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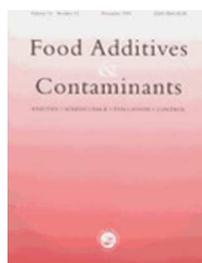
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Enzyme-assisted extraction for the HPLC determination of ochratoxin A in pork and dry-cured ham

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Food Types:	Animal products – meat
Abstract:	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 <i>o</i> -phosphoric acid. In this study, an innovative method was developed to extract ochratoxin A from pork and dry-cured ham samples. The method is based on an enzyme-assisted extraction with pancreatin in a pH 7.5 phosphate buffer. How pancreatin hydrolyses proteins, so that ochratoxin A, kept in the ionized form, is easily extracted by the aqueous solution. After purification through an immunoaffinity column, ochratoxin A is determined by HPLC with fluorimetric 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 0.060 and 0.120 µg kg ⁻¹ , respectively. A comparison between the new enzyme-assisted extraction and an established chloroform method was carried out on 6 pork and 40 dry-cured ham naturally contaminated samples; significantly higher (P<0.001) values of ochratoxin A were obtained on dry-cured ham samples by the enzyme-assisted method.

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6 **Enzyme-assisted extraction for the HPLC determination of ochratoxin A in pork**
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8 **and dry-cured ham**
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15 Amedeo Pietri, Alessia Gualla, Silvia Rastelli and Terenzio Bertuzzi*

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32
33 **Abstract**
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35 1 The extraction of ochratoxin A from meat products is generally carried out using chlorinated
36 2 organic solvents, such as chloroform or methyl chloride, acidified with hydrochloric or *o*-
37 3 phosphoric acid. In this study, an innovative method was developed to extract ochratoxin A from
38 4 pork and dry-cured ham samples. The method is based on an enzyme-assisted extraction with
39 5 pancreatin in a pH 7.5 phosphate buffer. Pancreatin hydrolyses the proteins, so that ochratoxin A,
40 6 kept in the ionized form, is easily extracted by the aqueous solution. After purification through an
41 7 immunoaffinity column, ochratoxin A is determined by HPLC with fluorescence detection. The
42 8 average recovery values were higher than 90.0 % and the relative standard deviations were below
43 9 5.5 %. The limits of detection and of quantification were 0.06 and 0.12 $\mu\text{g kg}^{-1}$, respectively. A
44 10 comparison between the new enzyme-assisted extraction and an established chloroform method was
45 11 carried out on 6 naturally contaminated samples of pork and 40 samples of dry-cured ham.
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2 12 Significantly higher ($P<0.001$) values of ochratoxin A were obtained on dry-cured ham samples by
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4 13 the enzyme-assisted method.
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9 15 *Keywords:* ochratoxin A; dry-cured ham; enzymatic extraction; pancreatin.
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12 13 17 **Introduction**

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15 18 Ochratoxin A (OTA) is a mycotoxin produced by various *Aspergillus* and *Penicillium*
16
17 19 species. Several studies have shown that the toxin has carcinogenic, nephrotoxic, immunotoxic,
18
19 20 teratogenic and possibly neurotoxic and genotoxic properties and it has also been associated with
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21 21 Balkan Endemic Nephropathy and the development of urinary tract tumours in humans (Marquardt
22
23 22 and Frohlich 1992; Pleština 1996; Schlatter et al. 1996). OTA has been classified by the
24
25 23 International Agency for Research on Cancer (IARC) into Group 2B as a possible human
26
27 24 carcinogen (IARC 1993). OTA contaminates many foods, such as cereals and derived products,
28
29 25 dried fruit, coffee, cocoa, some spices, liquorice, wine, grape juices, beer and ripened pork products
30
31 26 (Zimmerli and Dick 1996; Benford et al. 2001; Thirumala et al. 2001; Dall'Asta et al. 2010; Pietri et
32
33 27 al. 2010). Concerning EU legislation for human consumption, the European Commission fixed, in
34
35 28 Regulations (EC) 1881/2006 and 105/2010 (European Commission, 2006a; 2010), maximum
36
37 29 admissible levels for OTA in several foodstuffs and stated that, on the basis of the position adopted
38
39 30 by EFSA (EFSA 2004), it does not appear necessary for the protection of public health to set a
40
41 31 maximum level of OTA in dried fruit other than dried vine fruit, cocoa, liqueur wines and meat
42
43 32 products. In Italy, a guideline value of $1 \mu\text{g kg}^{-1}$ in pork meat and derived products has been
44
45 33 recommended by the Italian Ministry of Health since 1999 (Ministero della Sanità 1999). OTA can
46
47 34 occur in meat and meat products as a result both of indirect transmission from animals exposed to
48
49 35 naturally contaminated feed, and of direct contamination produced by moulds or by naturally
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51 36 contaminated spice mixtures used as ingredients (Gareis 1996). Among farm animals, the risk is
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2 37 limited to monogastric species, because ruminants can hydrolyze the amidic bond of OTA into
3
4 38 phenylalanine and ochratoxin α , which is generally considered to be non-toxic (Karlovskj 1999).
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6
7 39 Pigs are known to be particularly sensitive to OTA accumulation, with a tissue distribution
8
9 40 following the pattern: kidney > liver > muscle > fat (Galtier et al. 1981; Mortensen et al. 1983;
10
11 41 Lusky et al. 1995). OTA can also be produced by moulds growing on pork meat products during
12
13 42 ripening. *Penicillium nordicum*, a high OTA producer, has been proven to be able to grow on meat
14
15 43 (Battilani et al. 2007; Sorensen et al. 2008). OTA was found in hams sampled during the ripening
16
17 44 time (Chiavaro et al. 2002) and in dry-cured hams collected from retail outlets (Pietri et al. 2006).

19
20 45 The methods for OTA detection and quantification are based on different analytical
21
22 46 techniques, such as enzyme-linked immunosorbent assay (ELISA), high-performance liquid
23
24 47 chromatography (HPLC) with fluorescence detection (HPLC-FLD) or coupled with mass
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26 48 spectrometry (LC-MS/MS). All these techniques need an efficient sample extraction method. As
27
28 49 regards OTA determination in pig tissues and meat products, the most common extraction methods
29
30
31 50 are quite laborious. OTA is generally extracted by chlorinated solvents, such as chloroform or
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33 51 methyl chloride, acidified with hydrochloric or *o*-phosphoric acid. Successively, a liquid-liquid
34
35 52 partition with a sodium bicarbonate aqueous solution is carried out; the aqueous phase, containing
36
37 53 OTA, is cleaned up through a pre-packed or immunoaffinity column (Valenta 1998; Dragacci et al.
38
39 54 1999; Curtui et al. 2001) or analysed by HPLC without a purification step (Toscani et al. 2007). In
40
41 55 other methods, ethyl acetate (Monaci et al. 2005) or a NaHCO₃ solution: methanol mixture
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43 56 (Chiavaro et al. 2002) are used as an extraction solvents. In this study, an innovative and simple
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45 57 extraction method, based on an enzyme-assisted (EA) extraction without the use of organic
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47 58 solvents, has been developed. A comparison between the new EA and chloroform (CH) extraction
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49 59 method has been performed.
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62 **Materials and methods**

63 *Reagents*

64 Chemicals and solvents used for the extraction and clean-up solutions were ACS grade or
65 equivalent (Carlo Erba, Milan, Italy). For HPLC analysis, methanol, acetonitrile and acetic acid
66 were HPLC grade (VWR, Leuven, Belgium); water was purified through a Milli-Q treatment
67 system (Millipore, London, U.K.). Phosphate buffered saline (PBS) was prepared as per Vicam
68 (NaCl 8 g l⁻¹, KCl 0.2 g l⁻¹, Na₂HPO₄ 1.15 g l⁻¹, KH₂PO₄ 0.2 g l⁻¹; pH 7.4).

69 *Standard*

70 OTA standard (purity degree ≥ 98 %) was obtained from Sigma-Aldrich (St. Louis, MO,
71 USA). A stock solution of 40 µg ml⁻¹ was prepared in benzene: acetic acid (99+1 v/v) and stored at
72 -20 °C. The solution was calibrated spectrophotometrically at 333 nm using the value 5550 for the
73 absorption coefficient (AOAC 1995). The working standard solution (443.7 µg l⁻¹) was prepared
74 after evaporation under nitrogen of an aliquot (100 µl) of the stock solution and re-dissolution in the
75 HPLC mobile phase by ultrasonication. This solution was diluted with mobile phase to obtain eight
76 HPLC calibrant solutions at concentrations of OTA between 0.04 and 1.2 µg l⁻¹.

77 *Samples*

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

83 *Analysis for OTA*

84 *Preparation of slurry for dry-cured ham samples*

85 The distribution of OTA in pig tissues after an ingestion of OTA with contaminated feed is
86 assumed to be rather homogeneous; this is not the case for OTA produced by molds in pork meat

1
2 87 products during the ripening time. In fact, OTA can be produced on different parts of the ham and
3
4 88 consequently OTA contamination is inhomogeneous. In order to obtain an homogeneous sample, a
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6 89 slurry was prepared as follows: an aliquot (50 g) of minced dry-cured ham was weighed and
7
8 90 transferred to a commercial blender; under continuous mixing, a measured volume (generally 10
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10 91 ml) of distilled water was slowly added to bring the moisture of the slurry to between 60 and 65 %.
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12 92 From each slurry, eight aliquots (corresponding to 5 g of initial sample) were weighed: from four
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14 93 aliquots of these eight, OTA was extracted applying the CH method, from the other four, using the
15
16 94 new EA method. All eight extractions were carried out on the same day of the slurry preparation.
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18 95 Slurry preparation was not carried out for pork samples.
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20 96 *Chloroform method*

21
22 97 OTA was extracted according to the method reported by Dall'Asta et al. (2010), with slight
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24 98 modifications. In a 250 ml plastic centrifuge bottle, 100 ml of chloroform, acidified with 0.8 ml of
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26 99 concentrated (85 %) *o*-phosphoric acid, was added to 5 g of minced pork or to an aliquot
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28 100 (corresponding to 5 g of initial sample) of dry-cured ham slurry. Then, the mixture was
29
30 101 homogenized for 2 min using an Ultra-Turrax T25 homogenizer (T25, IKA Werke GmbH & Co,
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32 102 Staufen, Germany) at 9000 rpm. After centrifugation at 5500 g for 15 min at 4 °C and filtration
33
34 103 through a folded filter-paper, an aliquot of 50 ml was transferred into a separating funnel and a
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36 104 liquid-liquid partition with 50 ml of 0.5 M NaHCO₃ solution was performed. Partition was repeated
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38 105 with a further 25 ml of NaHCO₃ solution and the aqueous phases were recombined. Then, 5 ml of
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40 106 the aqueous extract was diluted with 5 ml of PBS and purified through an immunoaffinity column.
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46 107 *Enzyme-assisted method*

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48 108 In a 250 ml plastic centrifuge bottle, 100 ml of 1 % pancreatin (Sigma-Aldrich, St. Louis,
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50 109 MO, USA, cod P1750) solution, prepared in 0.2 M phosphate buffer (0.2 M NaH₂PO₄:0.2 M
51
52 110 Na₂HPO₄ 16+84 v/v, pH 7.5), was added to 5 g of minced pork or to an aliquot (corresponding to 5
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54 111 g of initial sample) of dry-cured ham slurry. The mixture was stirred with a magnetic stirrer in a
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3 112 thermostatic chamber at 37 °C for 3 hours, then centrifuged at 5500 g for 15 min at 4 °C and filtered
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5 113 through a folded filter-paper. Five ml of the filtrate was diluted with 5 ml of PBS and purified
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7 114 through an immunoaffinity column.
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10 115 ***Clean-up by immunoaffinity column***

11 116 The immunoaffinity column (Ochratest WB, Vicam, Watertown, MA, USA) was placed on
12
13 117 an SPE vacuum manifold (Visiprep, Supelco, Bellefonte, Pennsylvania, USA). The sample extract
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15 118 prepared as described above was applied to the column, followed by a washing with PBS (5 ml).
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17 119 Then, OTA was slowly eluted (0.5 ml min⁻¹) from the column with acetonitrile (3 ml) into a
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19 120 graduated glass vial; the eluate was concentrated under a gentle stream of nitrogen, brought to 1 ml
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21 121 with acetonitrile-2 % acetic acid aqueous solution (41+59 v/v) and vortex-mixed for a few seconds.
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23 122 The extract was filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 µm) before
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25 123 HPLC analysis.
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28 124 ***HPLC analysis***

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30 125 The HPLC system consisted of a Perkin Elmer 200 (Perkin Elmer, Norwalk, Connecticut,
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32 126 USA), equipped with a Jasco AS 1555 sampling system and a FP 1520 fluorescence detector (Jasco
33
34 127 Corporation, Tokyo, Japan) set at 333 nm excitation and 470 nm emission wavelength. The system
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36 128 was governed by a Borwin 1.5 software (Jasco). OTA was separated on a Phenyl-hexyl column (5
37
38 129 µm particle size, 150 x 4.6 mm i.d., Phenomenex, Torrance, CA, USA) at ambient temperature,
39
40 130 with a mobile phase gradient acetonitrile-2% acetic acid aqueous solution from 35:65 to 67:33 in 15
41
42 131 min; the flow rate was 1.0 ml min⁻¹. The injection volume for both standard solutions and sample
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44 132 extracts was 100 µl, corresponding to 16.7 or 25 mg of sample for CH and EA method,
45
46 133 respectively. For qualitative confirmation, derivatization of OTA through methylation with
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48 134 subsequent HPLC analysis was performed in 10 samples (Gareis 1999).
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54 135 ***Comparison between methods***

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2 136 Initially, a recovery test was performed for CH and EA method; the recovery values were
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4 137 determined by spiking a blank sample of pork or dry-cured ham with an appropriate volume of
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6 138 OTA standard solution, in order to have contamination levels of 0.50, 1.00 and 2.00 $\mu\text{g kg}^{-1}$. Three
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8 139 replicates were analyzed for each level and matrix. The recoveries values, converted into arcsine
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10 140 values according to Fowler et al. (1997), were compared using GLM procedure (SPSS 18.0, Inc.,
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12 141 Chicago, IL) considering as factors: method (EA and CH), matrix (pork and dry-cured ham) and
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14 142 level (0.50, 1.00 and 2.00 $\mu\text{g kg}^{-1}$).

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16
17 143 The limits of detection (LOD) and quantification (LOQ) were determined by the signal-to-
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19 144 noise approach, defined at those levels resulting in signal-to-noise ratios of 3 and 10, respectively.
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21 145 The analytical response and the chromatographic noise were both measured from the chromatogram
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23 146 of a purified blank sample extract (1 ml) to which between 10 and 50 μl of an OTA solution (0.80
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25 147 $\mu\text{g l}^{-1}$) had been added.

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27
28 148 Finally, OTA was extracted in quadruplicate by the CH and EA method from 6 pork and 40
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30 149 dry-cured ham samples. The results were compared using the paired *t*-test (SPSS 18.0) for pork and
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32 150 dry-cured ham samples separately.

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36 37 152 **Results and discussion**

38 39 153 *Development of the new method*

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42 154 For the analytical procedures, the character of OTA as a weak acid (pK_a 4.4 and 7.3 for the
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44 155 carboxyl and the hydroxyl group, respectively) is important. OTA can be extracted from a water
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46 156 phase into a less polar solvent not miscible with water only at $\text{pH} < 7$, as under neutral and alkaline
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48 157 conditions it is present in the dissociated form. Moreover, OTA extraction from blood or animal
49
50 158 tissue is hampered by OTA binding to proteins. Because of these difficulties, in most studies OTA
51
52 159 was extracted from blood or animal tissues by chloroform after acidification with a solution of
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54 160 hydrochloric or *o*-phosphoric acid (Valenta 1998). Two old methods for OTA determination in

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2
3 161 kidney included an enzymatic digestion with subtilisin A or papain prior to extraction (Hunt et al.
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5 162 1979; Scheuer et al. 1984), being higher concentrations of OTA measured in the samples after
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7 163 enzymatic digestion with subtilisin A (Hunt et al. 1979). However, enzymatic digestion was not
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10 164 applied in later studies and it was never used in pork analysis for OTA.

11
12 165 Because of the complexity of the published methods and of the use of chlorinated solvents
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14 166 for the extraction in the vast majority of them, we decided to develop a new EA method. Some
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16 167 proteolytic enzymes, like pepsin, are active in acid medium (pH 1.5-2.5), but this condition is not
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18 168 suitable for OTA, because the toxin is destroyed very quickly owing to the hydrolysis of the amide
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20
21 169 bond. On the contrary, pancreatin is active in neutral medium (pH 6-8) and it was chosen for the
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23 170 enzymatic extraction. In order to verify that OTA was not hydrolysed by pancreatin, two OTA
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25 171 standard solutions were prepared in 0.2 M phosphate buffer (pH 7.5): one containing pancreatin (1
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27 172 %), the other without the enzyme. Then, a stability kinetics of the standard solutions was performed
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29
30 173 in a thermostatic chamber at 37°C for 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 16 and 20 hours. The results
31
32 174 (Figure 1) showed that OTA was quite stable (recoveries >90 %) up to 6 hours in the presence of
33
34 175 pancreatin and up to 20 hours without the enzyme.

35
36 176 Then, aliquots (5 g) of pork and dry-cured ham were digested with pancreatin under the
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38 177 same experimental conditions; it was observed that the samples were completely hydrolysed
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40 178 (except adipose and connective tissue) after only 3 hours of enzymatic digestion; thus, this time
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42 179 was chosen for the EA method.

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45 180 [insert Figure 1 here]

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47 181 Successively, a recovery test on the immunoaffinity column was performed, in order to
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49 182 verify if the solution containing pancreatin might impair the antibodies in the column. The columns
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51 183 used in this study showed satisfactory recovery values (> 95 %); unexpectedly, columns from other
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53 184 companies showed lower values.

1
2 185 Finally, OTA was extracted from 4 contaminated dry-cured ham samples, using only 0.2 M
3
4 186 phosphate buffer (pH 7.5); after centrifugation, the filtrate was cloudy and the purification step
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6 187 through immunoaffinity column was not concluded for 2 samples (clogged column); for the other 2
7
8 188 samples, the results were lower than those obtained by the CH method.

11 189 *Performance of the methods*

13 190 The average recoveries were between 85.1 and 92.0 % (Table 1) with satisfactory relative
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15 191 standard deviation values (RSD), fulfilling completely the performance criteria fixed by Regulation
16
17 192 (EC) 401/2006 of the European Commission, i.e. recovery in the range 50-120% and 70-110% for
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19 193 levels < 1 and between 1-10 $\mu\text{g kg}^{-1}$, respectively (European Commission 2006b). The statistical
20
21 194 analysis showed that significantly higher mean recoveries were obtained by the EA compared to
22
23 195 CH method (90.9 vs. 86.6 %, $P=0.004$); for the other factors (matrix and level), the values were not
24
25 196 significantly different. No interactions among factors were observed.

27
28
29 197 [insert Table 1 here]

31 198 The LOD and LOQ were 0.090 and 0.180 $\mu\text{g kg}^{-1}$ using the CH method, 0.060 and 0.120 μg
32
33 199 kg^{-1} using the EA extraction. Concerning HPLC analysis, performance criteria for HPLC methods,
34
35 200 fixed by Decision 2002/657/EC, were fully fulfilled. Figure 2 shows the chromatograms of an OTA
36
37 201 standard solution and of a naturally contaminated dry-cured ham sample, extracted both by EA and
38
39 202 by CH method. The identity of OTA was confirmed by the preparation of its methyl ester in a
40
41 203 selection of 10 positive samples and by re-injecting the sample into the HPLC, according to the
42
43 204 method of Zimmerli and Dick (1995). The disappearance of the OTA peak, and the corresponding
44
45 205 appearance of a methyl-OTA peak was considered as positive identification.

48
49 206 [insert Figure 2 here]

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51 207

53 208 *OTA extraction by enzyme-assisted and chloroform methods from naturally contaminated*
54
55 209 *samples*

1
2 210 The mean values obtained by EA and CH extraction methods on naturally contaminated
3
4 211 pork and dry-cured ham samples, are shown in Table 2 and 3, respectively; the results were
5
6 212 corrected for recoveries (90.9 and 86.6% for EA and CH method, respectively). For 4 pork samples,
7
8 213 the average values of OTA contamination obtained by EA extraction, were slightly higher than
9
10 214 those obtained by CH method; for the other two samples (4 and 6), the values were similar. The
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12 215 statistical analysis using paired *t*-test showed that the difference between the average values was not
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14 216 significant ($P=0.09$).

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18 217 [insert Table 2 here]

19
20 218 As regards dry-cured hams, the values obtained by the EA method were much higher;
21
22 219 applying the paired *t*-test, the difference between the mean values was highly significant ($P<0.001$).
23
24 220 In some samples, the OTA concentrations obtained by the EA method were almost twice as high as
25
26 221 those by the CH method. These data indicated that a considerable OTA fraction was probably
27
28 222 associated with proteins and that this fraction was not completely extracted by acidified chloroform.
29
30 223 Moreover, the higher results found in dry-cured ham compared to pork samples, showed that the
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32 224 association with proteins is probably more relevant in the former ones, where OTA contamination
33
34 225 can also be due to toxigenic moulds growing on meat during the ripening time.

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38 226 [insert Table 3 here]

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41 42 228 **Conclusions**

43
44 229 Recent studies showed that pork meat products, particularly dry-cured ham, can be
45
46 230 contaminated by OTA. The level of the contamination seems not to be worrying, but should be
47
48 231 continually monitored. Current methods of analysis are quite elaborate and time-consuming; the EA
49
50 232 method proposed in this work is simple, easy to apply, shows satisfactory performance criteria and
51
52 233 gives better results in dry-cured ham samples, when compared with an established method. The EA
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54 234 method simulates part of the digestion process, therefore the OTA released and then quantified is

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2 235 probably closer to the amount really available for *in vivo* absorption. Moreover, the EA method
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4 236 does not use chlorinated solvents, achieving both considerable environmental and economic
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6 237 advantage.
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Table 1 - Average recovery (3 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.

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

Table 2 - OTA values ($\mu\text{g kg}^{-1}$, 4 replicates) in naturally contaminated pork ham muscle samples, analysed using the chloroform (CH) and the enzyme-assisted (EA) extraction.

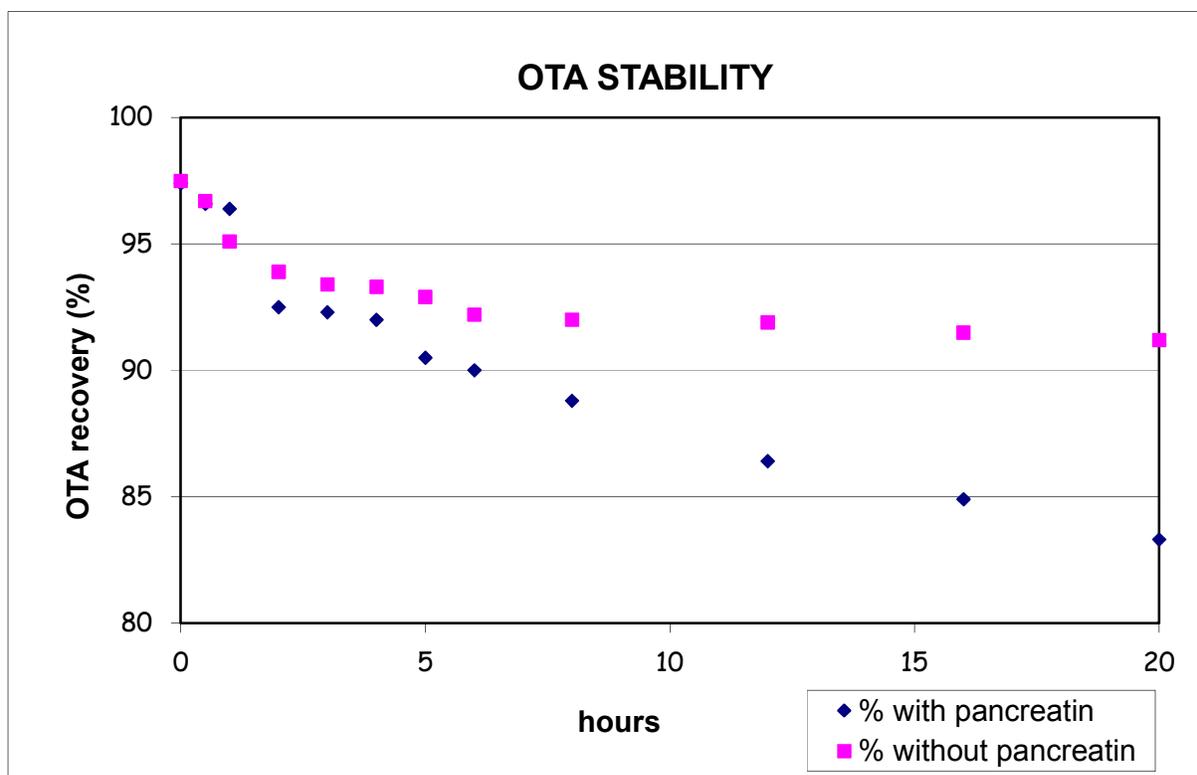
	CH extraction	EA extraction
pork ham muscle	Mean \pm SD	Mean \pm SD
1	1.37 \pm 0.08	1.71 \pm 0.10
2	1.05 \pm 0.08	1.3 \pm 0.11
3	4.20 \pm 0.11	4.47 \pm 0.13
4	1.44 \pm 0.06	1.39 \pm 0.10
5	2.59 \pm 0.12	3.60 \pm 0.13
6	2.02 \pm 0.13	2.09 \pm 0.10

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Table 3 - OTA values ($\mu\text{g kg}^{-1}$, 4 replicates) in naturally contaminated dry-cured ham samples, analysed using the chloroform (CH) and the enzyme-assisted (EA) extraction.

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



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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.

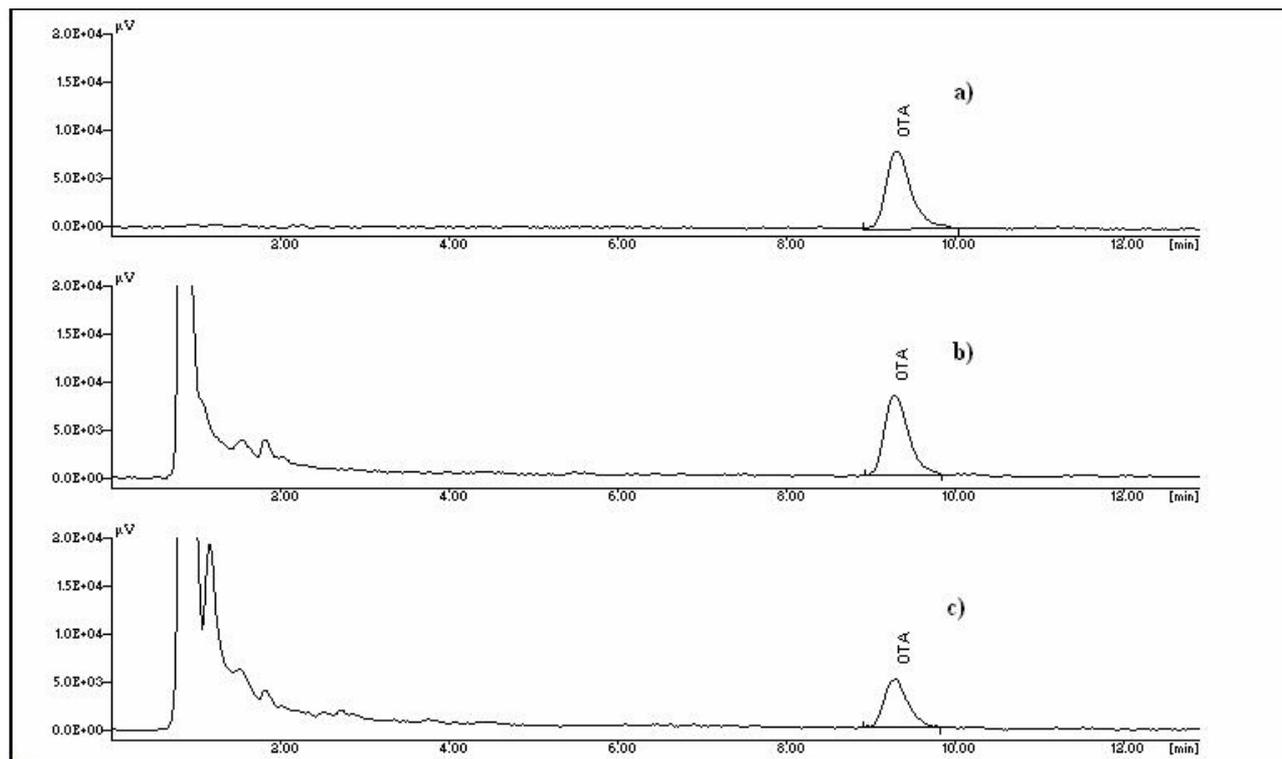


Figure 2 – Chromatograms of: a) an OTA standard solution ($0.48 \mu\text{g kg}^{-1}$) equivalent to 48 pg of OTA injected; b) a naturally contaminated dry-cured ham sample extracted by the enzymatic method, containing $2.07 \mu\text{g kg}^{-1}$ (equivalent to 51.75 pg of OTA injected); c) the same naturally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