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Short communication

Determination of ochratoxin A at part-per-trillion level in Italian salami by immunoaffinity clean-up and high-performance liquid chromatography with fluorescence detection

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Abstract

A fast high-performance liquid chromatography method has been devised for the determination of ochratoxin A (OTA) in Italian *salami* in the low part-per-trillion (pg/g) level. The samples were extracted with ethyl acetate and purified by immunoaffinity column (IAC). The IAC eluate could be directly injected or previously concentrated 10-fold. Recovery at 0.5 and 1 ng/g was 77 ± 4%. The between-day coefficient of variation measured over 5 days on samples spiked at 1 ng/g was 8%. The developed method required a relatively small volume of non-halogenated organic solvent and the whole procedure was simpler and faster compared to other existing procedures. The limit of detection was 0.06 ng/g that could be even lowered using a preconcentration step. A total of 30 *salami* samples were analysed using this procedure; the most contaminated sample was found to have OTA concentration at 0.4 ng/g level.

Keywords: Ochratoxin A; Mycotoxins; Salami; HPLC; Immunoaffinity; Fluorescence detection

1. Introduction

Ochratoxin A (OTA) is a known nephrotoxic [1] and carcinogenic [2] secondary metabolite produced by several *Aspergillus* species and by *Penicillium verrucosum*. OTA occurrence in cereals (barley, wheat, maize, oats), green coffee, cocoa beans and spices is well documented [3–6]. Human exposure to OTA is a cause of concern; the dietary intake of OTA by the population of EU member states has been assessed [7] (SCOOP) and a provisional tolerable weekly intake of 100 ng/kg bw has been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [8].

Animals fed with contaminated feed can also accumulate OTA in their organs. The highest amounts are found in blood, followed by kidney, liver, muscle and fat. Among the farmed animals, pigs are known to be particularly sensitive to this mycotoxin showing a relatively high serum half-life [9]. Then, products of animal origin can also contribute to OTA intake in humans, since processing procedures such as heating and ripening as well as storage have no effects on the reduction of the OTA levels in meat products [10]. Although the OTA content in several commodities is regulated in most countries, meat and meat products have received less attention; Denmark has enforced the limits of 10 and 25 ng/g in pig kidney for the condemnation of viscera and entire carcass, respectively; a guideline value of 1 ng/g in pork meat and derived products has been recommended in Italy since 1999.

Analytical methods developed for OTA detection in animal tissues are generally based on high-performance liquid chromatography (HPLC) coupled with fluorescence detection (FD) [11], tandem mass spectrometry (MS–MS) [12,13] and ion-trap sequential mass spectrometry [14]. Solvent (typically chloroform) extraction is required prior to extract

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purification by liquid–liquid partition and/or immunoaffinity column. Immunoaffinity clean-up possesses a very high selectivity (OTA is specifically recognised and bound to the immobilised antibody) and requires a small amount of non chlorinated solvent (typically 2–5 mL methanol) for elution [15,16]; however recovery values (43–68%) for meat products are typically lower than those observed for liquid samples (wine or beer).

A HPLC-FD procedure, for OTA determination in ham, based on immunoaffinity column (IAC) clean-up gave an average recovery of (74.8 ± 2.6%) and a detection limit of 0.04 ng/g was claimed starting from 25 g sample size [17]. Lower detection limits (0.01 ng/g) were obtained by Gareis and Scheuer [18] but their long procedure employed a large quantity of halogenated organic solvent for both OTA extraction (ca. 80 mL of $CHCl_3$) and extract purification (by partition with CH_2Cl_2) after clean up by IAC. Recovery was highly dependent on the analysed product varying from (37 ± 15%) to (88 ± 10%) as measured on spiked samples at 0.1 ng/g level. $CHCl_3$ extraction, followed by IAC clean-up was used for OTA determination in blood sausages with detection limits of 0.2 ng/g [19]. Pig liver derived pates required blending with 60% acetonitrile followed by hexane defatting and extract clean-up by solid-phase extraction (SPE) [20].

Very few data are available on the occurrence of OTA in meat derived products such as raw sausages known in Italy as *salami*. Italian salami composition varies from region to region and they are typically prepared from pork meat although a mix of lamb, pork and poultry meats can be found. Furthermore, the ripening process could favour growth of, e.g. *P. verrucosum* species, a potential producer of ochratoxin A.

The present paper aims at the development of a sensitive analytical procedure to detect traces of OTA in the low ppt range in salami samples. A total of 30 medium ripened salami samples were analysed using the present method.

2. Experimental

2.1. Apparatus and chromatographic conditions

The HPLC apparatus consisted of a Dionex P680 LPG pump equipped with a vacuum membrane degasser, a Rheodyne 7125 injection valve fitted with a 50 μL loop and a Supelcosil LC-18 DB (150 mm × 4.6 mm, 3 μm packing) chromatographic column. The fluorescence detector was a Jasco model FP-2020 Plus; fluorescence excitation and emission wavelengths were 334 and 460 nm, respectively. A mobile phase composed of H_2O/ACN/CH_3COOH (99/99/2, v/v/v) was used at a flow rate of 0.5 mL/min.

2.2. Chemicals

OTA was obtained from Sigma (St. Louis, MO, USA). Stock solutions were prepared in methanol at approximately 1 mg/ml; the actual concentration was calculated by UV spectrophotometry assuming a molar absorption coefficient of 6640 M^{-1} cm^{-1} at 333 nm [21]. Stock solution stored at −20 °C are stable for several years [22]. Working solutions were prepared by diluting stock solutions with mobile phase before use.

All the other chemicals used were of analytical grade.

Ochraprep (Rhone Diagnostic) IAC columns were obtained from OR-SELL (Carpi, Italy).

2.3. Sample preparation and immunoaffinity clean-up

A total of 30 salami specimens were collected from different local retailers. The procedure followed was a modification of the method developed by Monaci et al. [23]. Briefly, after addition of 6 mL of 1 M H_3PO_4 to the sample (10 g) it was homogenized in an Ultra Turrax T25 homogenizer for few minutes. An aliquot of 3 g was withdrawn from the homogenate and extracted twice with 5 mL of ethyl acetate containing 0.5 mol/L NaCl, the organic phases were combined and reduced to approximately 5 mL prior to back-extraction with 5 mL of 0.5 M $NaHCO_3$, pH 8.0. The aqueous extract was loaded onto a Ochraprep column and, after a washing step with 20 mL of water, the mycotoxin was eluted with 2.5 mL of a methanol/acetic acid (98/2, v/v) mixture. The eluate was evaporated to dryness under N_2 stream, the residue reconstituted in 250 μL of mobile phase and 50 μL injected on column.

Spiked samples were prepared by adding an appropriate volume of methanolic solution of the toxin to the homogenised tissue samples that were left, for at least 2 h, at room temperature in order to allow equilibration.

To study the precision of the method, OTA fortified (1 ng/g) samples, were analysed in triplicate for 5 days.

3. Results and discussion

Compared to our previous report [23], the detection capability offered by the present instrumental set-up is significantly improved; on column injection of 2.5 pg of OTA standard provided a signal-to-noise ratio > 15:1. This should imply that OTA at part-per-trillion (ppt) levels in *salami* samples could be detected provided a very effective extraction and clean-up procedure is devised.

As already reported, OTA shows a strong binding to proteins at neutral or alkaline pH that appears weakened with increasing ionic strength of the extraction solution and/or adjusting the pH to less than 2.0 [21]. In contrast to a previous method [18], in this work a shortened analytical procedure employing a less toxic solvent and immunoaffinity column clean up has been developed. Indeed, the choice of the less toxic ethyl acetate as extraction solvent, followed by back extraction into $NaHCO_3$, pH 8 solution has been investigated. Addition of 0.5 mol/L NaCl to ethyl acetate was found to further improve OTA extraction from

the investigated samples probably due to the higher ionic strength.

Immunoaffinity column with monoclonal antibodies, capable of recognizing OTA as specific antigen, was chosen for extract purification. This step contributes significantly to the high specificity offered by the whole analytical procedure (see later). A washing step of the IAC column with 20 mL of water was performed before elution of the toxin from the IAC column using 2.5 mL of a methanol/acetic acid mixture (98/2, v/v). The IAC washing/elution step gave a nearly quantitative recovery of (97 ± 2%), as measured using a standard solution of OTA in $NaHCO_3$, demonstrating that this step is not responsible for significant analyte loss. For the assessment of compliance to the Italian guideline value of 1 ng/g, no enrichment step was required; 50 μL of the eluate collected from the IAC column was directly injected and analysed. However, if required, the IAC column eluate could be enriched 10-fold by evaporation to dryness and reconstitution in 250 μL of mobile phase; this step did not significantly alter the recovery (see below).

The recovery of the overall procedure, as evaluated on four different salami samples spiked with 0.5 and 1 ng/g of OTA, was satisfactorily high: (77 ± 4%). Note that all the analysed samples had a fat content ranging between 35 and 47%; samples with higher fat content could not be extracted without a defatting step and were not considered in the present study.

A calibration curve was obtained spiking homogenised blank samples with OTA covering the range from 0.05 to 10 ng/g; 50 μL of the IAC column eluate was directly injected without any preconcentration step. The calibration curve was described by the following equation: peak area (a.u.) = (1.9 ± 5) + (217 ± 8)*C*, $R = 0.996$ where peak area was in arbitrary units and *C* was expressed as ng/g of the homogenate. The limit of detection (LOD) and the limit of quantitation (LOQ), calculated as 3- and 10-fold the standard deviation of the intercept, were 60 and 220 pg/g, respectively. The between-day coefficient of variation measured on samples spiked at 1 ng/g in the conditions described above was 8% (three replicates per day for 5 days).

The developed procedure is more than adequate for assessing compliance with the Italian guideline value of 1 ng/g. However, introducing the preconcentation step of the eluate from the IAC a further lowering of the detection/quantitation limits could be obtained. A chromatogram relevant to a 10-fold preconcentrated extract of a salami sample spiked with 20 pg/g is reported in Fig. 1. As can be seen, the detection capability is essentially limited by the chemical noise originating from matrix complexity that is further enhanced by the preconcentration step. OTA eluted in a reasonably noise-free region of the chromatogram and even considering the noise in the time window from 9 to 12 min, a signal-to-noise ratio still higher than 10 was obtained; then a LOD (at S/N = 3) of ca. 6 pg/g could be estimated.

The described procedure was applied to the screening of 30 pork raw sausages collected from local retailers in Southern Italy. None of the analysed samples was found contaminated at levels higher than the Italian guideline of 1 ng/g as shown in Table 1.

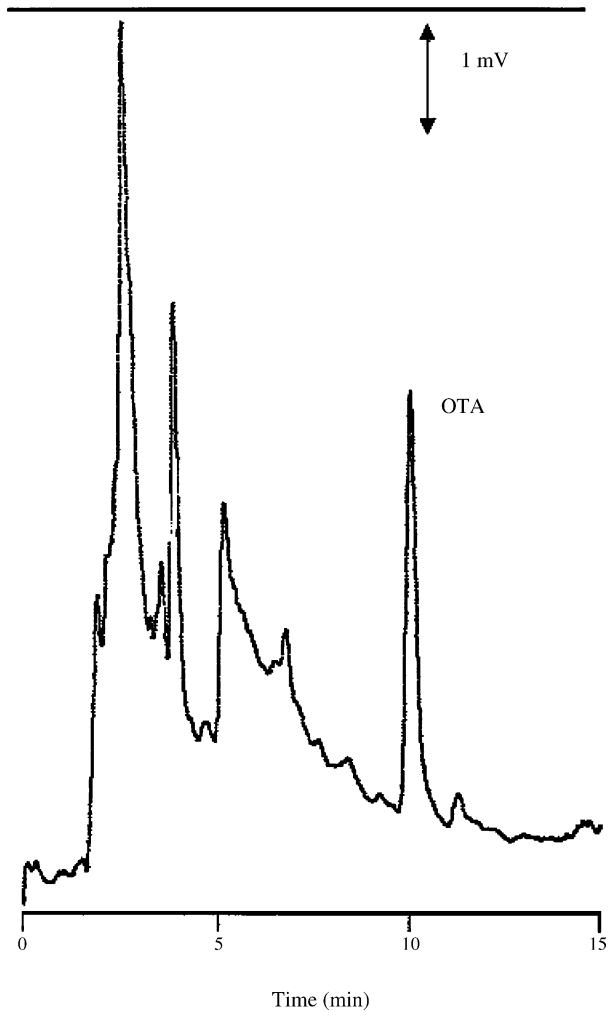

Fig. 1. Chromatogram obtained on the IAC purified extract of a *salami* sample spiked with OTA at 20 pg/g level. The IAC eluate was preconcentrated by a 10-fold factor as described in Section 2. For chromatographic and detection conditions see text.

One sample was found naturally contaminated at a concentration of 0.4 ng/g (see Fig. 2 for the relevant chromatogram) which was the highest value observed. Forty-three percent of the analysed samples were found contaminated with OTA at levels between 60 and 400 pg/g.

Table 1
Summary of results obtained on a survey of 30 salami samples

Range of contamination (ng/g)	Number of samples	Median (ng/g)	75th percentile (ng/g)
<LOD	16	–	–
0.006–0.06	9	0.011	0.02
0.06–1	5	0.08	0.16
>1	0	–	–

Table 2
Survey of HPLC-FD for OTA determination in meat derived products

Matrix (sample size)	Extraction	Clean-up	LOD (ng/g)	Recovery (%)	Reference
Meat products[a] (25 g)	$CHCl_3$ (80 mL) extraction; back-extraction in $NaHCO_3$ (eluate back extracted in CH_2Cl_2)	IAC	0.01	37–88	[18]
Ham (25 g)	100 mL (CH_3OH–1% $NaHCO_3$) extraction	IAC	0.04	75 ± 3	[17]
Blood sausages	H_3PO_4–$CHCl_3$	IAC	0.2	74 ± 6	[19]
Pig liver derived pates (10 g)	Blending in 60% acetonitrile (20 mL) defatting with hexane (10 mL)	SPE	0.56	85.7	[20]
Salami (3 g)	Ethyl acetate (10 mL); back extraction in $NaHCO_3$	IAC Eluate volume 10-fold reduced	0.06[b] 0.006[c]	77 ± 4	present work

[a] Pork, pig kidney, pig liver, raw sausages, bologna sausage, blood sausage, liver sausage.
[b] Evaluated at three times the standard deviation of the intercept of the calibration line.
[c] Evaluated at S/N = 3 from a chromatogram of a sample spiked at 20 pg/g level.

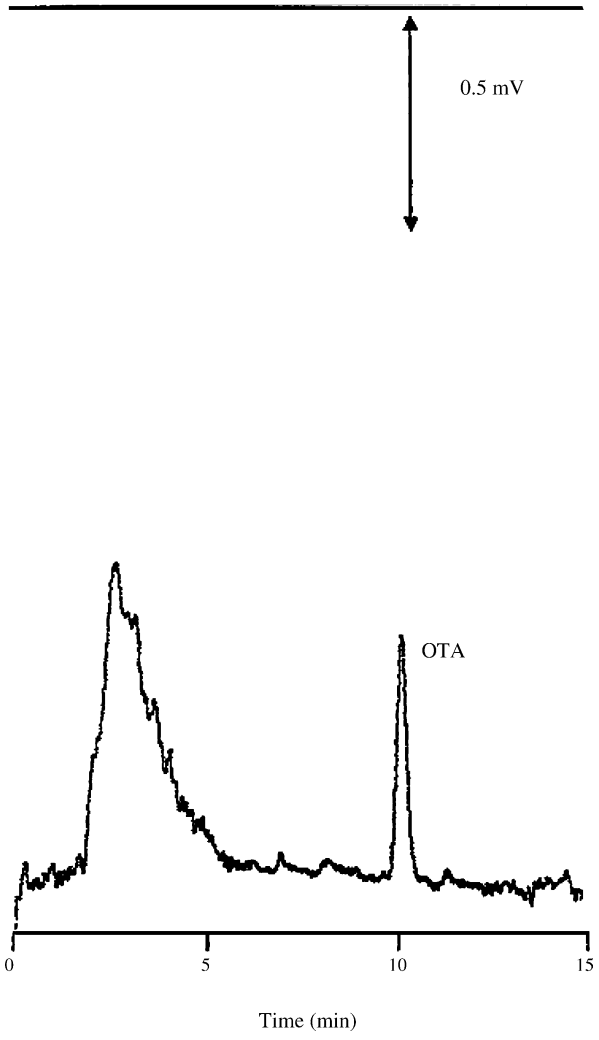

Fig. 2. Chromatogram obtained on the IAC purified extract of a *salami* sample found naturally contaminated with OTA at 0.4 ng/g level.

4. Conclusions

In this paper an improved highly sensitive analytical method for OTA determination in pork sausages, is presented. Compared to other existing methods (see Table 2) the present procedure requires only a reduced volume of non-chlorinated solvent and provides a remarkably low LOD of 0.06 ng/g that can be lowered by a 10-fold factor by a sample preconcentration step of the IAC eluate.

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