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Determination of zearalenone in corn by means of immunoaffinity clean-up and high-performance liquid chromatography with fluorescence detection

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Abstract

A rapid and accurate method to quantify zearalenone in corn is described. The method uses immunoaffinity chromatography for purification and high-performance liquid chromatography (HPLC) for detection and quantification of the toxin. Corn samples were extracted with acetonitrile–water (90:10, v/v) and the extract was diluted with water (1:10, v/v) and applied to a Vicam ZearalaTest immunoaffinity column. The column was washed with water and zearalenone was eluted with methanol and quantified by reversed-phase HPLC with fluorometric detection (λ_{ex}=274 nm, λ_{em}=440 nm) using acetonitrile–water–methanol (46:46:8, v/v) as mobile phase. Zearalenone recoveries from the ZearalaTest column were higher than 95%, and the column can hold a maximum of 4.0 μg of toxin. Average recoveries of zearalenone from corn spiked at levels of 0.1–10 μg/g ranged from 93 to 99.5%, with relative standard deviations of <6%. The detection limit was 3 ng/g based on a signal-to-noise ratio of 3:1. Comparative analysis of 14 naturally contaminated samples using this method and the AOAC official method 985.18 showed a reasonable correlation (r=0.87). Advantages of the immunoaffinity method as compared to the AOAC method are discussed.

Keywords: *Fusarium* spp.; Immunoaffinity chromatography; Sample preparation; Food analysis; Zearalenone; Mycotoxins; Toxins

1. Introduction

Zearalenone {6-[(10*S*)-10-hydroxy-6-oxo-*trans*-1-undecenyl]-β-resorcylic acid lactone} is one of the most widely distributed mycotoxins produced by *Fusarium* species, in particular *F. graminearum*, *F. culmorum* and *F. crookwellense*, responsible for important diseases of various cereals. It is associated mainly with corn but it occurs also in wheat, barley and sorghum among other commodities frequently used in human and animal diets [1]. Zearalenone is a strong estrogenic and anabolic compound that causes reproductive problems in farm animals, especially swine. Symptoms may include vaginal swelling (vulvovaginitis) and, in severe cases, vaginal and rectal prolapses, especially in immature gilts (swine). In young male swine it may cause testicular atrophy and enlargement of the mammary glands. Zearalenone ingestion may also cause prolonged estrus and reduced sex drive, infertility, fetal mummification, abortion, stillbirths and reduced litter size [2]. Serious impact on profit returns may be registered by swine producers and the hog industry as a consequence of reproductive failure related to the

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presence of zearalenone in corn [3]. Although further studies are required to confirm whether zearalenone should be considered a potential human carcinogen, positive evidence of carcinogenicity in experimental animals has been shown [1].

Presently six countries, namely Austria, Brazil, France, Romania, Russia and Uruguay, have specific regulations for zearalenone in foods or feeds, with maximum tolerated levels ranging from 30 to 1000 ng/g [4].

Current analytical methods for zearalenone include thin-layer chromatography [5–7], gas–liquid chromatography [5,8], gas chromatography–mass spectrometry [8,9], and HPLC [5,10–14]. Although several of these methods achieve low levels of detection, in practice they consume large amounts of time and solvent, and require one or more clean-up steps involving liquid–liquid partition or solid-phase extraction. Immunological methods, including line immunoblot [15], dipstick immunoassay [16] and enzyme-linked immunosorbent assay (ELISA) [17,18], have been used for zearalenone determination in combination with other mycotoxins. The ELISA method has also been tested collaboratively and approved by the AOAC as first action screening method for zearalenone concentrations higher than 800 ng/g [17].

During the past few years, a strong impulse to the improvement of mycotoxin analysis has been given by the introduction of an important clean-up technique using antibody-based immunoaffinity columns (IAC), which provides clean extracts and minimizes the interference of co-extracted compounds from complex matrices, such as cereals or cereal-based foods and feeds in which mycotoxins generally occur. IACs are available commercially for a number of mycotoxins, including aflatoxins, ochratoxin A, fumonisins, deoxynivalenol and zearalenone [19].

In this paper we describe the performances of a method for the determination of zearalenone in corn using HPLC with fluorescence detection coupled with a clean-up step using commercially available IAC, the Vicam ZearalaTest. The application of the method to the analysis of several naturally contaminated corn samples as compared to the liquid chromatographic AOAC official method is also described.

2. Experimental

2.1. Chemicals and materials

Zearalenone stock solution was prepared by dissolving in methanol (1 mg/ml) the solid standard purchased from Sigma (St. Louis, MO, USA). Zearalenone standard solutions for HPLC calibration or spiking purposes were prepared by dissolving in the mobile phase or methanol, respectively, adequate amounts of the stock solution, previously evaporated to dryness under nitrogen stream. Acetonitrile, methanol and water (HPLC grade) and potassium chloride (ACS) were purchased from Mallinckrodt Baker (Milan, Italy).

ZearalaTest immunoaffinity columns were obtained from Vicam L.P. (Watertown, MA, USA). Glass microfibre filters (Whatman GF/A) and paper filters (Whatman No. 1) were obtained from Whatman (Maidstone, UK).

The blank corn material used for recovery experiments was obtained from a retail store in Bari (Italy). Naturally contaminated corn samples, collected in northern Italy, were kindly provided by Dr. P. Sarzi Amadé (Safe Food, Viadana, Italy).

2.2. Apparatus

The HPLC apparatus consisted of a LKB 2150 isocratic pump (LKB, Bromma, Sweden) equipped with a Rheodyne Model 7125 injection valve (Rheodyne, Cotati, CA, USA), a Perkin-Elmer LC 240 fluorometric detector and a TURBOCHROM 4.0 data system (Perkin-Elmer, Norwalk, CT, USA). The analytical column was a C_{18} reversed-phase Supelcosil LC-18 (15 cm×4.6 mm, 5 μm particles) (Supelco, Bellefonte, PA, USA) preceded by a Rheodyne guard filter (0.5 μm).

2.3. Sample preparation and immunoaffinity clean-up

About 1 kg of corn sample was finely ground by a Model 2A Romer mill (Romer, Union, MO, USA) and homogenized. Subsamples (about 100 g) were taken and stored at −20°C until analysis. Ground sample (20 g) were weighed into a blender jar, 2 g

KCl added and extracted with 50 ml acetonitrile–water (90:10, v/v) by blending at high speed for 2 min (Sorvall Omnimixer). The extract was filtered through filter paper and 10 ml filtrate was collected and mixed with 90 ml distilled water. The diluted extract was filtered through Whatman GF/A glass microfibre filter and the filtrate collected. A 10-ml volume of diluted extract (equivalent to 0.4 g sample) was passed through the ZearalaTest immunoaffinity column at a flow-rate of about 1 drop/s, followed by 2×5 ml distilled water at 1–2 drops/s flow-rate. Zearalenone was then eluted with 1.5 ml methanol and collected in a clean vial. The eluted extract was evaporated under nitrogen stream at ~50°C and reconstituted with 250 μl of the HPLC mobile phase.

2.4. HPLC determination and confirmation of zearalenone

One hundred μl of reconstituted extract (equivalent to 0.16 g of sample) was injected into the chromatographic apparatus by full loop injection system. The mobile phase consisted of a mixture of acetonitrile–water–methanol (46:46:8, v/v) eluted at a flow-rate of 1.0 ml/min. Quantification of zearalenone was performed by measuring peak areas at zearalenone retention time, and comparing them with the relevant calibration curve.

The identity of zearalenone was confirmed in all positive samples by injecting sequentially sample extracts using 274 nm and 236 nm excitation wavelengths (440 nm emission wavelength) and comparing the peak area ratio (236 nm/274 nm) with that of zearalenone standard.

2.5. Comparison with the AOAC official method

In order to have a comparison with a well established method for the determination of zearalenone in corn, the naturally contaminated samples were analyzed by both the immunoaffinity method described herein and the AOAC official method 985.18 [20]. This method was applied according to the published protocol [20], with minor differences, i.e. the fluorescence detector was set at 274 nm (excitation) and 440 nm (emission) instead of 236 nm and 418 nm cut-off filter, respectively (see Section 3), and the mobile phase flow-rate was 1.3 ml/min (instead of 2 ml/min to limit high instrument back-pressure).

2.6. Column capacity

The capacity of the ZearalaTest columns was determined by comparing (duplicate measurements) the amount of zearalenone added to the immunoaffinity column with the amount bound. Different amounts of zearalenone, from 0.8 to 8.0 μg, were added to the immunoaffinity column by loading 10 ml (equivalent to 0.4 g matrix) of blank corn extract spiked with the corresponding amount of zearalenone.

2.7. Recovery experiments

Recovery experiments were performed in quadruplicate by spiking blank corn samples with zearalenone at levels of 0.1, 0.5, 1.0, 2.0, 4.0 and 10.0 μg/g. Spiked samples were left for 1 h, to allow solvent evaporation prior to extraction with acetonitrile–water.

3. Results and discussion

3.1. Choice of the excitation and emission wavelengths

The UV spectrum of zearalenone in methanol shows major absorption maxima at 236 nm (ϵ= 29 200 $l\,mol^{-1}\,cm^{-1}$) and 274 nm (ϵ=13 040 $l\,mol^{-1}\,cm^{-1}$), respectively, whereas the maximum of fluorescence emission occurs at 460 nm. Most publications use 236 nm or 274 nm as excitation wavelength and 418 nm cut-off filter or 440 nm emission for fluorescence detection of zearalenone [5,10,11,13,14,20]. Six excitation/emission wavelength combinations (236 and 274 nm excitation and 418, 440 and 460 nm emission) were tested herein to assess the optimum conditions for zearalenone detection. The emission wavelength at 418 nm was rejected because it generates fluorescence signals of low intensity. The highest fluorescence signals were

observed when using excitation at 236 nm and emission at 440 nm or 460 nm, but with an undesirable increase of the noise intensity that did not allow detection of zearalenone at levels <1 ng (absolute injected amount). The best results in terms of signal-to-noise ratio were obtained with the 274 nm excitation/440 nm emission wavelength combination; under these experimental conditions zearalenone could be detected at levels as low as 0.33 ng (signal-to-noise, 3:1). This wavelength combination was then used in the present study for zearalenone determination with both the immunoaffinity method and the AOAC official method.

3.2. Performance of the ZearalaTest immunoaffinity columns

The anti-zearalenone antibodies used by the manufacturer to prepare the columns bind zearalenone analogues, especially α-zearalanol; in particular, they showed 100% cross-reactivity to α-zearalanol and more than 80% cross-reactivity to the other zearalenone analogues, i.e. β-zearalanol, zearalanone and α- and β-zearalenol [21]. However, this does not represent a problem when zearalenone determination is performed by HPLC which provides a good separation of these compounds from zearalenone.

The ZearalaTest column capacity was found to be 4.0 μg of zearalenone. Above this level no increase of the fluorescence response was observed, indicating the saturation of zearalenone binding sites (Fig. 1). Zearalenone recoveries from the column below the saturation level were higher than 95%.

To evaluate the immunoaffinity column performances with respect to matrix interferences, different volumes of diluted blank corn extract, spiked with 1 μg/g zearalenone, were loaded onto the column (triplicate measurements). Up to 30 ml of corn extract (corresponding to 1.2 g of corn material) were used. Zearalenone recoveries decreased from 95 to 80% by increasing the loading volume from 2.5 to 15 ml, although a good precision (R.S.D.< 3%) was maintained. Optimal conditions were found with 10 ml of extract. When 20 ml or more extract was loaded onto the column, the elution was slowed and cloudy extracts were obtained. Therefore, the loading of such high volumes requires vacuum to allow elution of the extract and an additional filtration step before HPLC injection. Serious problems were encountered when filtration was performed with nylon filters (Micro-Spin Centrifuge Filter, Alltech, Deerfield, IL, USA) which resulted in the loss of zearalenone (up to 40%), in agreement with similar findings reported elsewhere [12].

Cloudy extracts (requiring filtration) were also found when a dilution factor 1:5 (v/v with water

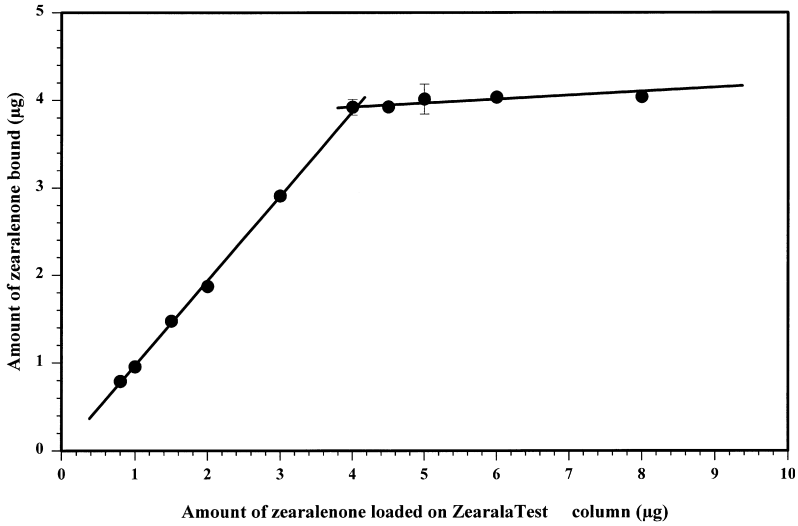

Fig. 1. Binding performance of anti-zearalenone antibodies used in the ZearalaTest immunoaffinity columns. Averages of duplicate measurements (±1S.D.) are represented.

before loading the extract onto column) was used instead of 1:10 in the attempt to increase the sensitivity of the method.

3.3. Performance of the analytical method

Results of the recovery experiments (quadruplicate measurements) of the full analytical procedure carried out on spiked corn samples are reported in Table 1. Within the spiking range 0.1–10 μg/g the overall average recovery was 95.6%, with minimum value at 92.8%, and the average R.S.D. was 2.9%, with maximum value at 5.4%. These results are better than those obtained from the AOAC collaborative study, based on liquid–liquid partition clean-up at different pH values [10], where recoveries of spiked samples (0.05 to 4.00 μg/g) averaged 86.3% (from 78.7 to 103%) and the average repeatability was 30% (ranging from 23 to 41%). The effect of the pH has not been taken into account in the present study due to the fact that a protocol of analysis is followed which does not imply any significant change of pH.

The limit of detection was 3 ng/g, based on a signal-to-noise of 3:1. The range of applicability of the method was from 3 to 10 000 ng/g of zearalenone in corn. For higher levels of contamination, the analysis should be repeated after appropriate dilution of the acetonitrile–water extract.

Although no collaborative validation of the method presented herein has been performed, the recovery and repeatability data reported above are within the criteria approved by CEN, the European Committee for Standardization, for the acceptability of analytical methods for zearalenone, i.e. recoveries between 70 and 100% and $R.S.D._r$ (within-laboratory relative standard deviation) <25% for zearalenone concentrations higher than 100 ng/g [22].

Table 1
Recoveries (and relevant R.S.D.s) obtained with the immunoaffinity method from blank corn spiked with zearalenone at different levels

Spiking level (μg/g)	Recovery±S.D.[a] (%)	R.S.D. (%)
0.1	98.6±5.3	5.4
0.5	94.2±1.8	1.9
1.0	95.4±3.7	3.9
2.0	99.5±2.5	2.5
4.0	92.8±2.9	3.1
10.0	92.8±0.4	0.4

[a] S.D.=standard deviation (n=4 replicates)

3.4. Analysis of naturally contaminated samples and comparison with the AOAC official method 958.15

The analysis of fourteen corn samples which originated from northern Italy, 1997 crop, revealed the occurrence of zearalenone in all tested samples, as shown in Table 2. The high incidence of zearalenone contamination of corn is not surprising, as similar results have been reported in several countries. In particular, a multiyear monitoring of Canadian grains showed 100% incidence (although at relatively low levels) of zearalenone contamination in corn produced in Ontario in 4 years and generally higher than 67% in other years [6]; in some Argentinian regions (over 2200 samples analyzed over a period of 10 years) contamination was found every year in corn with incidence up to 49% [23]; and 58% of corn samples were found contaminated by zearalenone in a survey involving 19 countries [24].

The results obtained with the immunoaffinity method were in good agreement with those obtained with the AOAC official method (Table 2). One false negative sample (2) was found with the AOAC method and only three samples (7, 8 and 11) showed R.S.D.s higher than 50% (Table 2). These results obtained with single measurements from two different methods are however better than those found in the AOAC collaborative study, which gave a repeatability of 44.4% from duplicate analysis using the same method.

The AOAC method vs. immunoaffinity method regression curve (Fig. 2) showed an R.S.D. of 0.87 and the slope of 0.75 indicated better recoveries of the immunoaffinity method. Sample 8 was removed from the data set used in Fig. 2 and the relevant statistical calculation because it proved to be an outlier when the 95% confidence interval was considered for the regression curve using all original data. Both the results of the analysis of naturally contaminated samples and the recoveries obtained with spiked materials are indicative of a better accuracy of the immunoaffinity method as compared

Table 2
Determination of zearalenone in naturally contaminated corn by the immunoaffinity and the AOAC official method 985.18

Sample	Immunoaffinity method (ng/g)	AOAC official method (ng/g)	R.S.D. (%)
1	45	64	24.7
2	9.5	N.d.	–
3	78	44	39.4
4	3.6	4	7.4
5	18	9.6	43.0
6	60	71	11.9
7	23	10.4	53.3
8	55	140	61.6
9	78	77	0.9
10	150	100	28.3
11	9	39	88.4
12	66	40	34.7
13	37	27	22.1
14	11	8.6	17.3
15	N.d.	N.d.	–

N.d.=not detected (<3 ng/g).

to the AOAC one, although a collaborative testing of the immunoaffinity method is necessary.

Figs. 3 and 4 show typical chromatograms of naturally contaminated samples containing levels of zearalenone close to the detection limit (Fig. 3) and to a contamination value commonly found in corn (Fig. 4). Fig. 4 shows clearly the absence of interfering signals at the zearalenone retention time in the immunoaffinity purified extract (right), whereas the sample analyzed by the AOAC method (left) shows an interfering peak. A similar interfering peak was also observed in sample 4 together with another more important peak at longer retention time (Fig. 3, left). These peaks are not present in the chromato-

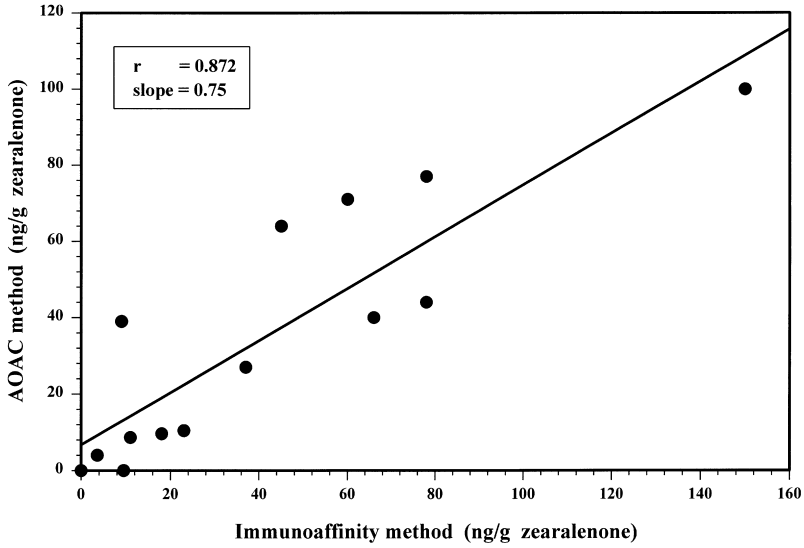

Fig. 2. Regression curve of immunoaffinity method vs. AOAC official method for the determination of zearalenone in a blank and 14 naturally contaminated corn samples.

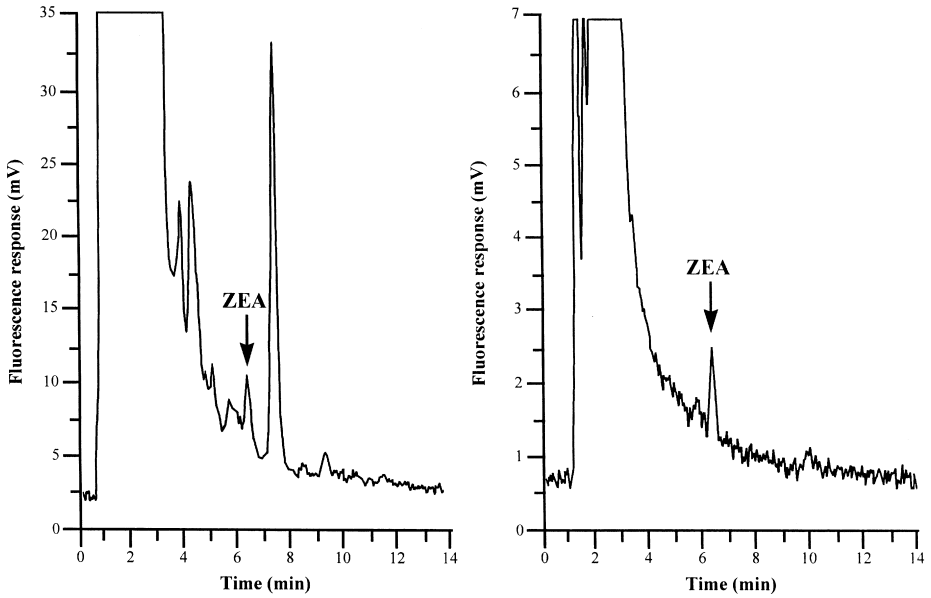

Fig. 3. Chromatograms relevant to a corn extract (sample 4) naturally contaminated at levels close to the detection limit: (left) AOAC official method and (right) immunoaffinity method. *AOAC method*. Injected extract: 20 μl (0.4 g sample equivalent); Column: Supelcosil LC-18 (150×4.6 mm I.D., 5 μm); mobile phase: acetonitrile–water–methanol (1.6:2.0:1.0, v/v); flow-rate: 1.3 ml/min; detection: fluorescence at λ_{ex}=274 nm and λ_{em}=440 nm (zearalenone concentration found=4 ng/g). *Immunoaffinity method*. Injected extract: 100 μl (0.16 g sample equivalent). Column: Supelcosil LC-18 (150×4.6 mm I.D., 5 μm); mobile phase: acetonitrile–water–methanol (46:46:8, v/v); flow-rate: 1.0 ml/min; detection: fluorescence at λ_{ex}=274 nm and λ_{em}=440 nm (zearalenone concentration found=3.6 ng/g).

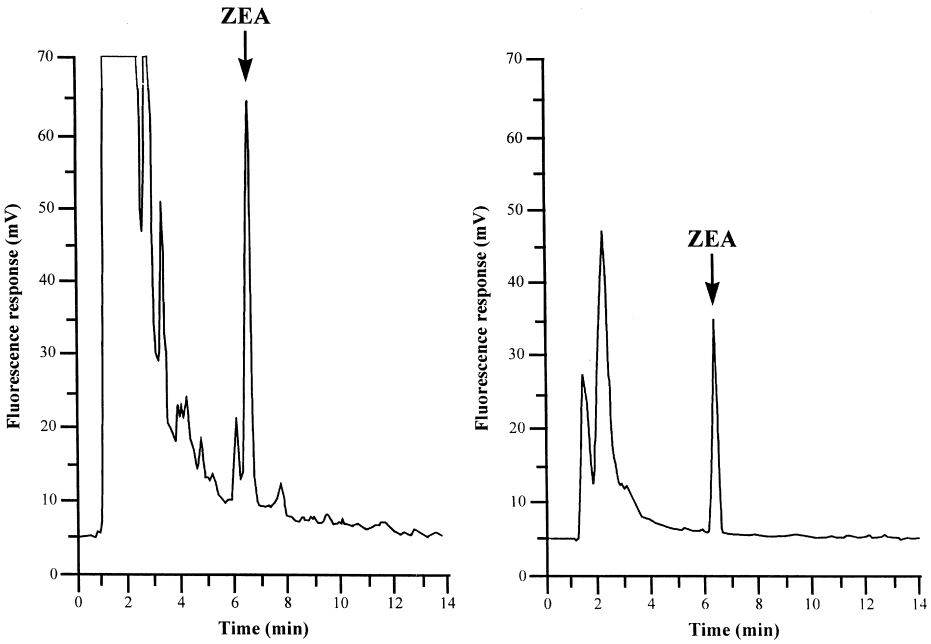

Fig. 4. Chromatograms relevant to a naturally contaminated corn extract (sample 9): (left) AOAC official method (zearalenone concentration found=77 ng/g) and (right) immunoaffinity method (zearalenone concentration found=78 ng/g). Chromatographic conditions as in Fig. 2.

gram of the immunoaffinity purified extract (Fig. 3, right).

In addition to the above mentioned advantages, i.e. accuracy and specificity, the immunoaffinity method avoids the use of very high amounts (~400 ml) of dangerous solvents such as chlorinated organic chemicals, and is time saving due to the absence of a number of liquid–liquid extraction and solvent evaporation steps which are included in the AOAC protocol.

4. Conclusions

The application of state of the art methods for the determination of zearalenone in foodstuffs and feedstuffs is highly desirable for surveillance and monitoring programs aimed at the prevention of undesired effects for animal and human health and for animal production with consequent serious economical losses. The use of immunoaffinity chromatography in the clean-up step provides a number of advantages as compared to other collaboratively tested methods, which include: (i) provision of clean extracts due to the specificity of the antibodies for the single toxin or a group of related toxins that can be easily separated by HPLC; (ii) optimal performances in terms of precision and accuracy within a wide range of concentrations which cover the field of practical interest; (iii) rapidity and, finally, (iv) a considerable reduction in the use of dangerous solvents, highly desirable and necessary for environmental protection.

Acknowledgements

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