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Enzyme-assisted extraction for the HPLC determination of ochratoxin A in pork and dry-cured ham

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The extraction of ochratoxin A from meat products is generally carried out using chlorinated organic solvents, such as chloroform or methyl chloride, acidified with hydrochloric or *o*-phosphoric acid. In this study, an innovative method was developed to extract ochratoxin A from pork and dry-cured ham samples. The method was based on an enzyme-assisted extraction with pancreatin in phosphate buffer pH 7.5. Pancreatin hydrolyses the proteins, so that ochratoxin A, kept in the ionised form, is easily extracted by the aqueous solution. After purification through an immunoaffinity column, ochratoxin A is determined by HPLC with fluorescence detection. The average recovery values were higher than 90.0% and the relative standard deviations were below 5.5%. The limits of detection and of quantification were 0.06 and 0.12 $\mu g\,kg^{-1}$, respectively. A comparison between the new enzyme-assisted extraction and an established chloroform method was carried out on six naturally contaminated samples of pork and on 40 samples of dry-cured ham. Significantly higher ($p < 0.001$) values of ochratoxin A were obtained on dry-cured ham samples by the enzyme-assisted method.

Keywords: animal products – meat; mycotoxins – ochratoxin A; extraction; chromatography – HPLC

Introduction

Ochratoxin A (OTA) is a mycotoxin produced by various *Aspergillus* and *Penicillium* species. Several studies have shown that the toxin has carcinogenic, nephrotoxic, immunotoxic, teratogenic, and possibly neurotoxic and genotoxic properties and it has also been associated with Balkan Endemic Nephropathy and the development of urinary tract tumours in humans (Marquardt and Frohlich 1992; Pleština 1996; Schlatter et al. 1996). OTA has been classified by the International Agency for Research on Cancer (IARC) into Group 2B as a possible human carcinogen (IARC 1993). OTA contaminates many foods, such as cereals and derived products, dried fruit, coffee, cocoa, some spices, liquorice, wine, grape juices, beer and ripened pork products (Zimmerli and Dick 1996; Benford et al. 2001; Thirumala-Devi et al. 2001; Dall'Asta et al. 2010; Pietri et al. 2010). Concerning European Union legislation for human consumption, the European Commission fixed, in Regulations (EC) 1881/2006 and 105/2010 (Commission of the European Communities 2006a, 2010), maximum admissible levels for OTA in several foodstuffs and stated that, on the basis of the position adopted by the European Food Safety Authority (EFSA) (2004), it does not appear necessary for the protection of public health to set a maximum level of OTA in dried fruit other than dried vine fruit, cocoa, liqueur wines and meat products. In Italy, a guideline value of 1 $\mu g\,kg^{-1}$ in pork meat and derived products has been recommended by the Italian Ministry of Health since 1999 (Ministero della Sanità 1999). OTA can occur in meat and meat products as a result both of indirect transmission from animals exposed to naturally contaminated feed, and of direct contamination produced by moulds or by naturally contaminated spice mixtures used as ingredients (Gareis 1996). Among farm animals, the risk is limited to monogastric species, because ruminants can hydrolyse the amidic bond of OTA into phenylalanine and ochratoxin α, which is generally considered to be non-toxic (Karlovsky 1999). Pigs are known to be particularly sensitive to OTA accumulation, with a tissue distribution following the pattern: kidney > liver > muscle > fat (Galtier et al. 1981; Mortensen et al. 1983; Lusky et al. 1995). OTA can also be produced by moulds growing on pork meat products during ripening. *Penicillium nordicum*, a high OTA producer, has been proven to be able to grow on meat (Battilani et al. 2007; Sorensen et al. 2008). OTA was found in hams sampled during the ripening time (Chiavaro et al. 2002) and in dry-cured hams collected from retail outlets (Pietri et al. 2006).

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The methods for OTA detection and quantification are based on different analytical techniques, such as enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC) with fluorescence detection (HPLC-FLD) or coupled with mass spectrometry (LC-MS/MS). All these techniques need an efficient sample extraction method. As regards OTA determination in pig tissues and meat products, the most common extraction methods are quite laborious. OTA is generally extracted by chlorinated solvents, such as chloroform or methyl chloride, acidified with hydrochloric or *o*-phosphoric acid. Successively, a liquid–liquid partition with a sodium bicarbonate aqueous solution is carried out; the aqueous phase, containing OTA, is cleaned up through a pre-packed or immunoaffinity column (Valenta 1998; Dragacci et al. 1999; Curtui et al. 2001) or analysed by HPLC without a purification step (Toscani et al. 2007). In other methods, ethyl acetate (Monaci et al. 2005) or a $NaHCO_3$ solution:methanol mixture (Chiavaro et al. 2002) are used as an extraction solvents. In this study, an innovative and simple extraction method, based on an enzyme-assisted (EA) extraction without the use of organic solvents, has been developed. A comparison between the new EA and chloroform (CH) extraction method has been performed.

Materials and methods

Reagents

Chemicals and solvents used for the extraction and clean-up solutions were ACS grade or equivalent (Carlo Erba, Milan, Italy). For HPLC analysis, methanol, acetonitrile and acetic acid were HPLC grade (VWR, Leuven, Belgium); water was purified through a Milli-Q treatment system (Millipore, London, UK). Phosphate-buffered saline (PBS) was prepared as per Vicam (NaCl $8\,g\,l^{-1}$, KCl $0.2\,g\,l^{-1}$, Na_2HPO_4 $1.15\,g\,l^{-1}$, KH_2PO_4 $0.2\,g\,l^{-1}$; pH 7.4).

Standard

OTA standard (purity degree $\geq 98\%$) was obtained from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of $40\,\mu g\,ml^{-1}$ was prepared in benzene:acetic acid (99 + 1 v/v) and stored at −20°C. The solution was calibrated spectrophotometrically at 333 nm using the value 5550 for the absorption coefficient (AOAC 1995). The working standard solution ($443.7\,\mu g\,l^{-1}$) was prepared after evaporation under nitrogen of an aliquot (100 µl) of the stock solution and re-dissolution in the HPLC mobile phase by ultrasonication. This solution was diluted with mobile phase to obtain eight HPLC calibrant solutions at concentrations of OTA between 0.04 and $1.2\,\mu g\,l^{-1}$.

Samples

In recent years (2007–2010), about 300 samples of pork and dry-cured ham taken in slaughterhouses and manufacturing plants located in northern Italy have been analysed in our laboratory for OTA. Since considerable OTA concentrations were found in several samples, 300 g of meat portions from these samples were minced using a mini-grinder (Illico, Moulinex, France) and kept at −20°C for this study.

Analysis for OTA

Preparation of slurry for dry-cured ham samples

The distribution of OTA in pig tissues after an ingestion of OTA with contaminated feed is assumed to be rather homogeneous; this is not the case for OTA produced by moulds in pork meat products during the ripening time. In fact, OTA can be produced on different parts of the ham and consequently OTA contamination is inhomogeneous. In order to obtain an homogeneous sample, a slurry was prepared as follows: an aliquot (50 g) of minced dry-cured ham was weighed and transferred to a commercial blender; under continuous mixing, a measured volume (generally 10 ml) of distilled water was slowly added to bring the moisture of the slurry to between 60 and 65%. From each slurry, eight aliquots (corresponding to 5 g of initial sample) were weighed: from four aliquots of these eight, OTA was extracted applying the CH method, from the other four, using the new EA method. All eight extractions were carried out on the same day of the slurry preparation. Slurry preparation was not carried out for pork samples.

Chloroform method

OTA was extracted according to the method reported by Dall'Asta et al. (2010), with slight modifications. In a 250 ml plastic centrifuge bottle, 100 ml of chloroform, acidified with 0.8 ml of concentrated (85%) *o*-phosphoric acid, was added to 5 g of minced pork or to an aliquot (corresponding to 5 g of initial sample) of dry-cured ham slurry. The mixture was then homogenised for 2 min using an Ultra-Turrax T25 homogeniser (T25, IKA Werke GmbH & Co, Staufen, Germany) at 9000 rpm. After centrifugation at 5500 *g* for 15 min at 4°C and filtration through a folded filter paper, an aliquot of 50 ml was transferred into a separating funnel and a liquid–liquid partition with 50 ml of 0.5 M $NaHCO_3$ solution was performed. Partition was repeated with a further 25 ml of $NaHCO_3$ solution and the aqueous phases were recombined. Then, 5 ml of the aqueous extract was diluted with 5 ml of PBS and purified through an immunoaffinity column.

Enzyme-assisted method

In a 250 ml plastic centrifuge bottle, 100 ml of 1% pancreatin (Sigma-Aldrich, St. Louis, MO, USA, code P1750) solution, prepared in 0.2 M phosphate buffer (0.2 M NaH_2PO_4:0.2 M Na_2HPO_4 16 + 84 v/v, pH 7.5), was added to 5 g of minced pork or to an aliquot (corresponding to 5 g of initial sample) of dry-cured ham slurry. The mixture was stirred with a magnetic stirrer in a thermostatic chamber at 37°C for 3 h, then centrifuged at 5500 *g* for 15 min at 4°C and filtered through a folded filter paper. A total of 5 ml of the filtrate was diluted with 5 ml of PBS and purified through an immunoaffinity column.

Clean-up by immunoaffinity column

The immunoaffinity column (Ochratest WB, Vicam, Watertown, MA, USA) was placed on an SPE vacuum manifold (Visiprep, Supelco, Bellefonte, PA, USA). The sample extract prepared as described above was applied to the column, followed by a washing with PBS (5 ml). Then, OTA was slowly eluted (0.5 ml min^{-1}) from the column with acetonitrile (3 ml) into a graduated glass vial; the eluate was concentrated under a gentle stream of nitrogen, brought to 1 ml with acetonitrile −2% acetic acid aqueous solution (41 + 59 v/v) and vortex-mixed for a few seconds. The extract was filtered (Millex-HV 0.45 µm, Millipore Corporation, Bedford, MA, USA) before HPLC analysis.

HPLC analysis

The HPLC system consisted of a Perkin Elmer 200 (Perkin Elmer, Norwalk, CT, USA), equipped with a Jasco AS 1555 sampling system and a FP 1520 fluorescence detector (Jasco Corporation, Tokyo, Japan) set at 333 nm excitation and 470 nm emission wavelength. The system was governed by a Borwin 1.5 software (Jasco). OTA was separated on a phenylhexyl column (5 µm particle size, 150 × 4.6 mm i.d., Phenomenex, Torrance, CA, USA) at ambient temperature, with a mobile phase gradient acetonitrile −2% acetic acid aqueous solution from 35:65 to 67:33 in 15 min; the flow rate was 1.0 ml min^{-1}. The injection volume for both standard solutions and sample extracts was 100 µl, corresponding to 16.7 or 25 mg of sample for CH and EA method, respectively. For qualitative confirmation, derivatisation of OTA through methylation with subsequent HPLC analysis was performed in ten samples (Gareis 1999).

Comparison between methods

Initially, a recovery test was performed for CH and EA method; the recovery values were determined by spiking a blank sample of pork or dry-cured ham with an appropriate volume of OTA standard solution, in order to have contamination levels of 0.50, 1.00 and 2.00 µg kg^{-1}. Three replicates were analysed for each level and matrix. The recoveries, converted into arcsine values according to Fowler et al. (1997), were compared using GLM procedure (SPSS 18.0, Inc., Chicago, IL, USA) considering as factors: method (EA and CH), matrix (pork and dry-cured ham) and level (0.50, 1.00 and 2.00 µg kg^{-1}).

The limits of detection (LOD) and quantification (LOQ) were determined by the signal-to-noise approach, defined at those levels resulting in signal-to-noise ratios of 3 and 10, respectively. The analytical response and the chromatographic noise were both measured from the chromatogram of a purified blank sample extract (1 ml) to which between 10 and 50 µl of an OTA solution (0.80 µg l^{-1}) had been added.

Finally, OTA was extracted in quadruplicate by the CH and EA method from six pork and 40 dry-cured ham samples. The results were compared using the paired *t*-test (SPSS 18.0) for pork and dry-cured ham samples separately.

Results and discussion

Development of the new method

For the analytical procedures, the character of OTA as a weak acid (pK_a 4.4 and 7.3 for the carboxyl and the hydroxyl group, respectively) is important. OTA can be extracted from a water phase into a less polar solvent not miscible with water only at pH < 7, as under neutral and alkaline conditions it is present in the dissociated form. Moreover, OTA extraction from blood or animal tissue is hampered by OTA binding to proteins. Because of these difficulties, in most studies OTA was extracted from blood or animal tissues by chloroform after acidification with a solution of hydrochloric or *o*-phosphoric acid (Valenta 1998). Two old methods for OTA determination in kidney included an enzymatic digestion with subtilisin A or papain prior to extraction (Hunt et al. 1979; Scheuer et al. 1984), being higher concentrations of OTA measured in the samples after enzymatic digestion with subtilisin A (Hunt et al. 1979). However, enzymatic digestion was not applied in later studies and it was never used in pork analysis for OTA.

Because of the complexity of the published methods and of the use of chlorinated solvents for the extraction in the vast majority of them, we decided to develop a new EA method. Some proteolytic enzymes, such as pepsin, are active in acid medium (pH 1.5–2.5), but this condition is not suitable for OTA, because the toxin is destroyed very quickly owing to the hydrolysis of the amide bond. On the contrary, pancreatin is active in neutral medium (pH

6–8) and it was chosen for the enzymatic extraction. In order to verify that OTA was not hydrolysed by pancreatin, two OTA standard solutions were prepared in 0.2 M phosphate buffer (pH 7.5): one containing pancreatin (1%), the other without the enzyme. Then, a stability kinetics of the standard solutions was performed in a thermostatic chamber at 37°C for 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 16 and 20 h. The results (Figure 1) showed that OTA was quite stable (recoveries >90%) up to 6 h in the presence of pancreatin and up to 20 h without the enzyme.

Then, aliquots (5 g) of pork and dry-cured ham were digested with pancreatin under the same experimental conditions; it was observed that the samples were completely hydrolysed (except adipose and connective tissue) after only 3 h of enzymatic digestion; thus, this time was chosen for the EA method.

Successively, a recovery test on the immunoaffinity column was performed, in order to verify if the solution containing pancreatin might impair the antibodies in the column. The columns used in this study showed satisfactory recovery values (>95%); unexpectedly, columns from other companies showed lower values.

Finally, OTA was extracted from four contaminated dry-cured ham samples, using only 0.2 M phosphate buffer (pH 7.5); after centrifugation, the filtrate was cloudy and the purification step through immunoaffinity column was not concluded for two samples (clogged column); for the other two samples, the results were lower than those obtained by the CH method.

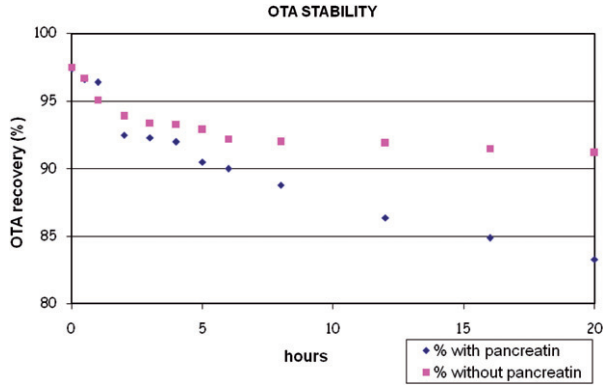

Figure 1. Stability kinetics of standard solutions of OTA in 0.2 M phosphate buffer (pH 7.5) at 37°C, with and without pancreatin.

Performance of the methods

The average recoveries were between 85.1% and 92.0% (Table 1) with satisfactory relative standard deviations (RSD), fulfilling completely the performance criteria fixed by Regulation (EC) 401/2006 of the European Commission, i.e. recovery in the range 50–120% and 70–110% for levels <1 and between 1 and 10 $\mu g\,kg^{-1}$, respectively (Commission of the European Communities 2006b). The statistical analysis showed that significantly higher mean recoveries were obtained by the EA compared with the CH method (90.9 versus 86.6%, $p = 0.004$); for the other factors (matrix and level), the values were not significantly different. No interactions among factors were observed.

LOD and LOQ were 0.090 and 0.180 $\mu g\,kg^{-1}$ using the CH method, 0.060 and 0.120 $\mu g\,kg^{-1}$ using EA extraction. Concerning HPLC analysis, performance criteria for HPLC methods, fixed by Decision 2002/657/EC, were fully fulfilled. Figure 2 shows the chromatograms of an OTA standard solution and of a naturally contaminated dry-cured ham sample, extracted by both the EA and CH methods. The identity of OTA was confirmed by the preparation of its methyl ester in a selection of 10 positive samples and by re-injecting the sample into the HPLC, according to the method of Zimmerli and Dick (1995). The disappearance of the OTA peak, and the corresponding appearance of a methyl-OTA peak was considered as positive identification.

Table 1. Average recovery (three replicates), standard deviation and relative standard deviation (RSD) of OTA from spiked blank pork ham muscle and dry-cured ham at different levels, using the chloroform (CH) and the enzyme-assisted (EA) extraction.

	Spiking level ($\mu g\,kg^{-1}$)					
	0.5		1		2	
Method	Recovery (%)	RSD	Recovery (%)	RSD	Recovery (%)	RSD
Pork ham muscle						
CH extraction	86.7 ± 5.6	6.4	87.7 ± 4.3	4.9	87.4 ± 3.9	4.5
EA extraction	90.6 ± 4.7	5.2	90.9 ± 4.1	4.5	90.8 ± 3.7	4.1
Dry-cured ham						
CH extraction	85.1 ± 5.3	6.2	86.6 ± 4.3	5.0	85.8 ± 3.9	4.5
EA extraction	91.1 ± 4.9	5.4	92.0 ± 3.8	4.1	90.2 ± 3.5	3.9

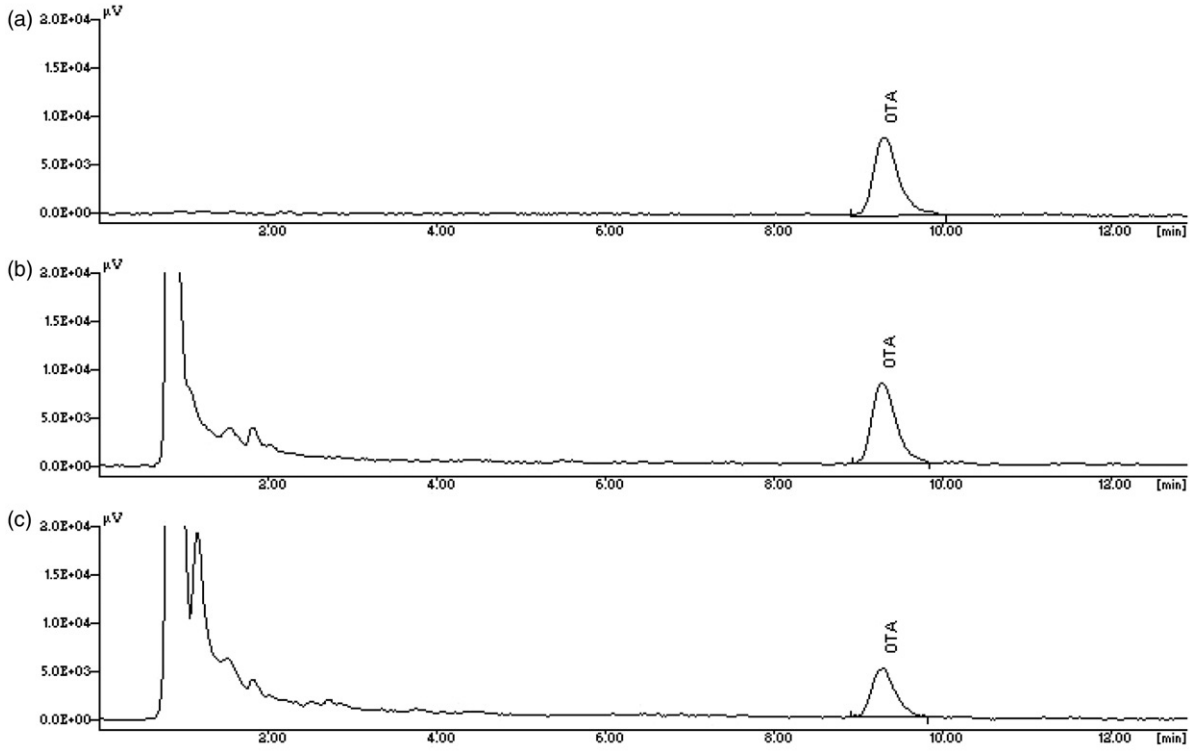

Figure 2. Chromatograms of: (a) an OTA standard solution (0.48 μg kg^{-1}) equivalent to 48 pg of OTA injected; (b) a naturally contaminated dry-cured ham sample extracted by the enzymatic method, containing 2.07 μg kg^{-1} (equivalent to 51.75 pg of OTA injected); and (c) the same naturally contaminated sample extracted by the chloroform method, containing 1.32 μg kg^{-1} (equivalent to 22 pg of OTA injected). Injection volume = 100 μl.

Table 2. OTA values (μg kg^{-1}, four replicates) in naturally contaminated pork ham muscle samples, analysed using the chloroform (CH) and the enzyme-assisted (EA) extraction.

	Mean ± SD	
Pork ham muscle	CH extraction	EA extraction
1	1.37 ± 0.08	1.71 ± 0.10
2	1.05 ± 0.08	1.3 ± 0.11
3	4.20 ± 0.11	4.47 ± 0.13
4	1.44 ± 0.06	1.39 ± 0.10
5	2.59 ± 0.12	3.60 ± 0.13
6	2.02 ± 0.13	2.09 ± 0.10

OTA extraction by enzyme-assisted and chloroform methods from naturally contaminated samples

The means obtained by the EA and CH extraction methods on naturally contaminated pork and dry-cured ham samples are shown in Tables 2 and 3, respectively; the results were corrected for recoveries (90.9% and 86.6% for the EA and CH methods, respectively). For four pork samples the averages of OTA contamination obtained by EA extraction were slightly higher than those obtained by the CH method; for the other two samples (4 and 6) the values were similar. The statistical analysis using a paired *t*-test showed that the difference between the averages was not significant ($p = 0.09$).

As regards dry-cured hams, the values obtained by the EA method were much higher; applying the paired *t*-test, the difference between the means was highly significant ($p < 0.001$). In some samples, the OTA concentrations obtained by the EA method were almost twice as high as those by the CH method. These data indicated that a considerable OTA fraction was probably associated with proteins and that this fraction was not completely extracted by acidified chloroform. Moreover, the higher results found in dry-cured ham compared with pork samples showed that the association with proteins is probably more relevant in the former ones, where OTA contamination can also be due to toxigenic moulds growing on meat during the ripening time.

Conclusions

Recent studies have shown that pork meat products, particularly dry-cured ham, can be contaminated by OTA. The level of the contamination seems not to be a worry, but should be continually monitored. Current methods of analysis are quite elaborate and time consuming; the EA method proposed here is simple, easy to apply, shows satisfactory performance criteria

Table 3. OTA values (μg kg^{-1}, four replicates) in naturally contaminated dry-cured ham samples, analysed using the chloroform (CH) and the enzyme-assisted (EA) extraction.

	Mean ± SD (μg kg^{-1})			Mean ± SD (μg kg^{-1})	
Dry-cured ham	CH extraction	EA extraction	Dry-cured ham	CH extraction	EA extraction
1	1.91 ± 0.10	3.85 ± 0.18	21	0.71 ± 0.08	1.66 ± 0.10
2	1.81 ± 0.09	3.68 ± 0.10	22	0.61 ± 0.05	1.48 ± 0.10
3	1.85 ± 0.09	2.94 ± 0.21	23	0.73 ± 0.04	1.95 ± 0.19
4	2.83 ± 0.07	4.48 ± 0.25	24	1.78 ± 0.13	3.51 ± 0.23
5	1.35 ± 0.08	2.41 ± 0.07	25	1.46 ± 0.14	3.27 ± 0.12
6	0.85 ± 0.09	2.02 ± 0.15	26	1.80 ± 0.09	3.73 ± 0.16
7	1.28 ± 0.12	2.69 ± 0.30	27	2.97 ± 0.14	4.56 ± 0.13
8	1.15 ± 0.06	2.65 ± 0.21	28	2.41 ± 0.09	4.06 ± 0.19
9	3.82 ± 0.12	6.08 ± 0.24	29	1.04 ± 0.07	1.23 ± 0.10
10	4.11 ± 0.11	6.16 ± 0.14	30	1.58 ± 0.09	3.59 ± 0.09
11	1.32 ± 0.09	2.07 ± 0.13	31	0.91 ± 0.06	1.47 ± 0.09
12	3.36 ± 0.19	4.43 ± 0.18	32	1.75 ± 0.10	2.32 ± 0.14
13	2.70 ± 0.11	3.88 ± 0.17	33	1.03 ± 0.07	2.50 ± 0.14
14	3.52 ± 0.09	5.03 ± 0.17	34	1.51 ± 0.10	3.79 ± 0.14
15	3.17 ± 0.18	6.29 ± 0.21	35	2.11 ± 0.11	3.53 ± 0.14
16	0.98 ± 0.08	2.37 ± 0.18	36	2.92 ± 0.13	4.75 ± 0.15
17	0.99 ± 0.04	2.56 ± 0.24	37	1.94 ± 0.13	3.59 ± 0.10
18	1.19 ± 0.07	2.62 ± 0.16	38	3.63 ± 0.17	5.01 ± 0.14
19	1.54 ± 0.10	3.01 ± 0.28	39	0.70 ± 0.04	1.14 ± 0.07
20	1.91 ± 0.09	3.07 ± 0.28	40	1.04 ± 0.12	1.50 ± 0.07

and gives better results in dry-cured ham samples when compared with an established method. The EA method simulates part of the digestion process. Therefore, the OTA released and then quantified is probably closer to the amount really available for *in vivo* absorption. Moreover, the EA method does not use chlorinated solvents, achieving both considerable environmental and economic advantage.

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