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## Validation data for HPLC/FLD determinations of ochratoxin A in red paprika and black pepper adopting a one step clean-up procedure

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### Abstract

Validation data for the determination of ochratoxin A (OTA) in two spice matrices, red paprika and black pepper were obtained for samples prepared with a simplified single step clean-up column Mycosep® 229. Extracts of finely ground samples of red paprika and black pepper were prepared and applied to a Mycosep® 229 Ochra clean-up column. The purified extract was then subjected to HPLC/FLD analysis. The relative standard deviation for repeatability (RSDr) of the method was 11.8% for red paprika and 9.9% for black pepper. The limit of detection (LOD) value (3 times the noise) was estimated as corresponding to the response of an extract derived from a blank matrix (previously washed) and spiked to 1.0 µg kg<sup>-1</sup>. The limit of quantitation (LOQ) (3 times LOD) was 3.0 µg kg<sup>-1</sup>. The performance of the one step column clean-up procedure appears to be a suitable alternative to commonly used clean-up techniques and allows the precise determination of OTA in two complex matrices.

**Keywords:** ochratoxin A (OTA), one step clean-up, Mycosep® 229 column, HPLC/FLD, red paprika, black pepper.

## Introduction

The contamination of various spices by aflatoxins and ochratoxin A (OTA) and the growth of the fungi responsible are dependent upon factors such as temperature, humidity, handling during harvesting and conditions of drying and storage. The European Commission EC set a legal limit only for aflatoxins, and the last Commission Regulation (Comm. Reg. EC, 2006) proposed a first legal limit for OTA in spices.

Only a few studies have been published concerning the occurrence of OTA in various spices such as black pepper, paprika, nutmeg, chili, coriander, ginger and turmeric (Thirumala-Devi et al., 2000; Fazekas et al., 2005; Jørgensen, 2005; Goryacheva et al.; 2006, Hierro et al., 2008). References are limited and there is a tendency to extrapolate the use of an analytical method, validated for a particular matrix, to the same mycotoxin in other commodities such as in spices. As a consequence, the procedures used for OTA estimation in various spices have been found to be influenced by substances present in different spice extracts, and methods suitable for particular spices need to be developed, particularly if the immunoaffinity column (IAC) sample clean-up or enzyme linked immuno-sorbent assay (ELISA) techniques are used. Moreover, although several "official methods" have been validated or adopted for several mycotoxins in a number of food commodities (Gilbert and Anklam, 2002; Monaci and Palmisano, 2004), particularly for aflatoxins, only a limited number of analytical methods for OTA have yet been validated, and are even more limited for spices.

The analytical methods that have been applied for OTA determination have included: HPLC methods, ELISA and GC/MS methods have been published (Scott, 2002; Monaci and Palmisano, 2004). The HPLC methods for OTA comprise reversed phase chromatography columns, mixtures of acetonitrile/water as mobile phases, and fluorescence detection, commonly referred to as HPLC/FLD. For sample extraction, a wide range of extraction solvents and their mixtures have been used: chloroform/ortho-phosphoric acid, dichloromethane/ethanol/ortho-phosphoric acid or other mixtures composed of ethyl acetate, toluene, methanol, acetonitrile, water, acetic acid or sodium bicarbonate. Post-column addition of ammonia enhance the fluorescence intensity (Langseth et al., 1993; Zimmerli and Dich, 1995), and with ESI interface and positive ionization, some LC-MS and LC-MS/MS methods have been proposed (Becker et al., 1998; Lau et al., 2000; Zollner et al., 2000).

Sample clean-up has been achieved by liquid-liquid extraction (LLE), solid-phase extraction (SPE) or immunoaffinity columns (IAC). The LLE is less acceptable because of the high solvent use and because of ecological concerns, and different methods have been published using various

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3 phase materials, like silica (Howell and Taylor, 1981), phenyl-phases (Dawlatana et al., 1996),  
4 cyano-phases (Cohen and Lapointe, 1986) and C18 phase (Terada et al., 1986), weak anion  
5 exchange and strong anion exchange (Biancardi and Riberzani, 1996; Akiyama et al., 1997;) and  
6 fluorosil (Seidel et al., 1993).  
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10 Two standardized extraction methods for the determination of OTA have been defined for  
11 cereals and cereal products (EN ISO1998 a,b) using an SPE column, the standard EN 14132 (2003  
12 a) is for barley and roasted coffee and adopts an IAC clean-up, while the fourth standard EN 14133  
13 (2003 b) for wine and beer also uses an IAC clean-up. Various studies have demonstrated that for  
14 wine and beer, the clean-up procedures are not necessary. (Bononi and Tateo, 2003; Bononi et al.,  
15 2004; Tateo et al., 1999, 2000; Tateo and Bononi, 2001, 2003a, 2003b).  
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21 Recently, a one step clean-up (OSCU) system has been adopted by Tateo and others to  
22 present validation data concerning aflatoxins in spices. The present study shows validation data  
23 concerning the determination of OTA in two spice matrices, red paprika and black pepper, using the  
24 simplified single step clean-up procedure with the Mycosep® 229 column, as previously proposed  
25 for cereals and foodstuffs (Buttinger et al., 2004).  
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## 31 **Materials and methods**

### 32 *Chemicals*

33 Acetonitrile and water (HPLC grade) were purchased from Sigma Aldrich (Milan, Italy).  
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37 High purity acetic acid glacial was purchased from Merck (Darmstadt, Germany).  
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39 Ochratoxin A ( $\geq 98\%$ ) at  $1024 \text{ ng mL}^{-1}$  in methanol was purchased from R-Biopharm Rhône Ltd  
40 (Milan, Italy). Mycosep® 229 Ochra clean up columns were provided from Romer Labs, (Union,  
41 MO, USA) and distributed by Orsell s.r.l. (Modena, Italy). For filtration, fluted filters Whatman 2  
42 were purchased from VWR International (Milan, Italy). Samples of each spice (paprika and pepper)  
43 derived from commodities purchased from various suppliers of the industry.  
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### 48 *Apparatus*

49 Liquid chromatography was performed with a HPLC Shimadzu system consisting of: a) System  
50 Controller SCL-10 A vp; b) two pumps LC-10AD vp; c) Rheodyne injection valve (7725i, 20  $\mu\text{L}$   
51 loop); d) fluorescence detector RF-10Ax1 using an excitation wavelength of 330 nm and an  
52 emission wavelength of 460 nm.; e) acquisition software Class VP-5 Shimadzu. Separation was  
53 achieved on a "GEMINI C18 110 A" column (250 x 4.6 mm, 5  $\mu\text{m}$ ) from Phenomenex (Bologna,  
54 Italy). Column temperature was set to 40°C. OTA was eluted under isocratic conditions with  
55 water/acetonitrile/acetic acid (72 + 27 +1 w/w/w) at a flow rate of  $1.5 \text{ mL min}^{-1}$ . A standard sample  
56 of OTA, the desired analyte, eluted at  $53 \pm 0.5 \text{ min}$  in this system.  
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3 LC-MS/MS experiments were carried out on a LC-ESI(+)-MS/MS system using an Agilent 1200  
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5 Series HPLC coupled to an Applied Biosystems API 2000 mass spectrometer (Toronto, Canada),  
6  
7 which was equipped with a TurbolonSpray Source (ESI). A stainless steel column (Macherey-  
8  
9 Nagel, EC/2 Nucleodur 100-5 C18) 150 mm x 2 mm in size with a 100 Å pore diameter (50 µm  
10  
11 particle size) was used. Water/5 mM ammonium acetate (Phase A) and methanol/5 mM ammonium  
12  
13 acetate (Phase B) were used for the mobile phase. The elution gradient was: from 20% to 100% B  
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15 in 7 min, isocratic for 8 min, returning to 20% B in 5 min for 5 min. Appropriate conditions for  
16  
17 multiple reaction monitoring (MRM) were adopted. The test solution was injected by continuous  
18  
19 infusion at 5 µL min<sup>-1</sup> in +Q1 mode and +MS<sup>2</sup> mode. The de-clustering potential (DP; 20V),  
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21 focusing potential (FP; 400V), entrance potential (EP; 10V), cell entrance potential (CEP; 15V),  
22  
23 collision energy (CE; 36V), and cell exit potential (CXP; 5V) were adopted to obtain the 401.1 to  
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25 239.0 m/z transition. The daughter ion at 239.0 m/z was used for the quantification and  
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27 identification by MRM. Probe temperature was 450°C.  
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#### 33 34 *Sample extraction and clean-up*

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36 Samples of each spice (paprika or pepper) were finely ground. An aliquot (25 g) of the pulverized  
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38 material was extracted with 100 mL of CH<sub>3</sub>CN/H<sub>2</sub>O 84:16 (w/w) in a 250 mL Pyrex® flask for 20  
39  
40 min in an ultrasonic bath. It was not necessary to acidify the solution because the pH value of the  
41  
42 extracting solvent results to be lower than 5.5 and after the addition of the matrix the pH value  
43  
44 results to be lower than 5.0. The extract was filtered through Whatman 2 filter paper.  
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46 Approximately 7 mL of the filtered crude extract were acidified with 70 µL of acetic acid and  
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48 transferred into the tube of a Mycosep® 229 Ochra clean-up column. The surface of the column  
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50 containing the adsorbent was pushed down so that about 4.5 mL of purified extract could be applied  
51  
52 on top of a single column. The purified extract (4 mL) was collected and diluted 1+2 with acidified  
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54 water (1% with acetic acid). For HPLC analysis, 20 µL of the solution was injected onto the  
55  
56 column.

#### 57 58 *Spiking and calibration curve*

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60 Samples of black pepper and of red paprika (25 g each) were spiked with various volumes (75-1250  
µL) of an OTA standard solution prepared in methanol (1024 µg L<sup>-1</sup>), and the added OTA  
concentrations ranged from 3.08- 51.25 µg kg<sup>-1</sup>. A series of purified extracts were prepared from  
the spiked samples of pepper and paprika, as described above. The standard addition studies were

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3 performed in triplicate. The extraction and clean up procedures was performed as described before  
4 and for HPLC analysis, 20  $\mu\text{L}$  of each solution was injected.

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7 The spiking operations were carried out by adding the standard solution to the spice, waiting for 1  
8 night in dark conditions in desiccator and under nitrogen headspace. At the end, vacuum was  
9 applied for 30 min to eliminate the residual methanol.

#### 10 11 *Comparison with LC-ESI(+)-MS/MS data*

12 To demonstrate the equivalence of this proposed method to alternative methods and in particular to  
13 demonstrate that the results obtained with this clean-up procedure are not altered from co-extracted  
14 interferences, the same samples were analyzed by LC-ESI(+)-MS/MS. For this purpose, the same  
15 samples were treated as described above and the calibration curve was obtained as described  
16 previously

### 17 18 19 20 21 22 23 24 **Results and discussion**

25 For extracts prepared from samples of black pepper or paprika which were spiked with increasing  
26 levels of OTA, there was excellent agreement between peak area and detected levels of OTA.  
27 Plotting the mean extracted OTA concentration as a function of the HPLC peak area values, yielded  
28 a linear regression line with regression coefficient 0.9971 and with a linear equation equal to:  
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$$30 \quad y = 6664.6 x + 30991$$

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38 The strict linear relationship indicates that the detection of OTA was not compromised by artifacts  
39 of the method at either the highest or the lowest levels of OTA. If the regression line was forced to  
40 start at the value  $x, y = 0$  and thereby eliminating the influence of endogenous OTA in the pepper or  
41 paprika matrix, the regression line results:  
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$$43 \quad y = 6664.6 x$$

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49 This linear relationship permits the calculation of the concentration of OTA from the peak area,  
50 including any correction depending on recovery and all extraction procedures. Thus, the  
51 instrumental responses expressed as peak area values were obtained, taking into account all  
52 extraction and clean-up conditions related to the method, including the matrix effect. Based on the  
53 data presented in Figures 1 and 2, the endogenous concentration of OTA in the presented sample of  
54 paprika, based on these two curves, may be calculated as  $4.6 \mu\text{g kg}^{-1}$ .

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60 The relative standard deviation for repeatability (RSDr) of the method was determined  
adopting the NMKL procedure n.5 (Nordic Committee on Food Analysis, 1997): the suggested



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3 method was applied to duplicate analyses of OTA in each sample and was applied to 10 different  
4 paprika samples with different contents of OTA, measured on different days. Data using these  
5 criteria are reported in Table 1 and the RSDr value result obtained for 10 samples of paprika equals  
6 11.8%. Repeating the experiments with 10 samples of pepper grains, we obtained an RSDr value of  
7 9.9%. For pepper, the endogenous levels of OTA, using the described method, ranged from 6.6 -  
8 18.7  $\mu\text{g kg}^{-1}$ .  
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15 The LOD value (3 times the noise) was estimated by direct experimental analysis, and indicated that  
16 the limit of detection corresponded to the response of an extract deriving from the blank matrix  
17 (previously washed) spiked with 1.0  $\mu\text{g kg}^{-1}$  OTA. LOQ (3 times LOD) results were recalculated as  
18 3.0  $\mu\text{g kg}^{-1}$ .  
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22 To demonstrate the absence of peaks interfering with OTA after clean up treatment also at the  
23 critical low concentrations, Figure 1 shows the clear identification of ion  $m/z = 239.0$  derived from  
24 the transition  $[\text{M}+\text{H}]^+ 404.1 \rightarrow 239.0 m/z$  in a red pepper sample not enriched and enriched with  
25 OTA at concentrations 1.2, 2.4 and 5.9  $\mu\text{g kg}^{-1}$ . The results derived from the LC-MS/MS method,  
26 surely not altered from interferences, were reported in comparison with the data obtained with the  
27 clean-up method: Table 2 demonstrated for all samples of Table 1 the accordance with LC-MS/MS  
28 data and than the absence of any interfering peak.  
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34 Table 1 contains only samples of market naturally contaminated. To give evidence of the  
35 cleanliness of the extracts in the range of r.t. including OTA, it can be useful to consider in Figure 2  
36 the blank sample obtained with the proposed clean-up FLD method. In the same figure are shown  
37 the traces obtained by spiking the blank sample to 20 and 60  $\mu\text{g kg}^{-1}$ : the quality of traces gives  
38 evidence of the cleanliness of the extracts and demonstrate the efficiency of the extraction. The  
39 method application, as before described, take in account any effect matrix influencing the extraction  
40 and particularly the recovery. In the concentration range experimented, in all cases the recovery  
41 evaluated by spiking technique results to be not less than 85%.  
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50 The performance of the one step clean-up system appears to be a suitable alternative to the  
51 commonly used clean-up techniques and allows the precise determination of OTA in two complex  
52 matrices, red paprika and pepper, for which maximum levels of 10 – 20  $\mu\text{g kg}^{-1}$  have been  
53 established for the commercial transactions within the international spice trade (Thirumala-Devi et  
54 al., 2001; Almela et al., 2007). Actually no limit for OTA in spices is included in EU Regulation  
55 1881/2006. (European Commission, 2006). Considering the data reported for *Capsicum* spp. and  
56 *Piper* spp. previously cited in the literature, our proposed method using the OSCU with the Micosep  
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229 column may be considered to be an useful alternative. Furthermore, the present procedure offers the benefits of limited procedural manipulations and lower costs.

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Table 1. Repeatability data and evaluation of RSDr for OTA in specimens of paprika, following the NMKL protocol.

Samples	Date	Results ( $\mu\text{g}/\text{kg}$ )		$x_{i(\text{mean})}$	$(a_i - b_i) / x_{i(\text{mean})}$	$[(a_i - b_i) / x_{i(\text{mean})}]^2$
		$a_i$	$b_i$			
1	11.06.08	5.145	4.658	4.902	0.099	0.010
2	13.06.08	12.348	7.450	9.899	0.495	0.245
3	16.06.08	13.393	13.428	13.410	-0.003	0.000
4	19.06.08	30.678	30.837	30.757	-0.005	0.000
5	23.06.08	49.070	50.803	49.937	-0.035	0.001
6	25.06.08	12.653	13.832	13.242	-0.089	0.008
7	27.06.08	6.269	5.951	6.110	0.052	0.003
8	01.07.08	23.201	21.944	22.572	0.056	0.003
9	03.07.08	31.894	32.345	32.120	0.014	0.000
10	04.07.08	8.432	9.190	8.811	-0.086	0.007

$$\sum [(a_i - b_i) / x_{i(\text{mean})}]^2 = 0.277$$

$$\text{RSDr} = \sqrt{[\sum [(a_i - b_i) / x_{i(\text{mean})}]^2 / 20]} = 0.118$$

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3 Table 2. Comparison between results (mean values) obtained for OTA in specimens of paprika by  
4 HPLC/FLD and LC-ESI(+)-MS/MS methods.  
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Samples	HPLC/FLD ( $\mu\text{g kg}^{-1}$ )	LC-ESI(+)-MS/MS ( $\mu\text{g kg}^{-1}$ )	
10			
11			
12			
13	1	4.9	4.6
14	2	9.9	10.5
15	3	13.4	13.9
16	4	30.7	32.0
17	5	49.9	52.2
18	6	13.2	14.0
19	7	6.1	5.5
20	8	22.6	23.2
21	9	32.1	33.1
22	10	8.8	8.6
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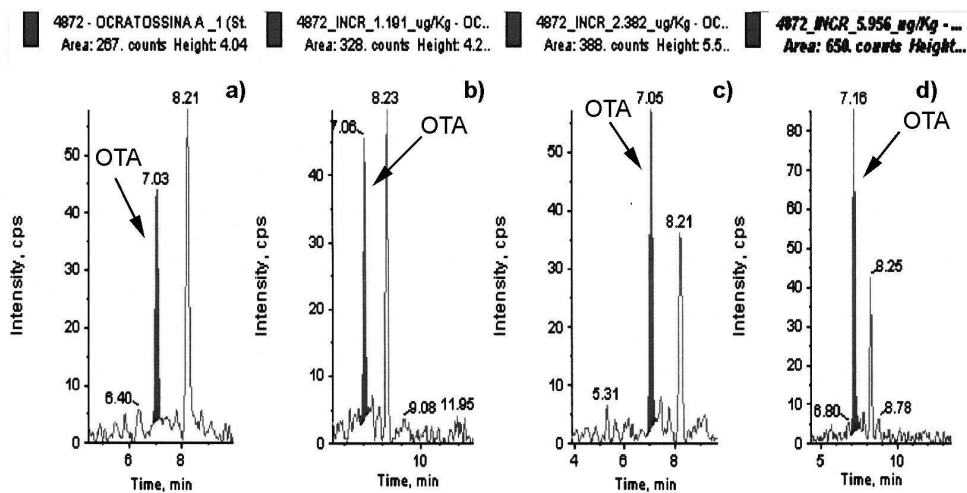


Figure 1. Transition  $[M+H]^+$  404.1  $\rightarrow$  239.0  $m/z$  (peak at r.t. 7.0 – 7.1 min.) in a red pepper sample not enriched (a) and enriched with OTA at concentrations 1.2 (b), 2.4 (c) and 5.9 (d)  $\mu\text{g kg}^{-1}$

149x90mm (285 x 285 DPI)



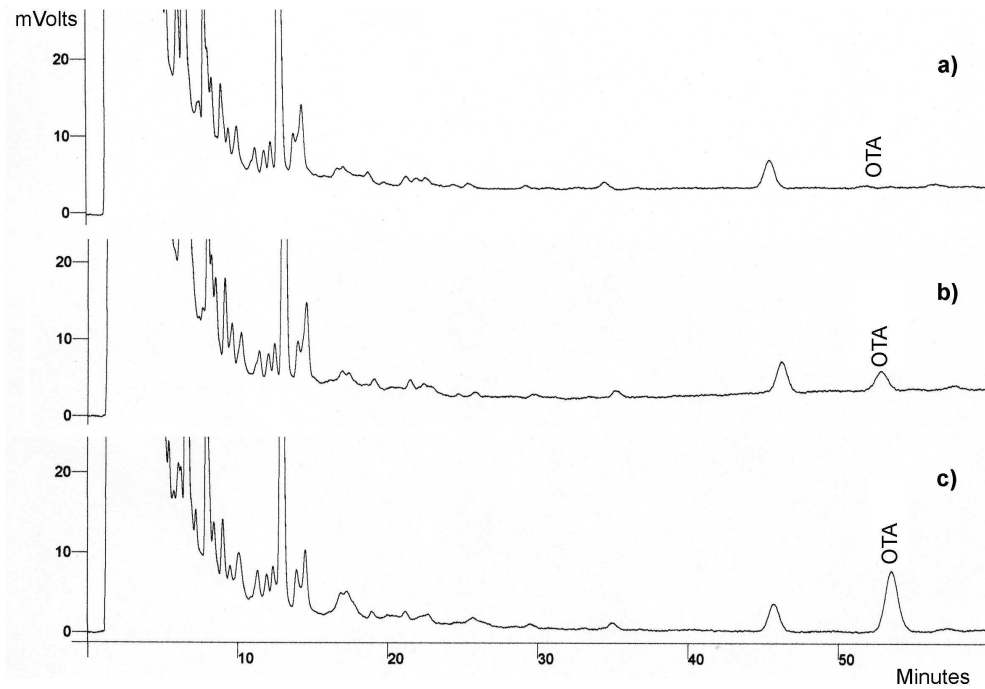


Figure 2. HPLC/FLD traces obtained for a blank red pepper sample (a) and by spiking the same sample to 20 (b) and 60 (c)  $\mu\text{g kg}^{-1}$  after cleanliness of the extract.  
149x102mm (403 x 403 DPI)