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Use of various clean-up procedures for the analysis of ochratoxin A in cereals

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Abstract

A rapid and reliable procedure has been developed for the determination of ochratoxin A in wheat and oats. The method consists of extraction of the sample with acidic chloroform, followed by defatting with *n*-hexane and finally, HPLC determination with fluorometric detection. Mean recoveries for wheat and oats spiked at levels between 1 and 100 μg/kg ranged from 80 to 104%. The limit of determination (field blank $+6\sigma$) was 0.8 μg/kg and the precision (within-laboratory relative standard deviation) ranged from 3 to 7%. The method was tested on 34 wheat and 34 oats samples. Ochratoxin A was confirmed in some positive samples by methyl ester formation and/or by clean-up of the extracts with immunoaffinity columns. The method was not appropriate for the analysis of barley (45 tested samples), rye (69 samples) or trout feed (13 samples). A false positive was recorded within the four positive barley samples and 18 false positives were recorded within the 21 positive rye samples whereas trout feed samples could not be analysed due to insufficient clean-up. The use of immunoaffinity columns made the analysis of trout feed and rye samples possible, providing excellent clean-up of the extracts with no false positive results and a good limit of determination (0.2 μg/kg).

Keywords: Food analysis; Sample preparation; Ochratoxins; Mycotoxins; Toxins

1. Introduction

Ochratoxin A (OTA), due to its worldwide occurrence and toxicity, is one of the most important mycotoxins together with aflatoxins, fumonisins, trichothecenes and zearalenone. OTA occurs in plant products such as cereals, beans, groundnuts, coffee, and wine, as well as pig blood and kidney, cereals (mainly wheat, barley and oats) being the main source for human exposure [1,2]. The carcinogenicity of OTA has been evaluated by the IARC in a recent monograph where OTA is classified as Group 2B, possible human carcinogen [2]. This mycotoxin has also been suspected of involvement in the aetiology of BEN (Balkan endemic nephropathy), and associated with urinary tract tumour due to the similarity between porcine nephropathy, caused by OTA, and BEN [3]. This possibility seems to be supported by the higher levels of OTA found in blood collected from humans in the Balkan endemic area compared to the levels found in nonendemic areas [4] and by the high levels of OTA found in human blood in other countries with a high incidence of human nephropathy [5]. In contrast, 100% incidence of positive samples (at low concentration) has been reported in human blood collected in southern Italy from healthy individuals as well as from individuals suffering from different kidney diseases [6]. The widespread occurrence of OTA in human blood reported by several authors, particularly in

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Europe [2,6], gives evidence of a worrying degree of exposure to this mycotoxin through the ingestion of contaminated foods. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) after evaluation of the toxic effect of OTA, proposed a provisional maximum tolerable weekly intake of 0.1 μg/kg body mass for this mycotoxin [7].

Currently eight countries have specific regulations for OTA in one or more commodities at levels ranging from 1 to 50 μg/kg for foods and from 5 to 300 μg/kg for animal feeds [8]. Within the EU, tolerance levels for OTA of 1 μg/kg have been suggested for infant foods and 5 μg/kg for cereals [9].

Several published analytical methods for the determination of OTA in cereals based on HPLC include a solid-phase extraction (SPE) clean-up step using C_{18}, Si 60, or immunoaffinity columns [10–12]. Some of these methods have also been tested by inter-laboratory studies at OTA contamination levels >2 μg/kg [12–15]. Recently CEN (European Committee for Standardization) has adopted two LC methods for determination of OTA in cereals and cereal products. The methods use silica gel or C_{18} cartridges for the clean-up and reversed-phase HPLC with fluorometric detection. The first method was validated by a inter-laboratory study for wheat containing 0.4 μg/kg and 1.2 μg/kg OTA, and the second one for barley (2.9–14.4 μg/kg OTA), maize (8.2 and 16.3 μg/kg OTA), and wheat bran (3.8 and 4.5 μg/kg OTA) [14,15]. Certified reference materials are made available by the European Commission SMT Programme relevant to blank wheat (CRM 471, <0.6 μg/kg OTA) and contaminated wheat (CRM 472, 8.2±1.0 μg/kg OTA) [16].

The development and validation of new analytical methods for OTA is still in progress, as evidenced by some recent publications [17–19] and an ongoing inter-laboratory study sponsored by the European Commission SMT Programme for the validation of analytical methods for determination of aflatoxin, ochratoxin A and patulin in foodstuffs of vegetable origin.

In the present paper we describe a rapid procedure based on liquid–liquid defatting instead of the SPE clean-up for the determination of OTA in cereals. This procedure has been shown to be reliable for wheat and oats, but not for barley, rye and mixed cereal matrices such as trout feed. An alternative method using immunoaffinity column clean-up is proposed for the analysis of these cereals as well as for confirmation of positive results obtained with other analytical methods.

2. Materials and methods

A stock solution (1.0 mg/ml) of OTA (Sigma, St. Louis, MO, USA) was prepared in toluene–acetic acid (99:1, v/v). Recovery experiments on wheat were performed by spiking blank samples (10 g) with 100 μl of spike solutions (prepared by appropriate dilution of the stock solution) containing 10, 30, 50, 100, 200 and 1000 ng OTA. Spiked samples were left for 1 h, to allow solvent evaporation prior to extraction with chloroform–aq. phosphoric acid. Similarly, recovery experiments on oats were performed at a spiking level of 5.0 μg/kg. OTA calibration standard solutions for HPLC determination were prepared by dissolving in acetonitrile–water–acetic acid (41:58:1, v/v) appropriate amounts of stock solution, evaporated under dry nitrogen, to obtain final concentrations of 0.5, 1.0, 3.0, 10.0 and 30.0 ng/ml.

The following numbers of samples were analyzed in the present study: 34 wheat, 34 oats, 45 barley and 27 rye, kindly provided by Dr. A. Rizzo (National Veterinary and Food Research Institute, Helsinki, Finland), 13 trout feed samples kindly provided by Dr. M. Caramelli (Experimental Institute for Zooprophylaxis of Piedmont, Liguria and Aosta Valley, Italy), and 42 rye samples collected in Italy.

Cereal and trout feed samples were ground using a Romer mill (Subsampling mill Model 2A, Romer Labs., Union, MO, USA) to obtain a 20-mesh sieve size. The ground samples were fully mixed and 10 g of each sample were extracted with 60 ml chloroform and 5 ml 0.1 *M* phosphoric acid by shaking for 30 min, and the extract filtered through a fluted filter paper. A 10-ml volume of filtrate was collected in a 10-ml vial and evaporated under a stream of nitrogen at ~60°C. The residue was quickly reconstituted in 600 μl of acetonitrile–water–acetic acid (41:58:1, v/v) by vortexing for 1 min, and the extract defatted with 1 ml *n*-hexane by vortexing again for 1 min and centrifuged at 4000 rpm (3290 *g*) for 10 min. The

lower phase (100 μl equivalent to 0.277 g of matrix) was injected into HPLC with full loop technique. OTA was quantified by measuring peak areas and comparing them with OTA external standards. The centrifugation step provided cloudless extracts suitable for HPLC analysis, although for some samples an additional filtration step (4-mm nylon syringe filter 0.45 μm, Alltech, Deerfield, IL, USA) was necessary.

Confirmation of the identity of OTA by methyl ester formation was performed for 11 selected positive cereal samples by derivatizing the extracts with 14% BF_3–methanol as described elsewhere [10]. In addition, 14 selected positive cereal samples were also extracted and purified through silica gel mini columns according Langseth et al. [11], and trout feed and some selected positive cereal samples were also purified by immunoaffinity clean-up prior HPLC analysis.

2.1. Immunoaffinity clean-up

This clean-up procedure was used both for confirmation of 15 positive cereal sample extracts resulting from the above screening and for purification of trout feed extracts for which inadequate clean-up was obtained with the above described procedure. The extract (450 μl, obtained as reported above), was diluted with 4.55 ml of acetonitrile–water–acetic acid (41:58:1, v/v), mixed by vortexing for 30 s followed by dilution with 20 ml water and further mixing. After filtration through 90-mm diameter GF/A glass microfibre filter (Whatman, Maidstone, UK), 10 ml of extract (equivalent to 0.5 g sample) were passed through the OchraTest immunoaffinity column (Vicam, Watertown, MA, USA) at a steady slow flow-rate of about 1–2 drops/s, followed by 10 ml of washing solution (25 g/l NaCl, 5 g/l $NaHCO_3$, 0.1 ml/l Tween-20) and 10 ml distilled water at flow-rate of 1–2 drops/s. OTA was eluted from the OchraTest column with 1.5 ml methanol, which was evaporated under a stream of nitrogen at ~60°C, reconstituted in 200 μl of acetonitrile–water–acetic acid (41:58:1, v/v), and 100 μl (equivalent to 0.25 g sample) were injected into the HPLC apparatus. The sample extracts were stored at +4°C prior to HPLC analysis.

2.2. HPLC determination

The HPLC system consisted of an isocratic pump (LKB 2150, Bromma, Sweden) connected to a fluorometric detector, MPF-44B or LC 240, (λ_{ex} = 340, λ_{em} = 460 nm) and a TURBOCHROM 4.0 data system (Perkin-Elmer, Norwalk, CT, USA). Separation was achieved on a C_{18} reversed-phase Supelcosil LC 18 column (15 cm×4.6 mm, 5 μm particles), with a guard column (20×4.6 mm) of the same packing material (Supelco, Bellefonte, PA, USA). The mobile phase consisted of a mixture of acetonitrile–water–acetic acid (99:99:2, v/v) at a flow-rate of 1 ml/min [10]. OTA was quantified by measuring peak areas, and comparing them with the relevant calibration curve.

3. Results and discussion

HPLC analysis of wheat and oats extracts after a simple defatting step with *n*-hexane, without any additional purification, gave clean extracts. Figs. 1 and 2 show two chromatograms typical of blank wheat and oats samples spiked with OTA at levels of 1.0 μg/kg and 5.0 μg/kg, respectively. No broadening of the OTA peak due to the high injection volume (100 μl) was observed because the higher polarity of the injection solvent (58% water compared to 49.5% of the mobile phase) allows OTA to concentrate on the top of the HPLC column shortly after the injection.

Recoveries from wheat spiked with OTA at various levels in the range 1–100 μg/kg were consistently equal to, or higher than 80% and within-laboratory relative standard deviation ($R.S.D._r$) values were lower than 7% (Table 1). The recovery from oats spiked at levels of 5.0 μg/kg (three replicates) was 96%, with $R.S.D._r$=4%. The good recoveries obtained with this method are related to the reduced number of steps, as compared to other methods, prior to HPLC analysis. The limit of determination was calculated by measuring the field blank $+6\sigma$, where σ is the standard deviation of the field blank value signal measured on three replicates. The limit of determination of the method was 2 μg/kg, or 0.8 μg/kg using the Perkin-Elmer MPF 44B or LC 240, fluorometric detector, respectively.

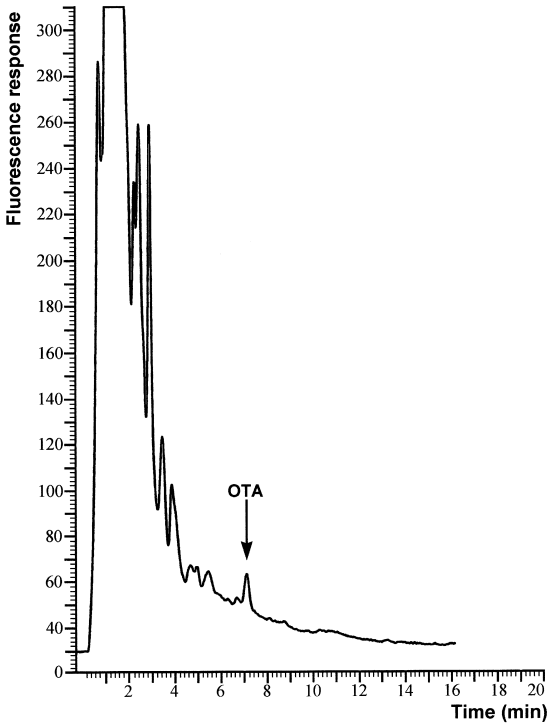

Fig. 1. Chromatogram of blank wheat spiked with OTA at a level of 1.0 μg/kg; injected volume, 100 μl equivalent to 277 mg sample material. Column, Supelcosil LC 18 (150×4.6 mm I.D.); mobile phase, acetonitrile–water–acetic acid (99:99:2, v/v); flow-rate, 1 ml/min; detection, fluorescence at 340 nm excitation and 460 nm emission.

A satisfactory HPLC calibration curve was obtained in the range of 0.05–3.0 ng OTA, and the linearity of the curve conformed to the criteria of van Trijp and Roos [20]. In particular the coefficient of correlation *r*, slope and *y*-intercept values of the calibration graph were 0.99989, −441.00 and 858 219.46 respectively. Minimum and maximum values of the percent of the *y*/*x* ratio obtained in the five point calibration curve were between 90 and 110%, indicative of a good calibration curve [20].

The use of a simple liquid–liquid partition instead of a SPE clean-up makes this method more rapid and inexpensive. The method has been successfully used by our laboratory during a certification exercise for OTA in wheat materials, sponsored by the European Commission SMT Programme. Of the fifteen laboratories participating in this study, only ten produced results that were accepted for setting the certification value as they fulfilled the required criteria [17]. In particular, the results obtained by our laboratory by using this method gave 92–98% recoveries, and within-day and between-day repeatability (R.S.D.$_r$) of results were ≤7% based on replicate analyses of a contaminated wheat sample performed on 2 days.

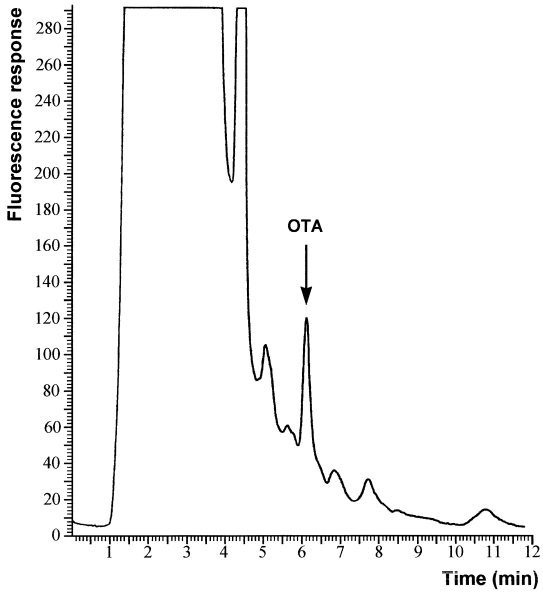

Fig. 2. Chromatogram of blank oat spiked with OTA at level of 5.0 μg/kg; injected volume, 100 μl equivalent to 277 mg sample material; chromatographic conditions as in Fig. 1.

The reliability of this method was confirmed by the analysis of 34 samples of wheat and 34 samples of oats, resulting in no false positives when the positive samples were re-extracted and analyzed with

Table 1
Recoveries of OTA added to blank wheat obtained with the liquid–liquid clean-up method

Spiking level (μg/kg)	Recoveries[a] (%)	R.S.D. (%)
1.0	80	7
3.0	82	6
5.0	84	5
10.0	98	4
20.0	104	3
100.0	83	6
Mean recovery±1S.D.	89±9	

[a] Number of replicates=3.

Table 2
Results obtained on analysing positive samples by different analytical methods

Matrix (positive/total)	No.	OTA (μg/kg)			
		Liquid–liquid clean-up	Immunoaffinity clean-up	Methyl ester formation	Silica clean-up
Wheat	24	430		350	
(2/34)	10	188		170	
Oats	72	56.6	67.0		
(2/34)	89	1.4	0.9		
Barley	2	11.7	12.1		
(3/45)	87	12.3	13.1		
	80	0.9	0.3		
	68	21.8		0	0.4
Trout feed	7		1.9		
(4/13)	5		0.4		
	6		0.7		
	13		0.7		

immunoaffinity clean-up or methyl ester formation as reported in Table 2. Moreover, the quantitative results obtained with the methyl ester formation or immunoaffinity clean-up were similar to those obtained with the proposed method. Table 2 also reports the results obtained with barley and trout feed samples. The trout feed samples were analysed by using immunoaffinity clean-up since the method described above did not produce sufficiently clean extracts. Three out of the four positive results found from the analysis of the 45 barley samples were confirmed with immunoaffinity clean-up whereas the fourth was found to be a false positive as no OTA methyl ester was detected when the sample was re-extracted, derivatized with BF_3–methanol and analyzed by HPLC. However, a low level of OTA contamination (0.4 μg/g) was detected when this sample was re-extracted using a silica gel mini column for the clean-up [11].

Twenty-one out of 69 rye samples analyzed by liquid–liquid clean-up were found positive for OTA, at levels ranging from 1.0 to 105.9 μg/kg, as shown in Table 3. However, when these positive samples were analyzed by different methods, eight of them (Nos. 125, 128, 136, 140, 139, 126, 200 and 201) were found to be truly false positives, whereas ten samples (Nos. 75, 74, 121, 124, 127, 129, 135, 130, 131 and 137) were found positive, but at levels lower than expected (see Table 3). The results matched very well for only two samples (Nos. 37 and 77), whereas for one sample (No. 120) the results obtained with immunoaffinity clean-up and OTA methyl ester formation were higher. The liquid–liquid clean-up is not suitable for analysis of OTA in rye. Immunoaffinity clean-up proved to be a good alternative because the specificity of the OTA antibody allows elimination of interfering compounds which could give rise to false positive results. Clean extracts were obtained with this procedure also when complex matrices such as trout feed samples were analyzed, as shown in Fig. 3 reporting the chromatogram of the trout feed extract found contaminated with OTA at level of 1.9 μg/kg. The same sample analysed by using the liquid–liquid extraction gave a dirty extract which produced an unreadable chromatogram with a profile that masked the OTA peak giving rise to false negative result. Four out of thirteen samples of trout feed analyzed were found contaminated with OTA at levels ranging from 0.4 to 1.9 μg/kg, as reported in Table 2.

The accuracy of the immunoaffinity clean-up was tested on wheat reference material, CRM 472, containing 8.2 μg/kg OTA, with an uncertainty of 1.0 μg/kg. The level of OTA found was 8.05 μg/kg, confirming the good performance of this type of clean-up. The limit of determination of 0.2 μg/kg, calculated as reported above, can be obtained by injecting 100 μl extract, equivalent to 0.25 g sample.

In a recent AOAC collaborative study of the LC method applied to corn, barley, and tissue, 15 of the

Table 3
Results obtained on analysing positive rye samples by different analytical methods

Sample (positive/total)	No.	OTA (μg/kg)			
		Liquid–liquid clean-up	Immunoaffinity clean-up	Methyl ester formation	Silica clean-up
Rye	75	105.9		62.3	
(13/69)	77	1.3	1.0		
	37	1.3	1.1		
	74	17.2	0.7		0.6
	120	7.0	12.8	12.8	
	121	9.3	0.1		
	124	2.2	0.1		1.2
	125	1.3	0		0
	127	23.9	0.6		0
	129	66.4	0.1	0	0
	135	16.0	0.21		1.1
	130	3.7			0.5
	131	5.7			0.8
	128	5.9			0
	136	1.2			0
	137	3.5			0.7
	140	4.2		0	
	139	3.1		0	
	126	1.3		0	
	200	1.0		0	
	201	1.0		0	

41 blanks were reported to contain OTA and in three cases the false positives were even confirmed after derivatization to the methyl ester [10]. The LC method employed in the AOAC study comprised the purification on C_{18} cartridge of sodium hydrogencarbonate extract obtained after liquid–liquid partitioning of chloroform extract. In addition, serious difficulties with false positives were encountered by Zimmerli and Dick [17] using several different silica gel adsorbents for clean-up. The same authors found that immunoaffinity columns, different from those used in the present study, contained up to 0.5 ng OTA per cartridge [17]. We did not encounter this OTA background problem with the Vicam immunoaffinity columns as demonstrated by the several samples found negative for OTA in the present study. Therefore the use of immunoaffinity columns could be a useful tool for confirmation of positive results, provided that the absence of OTA in the columns is previously ascertained.

The possibility that false negative results could occur with the procedures described in this paper (liquid–liquid clean-up) can be reasonably excluded because the method provides adequate sensitivity and recoveries at the level of application.

4. Conclusions

The use of a simple liquid–liquid defatting clean-up step followed by reversed-phase HPLC with fluorometric detection provides good results in terms of rapidity, expense, accuracy, repeatability and sensitivity for the analysis of OTA in wheat and oats.

Barley samples could also be screened by this method but confirmation of positive samples is needed by using alternative clean-up procedures, such as immunoaffinity. The use of immunoaffinity columns provides satisfactory results in terms of extract clean-up, accuracy and sensitivity also for analysis of rye and other complex matrices, such as trout feed samples. This procedure could also be used as a confirmation of wheat and oat samples found positive with the liquid–liquid clean-up procedure.

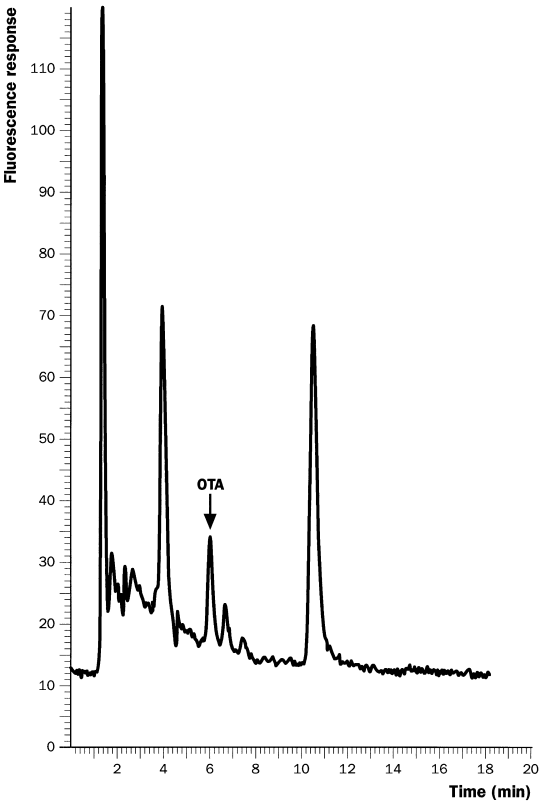

Fig. 3. Chromatogram of trout feed, naturally contaminated with 1.9 μg/kg OTA, after clean-up by immunoaffinity column. Injected volume, 40 μl equivalent to 100 mg feed. Chromatographic conditions as in Fig. 1.

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References

[1] R. Battaglia, T. Hatzold, R. Kroes, Food Addit. Contam. 13 (1996) 1–3.
[2] IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occurring Substances; Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins, Vol. 56, International Agency for Research on Cancer, Geneva, 1993, pp. 489–521.
[3] P. Krogh, in: A. Puchlev, I.V. Dinev, B., Doichinov, D., (Editors), Endemic Nephropathy, Bulgarian Academy of Sciences, Sofia, 1974, pp. 266–270.
[4] T. Petkova-Bocharova, M. Castegnaro, in: M. Castegnaro, R. Plèstina, G. Dirheimer, I.N. Chernozemsky, H. Bartsch (Editors), Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours, IARC Scientific Publication No. 115, IARC, Lyon, 1991, pp. 135–137.
[5] K. Maaroufi, A. Achour, A.M. Betbeder, M. Hammami, F. Ellouz, E.F. Creppy, H. Bacha, Arch. Toxicol. 69 (1995) 552–558.
[6] A. Breitholtz-Emanuelsson, F. Minervini, K. Hult, A. Visconti, Nat. Toxins 2 (1994) 366–370.
[7] Food and Agriculture Organization of the United Nations, Worldwide Regulations for Mycotoxins 1995, a Compendium, FAO Food and Nutrition Paper, 64, Rome, 1997, pp. 6–36.
[8] H.P. van Egmond, in: R. Battaglia, T. Hatzold, R. Kroes (Editors), Occurrence and Significance of Ochratoxin A in Food, Food Addit. Contam. 13, Supplement, 1996, pp. 11–13.
[9] G. Verardi, H. Rosner, Nat. Toxins 3 (1995) 337–340.
[10] S. Nesheim, M.E. Stack, M.W. Trucksess, R.M. Eppley, J. AOAC Int. 75 (1992) 481–487.
[11] W. Langseth, Y. Ellingsen, U. Nyomoen, E.M. Okland, J. Chromatogr. 478 (1989) 269–274.
[12] M. Sharman, S. MacDonald, J. Gilbert, J. Chromatogr. 603 (1992) 285–289.
[13] K. Larsson, T. Möller, J. AOAC Int. 79 (1996) 1102–1105.
[14] European Committee for Standardization, CEN/TC 275/WG 5 Food analysis — Horizontal Methods Biotoxins, prEN 15141-1 Document No. 156, 1997.
[15] European Committee for Standardization, CEN/TC 275/WG 5 Food analysis — Horizontal Methods Biotoxins, prEN 15141-2 Document No. 157, 1997.
[16] G.M. Wood, S. Patel, C. Entwisle, A.C. Williams, A. Boenke, P.J. Farnell, Food Addit. Contam. 14 (1997) 237–248.
[17] B. Zimmerli, R. Dick, J. Chromatogr. B 666 (1995) 85–99.
[18] I. Studer-Rohr, D.R. Dietrich, J. Schlatter, C. Schlatter, Food Chem. Toxicol. 33 (1995) 341–355.
[19] B.G. Osborne, F. Ibe, G.L. Brown, F. Petagine, K.A. Scudamore, J. Banks, M.T. Hetmanski, C.T. Leonard, Food Addit. Contam. 13 (1996) 141–153.
[20] J.M.P. van Trijp, A.H. Roos, RIKILT Report 91.02; RIKILT-DLO, Wageningen, 1991.