and in the Chronic Interstitial Nephropathy (Bayman & Baker, 2006; Pena, Cerejo, Lino, & Silveira, 2005; Bayman and Baker, 2006; Pena et al., 2005).

Cereals and derivatives have been reported to be the main sources of OTA, even though it has been detected in various other food items as wine, coffee, beer and vegetables as beans and dried fruits (Battilani, Magan, & Logrieco, 2006; Gauchi & Leblanc, 2002; Monaci & Palmisano, 2004; Visconti, Pascale, & Centonze, 2000; Zheng et al., 2005; Battilani et al., 2006; Gauchi and Leblanc, 2002; Monaci and Palmisano, 2004; Visconti et al., 2000; Zheng et al., 2005). In animal products, such as poultry and pork meat, OTA can be present both because of direct moulds contamination and because of a carry-over from contaminated feed stuffs (Bayman & Baker, 2006; Monaci, Tantillo, & Palmisano, 2004; Valenta, Khun, & Rohr, 1993; Bayman and Baker, 2006; Monaci et al., 2004; Valenta et al., 1993). Traces of OTA have been also found in human milk (Mantle, 2002; Turconi et al., 2004; Mantle, 2002, Turconi et al., 2004). With regard to ruminants' milk, several studies have demonstrated that OTA can be hydrolysed by rumen microflora and rumen pH to a less toxic metabolite, the ochratoxin  $\alpha$  (Boudra & Morgavi, 2006; Sørensen & Elbæk, 2005; Boudra and Morgavi, 2006; Sørensen and Elbæk, 2005). So bovine milk should not be considered as an important source of OTA.

However, a 2002 report on "Assessment of dietary intake of Ochratoxin A by the population of EU member states" has focused the attention on the possible presence of OTA in milk (SCOOP, 2002). More recently, other authors have considered the same matter (Boudra & Morgavi, 2006; Sørensen & Elbæk, 2005; Boudra and Morgavi, 2006; Sørensen and Elbæk, 2005).

In conventional husbandry, 75% of the ration consists of concentrates and silage. In organic farming, on the contrary, more than 50% of the ration is represented by hay, pasture and root crops (Lund & Algers, 2003). This difference in the amount of available energy leads to a lower protozoa density. Since protozoa are partially responsible for the OTA degradation to the less toxic metabolite ochratoxin  $\alpha$  (Özpinar, Augonyte, & Drochner, 1999), it could be reasonably supposed that this process is impaired in case of an "organic" diet. Similarly, such a diet, when fed to dairy cows with high genetic potential for milk production could induce a lowering of rumen pH which, in its turn, may also influence the rate of OTA degradation (Hovi, Sundrum, & Thamborg, 2003; Sundrum, 2001, Hovi et al., 2003; Sundrum, 2001).

On the other hand, the concern for the dangerousness of feedstuff parasited by toxin-producing moulds accounts for the use of fungicide additives during their manufacturing. Such a practice is not allowed for cereals and meals used in "organic" farms; as a consequence, the presence of toxin-producers *Aspergillus* spp. and *Penicillium* spp. in organic food could be reasonably supposed (Hoogenboom et al., 2008). Pfol-Leszkowicz and Manderville (2007) compared the OTA content in crops from organic farms, in which no pesticides or fungicides were used, with that in crops from

traditional farms finding a considerably higher contamination (up to 5 times more) in organic feedstuff.

For the detection and quantification of OTA, in addition to methods such as ELISA (Mattarella, Monaci, Milillo, Palmisano, & Tantillo, 2006; Zheng et al., 2005; Mattarella et al., 2006; Zheng et al., 2005) or tandem mass spectrometry (Boudra & Morgavi, 2006; Lindenmeier, Schieberle, & Rychlik, 2004; Pena et al., 2005; Boudra and Morgavi, 2006; Lindenmeier et al., 2004; Pena et al., 2005), HPLC with fluorescence detection is extensively applied (Boudra & Morgavi, 2006; Monaci & Palmisano, 2004; Monaci et al., 2004; Boudra and Morgavi, 2006; Monaci and Palmisano, 2004; Monaci et al., 2004).

The confirmation of the presence of OTA could be done by the aid of LC–MS–MS, or HPLC after methylation process (González-Osnaya, Soriano, Moltó, & Mañes, 2008; Monaci & Palmisano, 2004; Monaci et al., 2004; Pena et al., 2005; González-Osnaya et al., 2008; Monaci and Palmisano, 2004, Monaci et al., 2004; Pena et al., 2005).

The aim of this work has been the evaluation of the possible presence of OTA in milk sample coming from organic farms. The OTA quantification has been carried applying a HPLC assay (Sørensen & Elbæk, 2005) with some modifications in the extraction and purification steps.

## 2. Materials and methods

### 2.1. Samples

63 Samples (39 bovine, 15 goats and 9 sheep) coming from organic farms, as defined by the Reg. EEC 2092/1991 and following supplements and 20 samples of bovine milk not labelled as “organic” from the retail market were analysed.

### 2.2. Reagents

The OTA standard was purchased from Supelco (Bellefonte, PA, USA) and stored at  $-40.0\text{ }^{\circ}\text{C}$ . The OTA working solutions in methanol were prepared weekly and stored at  $-40.0\text{ }^{\circ}\text{C}$  too. All the solvents as acetonitrile, acetic acid, methanol and hexane were HPLC grade (Merck, Darmstadt, Germany). The water for the mobile phase was produced by a MilliQ (Millipore, Billerica, MA, USA) and the reagent for the methylation step was  $\text{BF}_3$  (Merck, Darmstadt, Germany).

### 2.3. Extraction of OTA in milk samples

All glassware was washed with methanol in order to avoid a loss of OTA from solvents by salt formation, precipitation and/or adsorption to glassware (Pena et al., 2005). A volume of 2.5 mL milk was mixed with 20  $\mu\text{L}$  of sulphuric acid 18% (v/v) to obtain a pH of  $2.0 \pm 0.5$ . Then the defatting was carried out by adding 10 mL of hexane to 2.5 mL of milk and mixed for 1 min. After centrifugation at 4000 rpm  $\times$  15 min at room temperature the hexane phase was removed. A volume of 5 mL acetonitrile was added to the milk and shaken horizontally for 30 min (600 strokes/min). Acetonitrile was filtered through regenerate cellulose filters – pore size 0.45  $\mu\text{m}$  – (Chemtek, Anzola Emilia, Italy). Both the tube and the filter were washed twice with 1 mL of acetonitrile each time. A volume of 1.5 mL of the extract was evaporated to dryness at  $60\text{ }^{\circ}\text{C}$  and stored until analysis, when 300  $\mu\text{L}$  mobile phase were added.

### 2.4. Chromatographic conditions

The HPLC apparatus consisted of a Merck-Hitachi L-7100 pump equipped with a L-7614 vacuum membrane degasser (Merck, Darmstadt, Germany), a Rheodyne 7725i injection valve fitted with a 50  $\mu$ L loop and connected to a Merck PuroSpher Star RP-18 endcapped 250 $\times$ 4 mm $\times$ 5  $\mu$ m (Merck, Darmstadt, Germany).

The Fluorescence detector was a Merck-Hitachi L-7480 (Merck, Darmstadt, Germany); fluorescence excitation and emission wavelengths were 333 nm and 460 nm, respectively. The mobile phase was composed of: H<sub>2</sub>O:acetonitrile:acetic acid (49.5:49.5:1), flow rate 1.0 mL/min (Visconti et al., 2000).

### 2.5. Confirmation of OTA by methyl ester formation

For confirmation, OTA was converted into its methyl ester. The extracts were evaporated to dryness 70  $\mu$ L BF<sub>3</sub> (20% methanolic solution) were added, and the mixture was heated at 80 C for 15 min. After evaporation the residue was dissolved in 300  $\mu$ L of mobile phase and 50  $\mu$ L were injected (Monaci et al., 2004).

### 3. Results and discussion

To evaluate the OTA in milk samples considered in the present paper, the method described by Sørensen and Elbæk (2005) (defatting with hexane and extraction with acetonitrile) has been initially applied. In a second time, to verify the absence of compounds potentially interfering with the retention time of OTA, a number of representative blank milk samples of different origin were analysed. These blank samples were obtained from farms which fed the animals with feedstuff carefully checked for the presence of OTA. No interferences were observed in the elution time of OTA. So, a subsequent purification in solid phase was considered unnecessary.

The recovery tests were repeated three times, each one with 7 replicates. The data are shown on Table 1. The recoveries were always more than 87% at 1 ppb spiked level and more than 83% at 5 ppb spiked level. Standard deviations and relative standard deviations were always less than 10%.

Table 1: OTA mean recovery from milk ( $n = 3$ ).

Milk	Recovery $\pm$ SD at spiking level 1 ppb (%)	RSD (%)	Recovery $\pm$ SD at spiking level 5 ppb (%)	RSD (%)
WM	0.876 $\pm$ 0.08 ppb (87.6 $\pm$ 8.3)	9.5	4.16 $\pm$ 0.22 ppb (83.2 $\pm$ 4.4)	5.2
PSM	0.91 $\pm$ 0.08 ppb (91.2 $\pm$ 8.7)	9.5	4.36 $\pm$ 0.36 ppb (87.3 $\pm$ 7.3)	8.3
SM	0.93 $\pm$ 0.06 ppb (92.7 $\pm$ 6.6)	7.2	4.29 $\pm$ 0.33 ppb (85.9 $\pm$ 6.7)	7.8

Legenda: each recovery test done with seven replicates and repeated three times ( $n$ ). SD, standard deviation; RDS, relative standard deviation; WM, whole milk; PSM, partially skimmed milk; SM, skimmed milk.

The calibration curve on 6 points was linear in the range considered (from 0.05 to 5 ppb) with  $r^2=0.9988$ . The limit of quantification (LOQ) was evaluated applying the method described by Boudra and Morgavi (2006) for the quantification of OTA in plasma and milk. This method consists in the addition of decreasing amounts of OTA to negative partially skimmed milk samples. LOQ in milk samples was 0.05 ppb.

After having tested the reliability of the procedure of extraction and quantification, 63 milk samples from organic farms were analysed. Only 3 out of 63 samples resulted positive for OTA, with amounts comprised between 0.07 and 0.11 ppb. This result was confirmed by the methyl ester formation step. No “non organic” sample resulted positive. The results are summarised in Table 2 together with the few data referred to some European Countries published in the 2002 Report “Assessment of dietary intake of Ochratoxin A by the population of EU member states” referred to Germany, Sweden and Norway, and in the paper of González-Osnaya et al. (2008).

Table 2: Positive samples of OTA contaminated milk.

Country	Number of samples	Positive samples	Percentage of positive samples (%)
Present study			
Organic milk	63	3	4.8
Conventional milk	20	0	0
Denmark <sup>a</sup>	42	0	0
Spain <sup>b</sup>	39	0	0
Norway <sup>b</sup>	36	4	12
Germany <sup>c</sup>	69	0	0
Norway <sup>c</sup>	165	13	7.9
Sweden <sup>c</sup>	36	5	13.8

a Sørensen and Elbæk (2005).

b González-Osnaya et al. (2008).

c SCOOP (2002).

A critical comparison between the data reported in this paper and the data of the literature is difficult since in many cases the origin of the samples is not known.

For what the data reported in this paper, the amount of OTA detected does not seem dangerous. In fact, assuming an average milk consumption for the adult of 1 L/week (data referred to Italy – CLAT, 2010), the Total Weekly Intake (TWI) of OTA would be nearly 1.52 ng/kg b.w. This intake is considerably lower than the maximum tolerable TWI established by the European Food Safety Authority (EFSA) which is 120 ng/kg b.w. (Caloni & Nebbia, 2009).

At the moment, no evaluation of the concentration factor of OTA when a contaminated milk is processed to make cheese is has been detected. Manetta et al. (2009) has calculated a concentration factor of 4.5 for Aflatoxin M<sub>1</sub> in hard cheese; considering the same factor for OTA, the presumable intake of the mycotoxin, assuming the consumption calculated by CLAT (2010) would be double than that calculated for milk. However also in this case, the intake would result considerably lower than that calculated by EFSA (Caloni & Nebbia, 2009).

The method described in this Paper allows to determine only free OTA and not OTA bound to glucuronic acid. On the other hand at the moment no evidence of glucuronide formation was found in cow’s milk (Monaci et al., 2004; Valenta & Goll, 1996; Valenta et al., 1993; Monaci et al., 2004, Valenta et al., 1993; Valenta and Goll, 1996).

#### 4. Conclusions

In spite of what one could presume, milk from organic farms, can sometimes contain detectable amounts of OTA. As pointed out in Section 1, several factors have been supposed to explain this presence: lower energy rate of the pasture and subsequent lower protozoan flora, changes in ruminal pH, presence of a significant mycotoxin contamination in feed or a sum of all these factors (Hovi et al., 2003; Lund & Algers, 2003; Pfol-Leszkowicz & Manderville, 2007; Özpınar et al., 1999; Hovi et al., 2003; Lund and Algers, 2003; Özpınar et al., 1999; Pfol-Leszkowicz and Manderville, 2007).

With regard to the samples examined, the data confirm that the contribution of organic milk to the intake of OTA in humans is negligible compared to other food items such as cereals, dried fruits, wine and coffee (Battilani et al., 2006; Gauchi & Leblanc, 2002; Zheng et al., 2005; Battilani et al., 2006; Gauchi and Leblanc, 2002; Zheng et al., 2005; Pena et al., 2005).

However, at the moment, few data are available but the possibility of a dangerous contamination shouldn't be neglected (González-Osnaya et al., 2008). It is clear the need for stricter surveillance of feedstuff control and food chain also in organic production.

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#### References

Battilani et al., 2006 Battilani P., Magan N., Logrieco A., European research on ochratoxin A in grapes and wine, *International Journal of Food Microbiology*, Volume: 111, (2006), pp. S2-S4

Bayman and Baker, 2006 Bayman P., Baker J.L., Ochratoxins: A global perspective, *Mycopathologia*, Volume: 162, (2006), pp. 215-223

Boudra and Morgavi, 2006 Boudra H., Morgavi D.P., Development and validation of a HPLC method for the quantitation of ochratoxins in plasma and raw milk, *Journal of Chromatography B*, Volume: 843, (2006), pp. 295-301

Caloni and Nebbia, 2009 Caloni F., Nebbia C., Micotossine, *Residui di farmaci e contaminanti ambientali nelle produzioni animali*, (2009), EdiSES, Napoli. pp. 453-480

Council Regulation, 1991 Council Regulation (EEC) No 2092/91 of June 1991 on organic production of agricultural products and indications referring thereto on agricultural products and foodstuffs. Official Journal L 198 22/07/1991: 1-15.

Gauchi and Leblanc, 2002 Gauchi J.P., Leblanc J.C., Quantitative assessment exposure to the mycotoxin Ochratoxin A in food, *Risk Analysis*, Volume: 2, Issue: 22 (2002), pp. 219-234

González-Osnaya et al., 2008 González-Osnaya L., Soriano J.M., Moltó J.C., Mañes J., Simple liquid chromatography assay for analyzing ochratoxin A in bovine milk, *Food Chemistry*, Volume: 108, (2008), pp. 272-276

Hoogenboom et al., 2008 Hoogenboom L.A.P., Bokhorst J.G., Northolt M.D., Van De Vijer L.P.L., Broex N.J.G., Mevius D.J., et al. Contaminants and microorganisms in Dutch organic food products: A comparison with conventional products, *Food Additives and Contaminants*, Volume: 25, Issue: 10 (2008), pp. 1195-1207

Hovi et al., 2003 Hovi M., Sundrum A., Thamborg S.M., Animal health and welfare in organic livestock production in Europe: Current state and future challenges, *Livestock Production Science*, Volume: 80, (2003), pp. 41-53

CLAT, 2010 CLAT [internet]. Available at < [http://www.clal.it/index.php?section=quadro\\_italia](http://www.clal.it/index.php?section=quadro_italia) >. Accessed 19.03.10.

Lindenmeier et al., 2004 Lindenmeier M., Schieberle P., Rychlik M., Quantification of ochratoxin A in foods by a stable isotope dilution assay using high-performance liquid chromatography–tandem mass spectrometry, *Journal of Chromatography A*, Volume: 1023, (2004), pp. 57-66

Lund and Algers, 2003 Lund V., Algers B., Research on animal health and welfare in organic farming – A literature review, *Livestock Production Science*, Volume: 80, (2003), pp. 55-68

Magan, 2006 Magan N., Mycotoxin contamination of food in Europe: Early detection and prevention strategies, *Mycopathologia*, Volume: 262, (2006), pp. 245-253

Manetta et al., 2009 Manetta C.A., Giammarco M., Di Giuseppe L., Fusaro I., Gramenzi A., Formigoni A., et al. Distribution of aflatoxin M<sub>1</sub> during Grana Padano cheese production from naturally contaminated milk, *Food Chemistry*, Volume: 113, (2009), pp. 595-599

Mantle, 2002 Mantle P.G., Risk assessment and the importance of ochratoxins, *International Biodeterioration & Biodegradation*, Volume: 50, (2002), pp. 143-146

Mattarella et al., 2006 Mattarella R., Monaci L., Milillo M.A., Palmisano F., Tantillo M.G., Ochratoxin A determination in paired kidneys and muscle samples from swine slaughtered in southern Italy, *Food Control*, Volume: 17, (2006), pp. 114-117

Monaci and Palmisano, 2004 Monaci L., Palmisano F., Determination of ochratoxin A in foods: State-of-art and analytical challenges, *Analytical and Bioanalytical Chemistry*, Volume: 378, (2004), pp. 96-103

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

Muscarella et al., 2004 Muscarella M., Palermo C., Rotunno T., Quaranta V., D'Antini P., Survey of Ochratoxin A in cereals from Puglia and Basilicata, *Veterinary Research Communications*, Volume: 28, (2004), pp. 229-232

Özpinar et al., 1999 Özpinar H., Augonyte G., Drochner W., Inactivation of ochratoxin in ruminal fluid with variation of pH-value and fermentation parameters in an in vitro system, *Environmental Toxicology and Pharmacology*, Volume: 7, (1999), pp. 1-9

Pena et al., 2005 Pena A., Cerejo F., Lino C., Silveira I., Determination of ochratoxin A in portuguese rice samples by high-performance liquid chromatography with fluorescence detection, *Analytical and Bioanalytical Chemistry*, Volume: 382, (2005), pp. 1288-1293

Pfol-Leszkowicz and Manderville, 2007 Pfol-Leszkowicz A., Manderville R.A., Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans, *Molecular Nutrition and Food Research*, Volume: 51, (2007), pp. 61-99

SCOOP, 2002 SCOOP, Task 3.2.7. (2002). Report of Experts participating in Task 3.2.7. Assessment of dietary intake of Ochratoxin A by the population of EU Member States < [http://ec.europa.eu/food/fs/scoop/3.2.7\\_en.pdf](http://ec.europa.eu/food/fs/scoop/3.2.7_en.pdf) >. Accessed 18.03.10.

Sørensen and Elbæk, 2005 Sørensen L.K., Elbæk T.H., Determination of mycotoxins in bovine milk by liquid chromatography tandem mass spectrometry, *Journal of Chromatography B*, Volume: 820, (2005), pp. 183-196

Sundrum, 2001 Sundrum A., Organic livestock farming. A critical review, *Livestock Production Science*, Volume: 67, (2001), pp. 207--215 Bibliographic Page Full text

Turconi et al., 2004 Turconi G., Guarcello M., Livieri C., Comizzoli S., Maccarini L., Castellazzi A.M., et al. Evaluation of xenobiotics in human milk and ingestion by the newborn, *European Journal of Nutrition*, Volume: 43, (2004), pp. 191-197

Valenta et al., 1993 Valenta H., Khun I., Rohr K., Determination of ochratoxin in urine and feces of swine by high-performance-liquid-chromatography, *Journal of Chromatography B*, Volume: 613, (1993), pp. 295-302

Valenta and Goll, 1996 Valenta H., Goll M., Determination of ochratoxin-A in regional samples of cow milk in German, *Food Additives and Contaminants*, Volume: 13, (1996), pp. 669-676

Visconti et al., 2000 Visconti A., Pascale M., Centonze G., Determination of Ochratoxin A in domestic and imported beers in Italy by immunoaffinity clean-up and liquid chromatography, *Journal of Chromatography A*, Volume: 888, (2000), pp. 21-26

Zheng et al., 2005 Zheng Z., Hanneken J., Houchins D., King R.S., Lee P., Richard J.L., Validation of an ELISA test kit for the detection of ochratoxin A in several food commodities by comparison with HPLC, *Mycopathologia*, Volume: 159, (2005), pp. 265-272