

HPLC fluorescence determination of ochratoxin A utilizing a double internal standard and its application to poultry feed

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Abstract: A validated liquid chromatography method employing a fluorescence detector for the determination of ochratoxin A (OTA) was developed with double internal standard and it was applied to ten different poultry feeds. The analysis was performed in an octadecyl silane column using a solvent system [ACN:water:formic acid (50:50:1.25, v/v/v)] by isocratic elution. The flow rate and injection volume were 1 mL min⁻¹ and 12 μL, respectively. Signals were detected at 278(λ_{ex})/315(λ_{em}) and 330(λ_{ex})/450(λ_{em}) nm between 0 and 8, and 8.01 and 20.0 min, respectively. The method was validated with precision, linearity, accuracy, limit of detection, limit of quantification, robustness, and stability. Good linearity (r² = 0.9998–0.9999) was achieved over a concentration range of 1.60 × 10⁻⁸ M to 6.40 × 10⁻⁶ M for OTA. LOD and LOQ values were 7.83 × 10⁻¹⁰ M and 2.37 × 10⁻⁹ M, and 2.01 × 10⁻⁹ M and 6.10 × 10⁻⁹ M for internal standard 1 (IS1) and internal standard 2 (IS2), respectively, on an interday basis. The method was applied to poultry feed samples. Good recovery data ranged between 79.10% and 85.57%, and 71.98% and 76.66%, and the RSD% values were in the range of 1.36–11.70 and 2.07–2.34 for IS1 and IS2, respectively.

Key words: Animal feed and poultry feed, fluorescence detector, HPLC, mycotoxin, ochratoxin A

1. Introduction

Ochratoxin A (OTA) is a toxic secondary ubiquitous mycotoxin, naturally produced by several *Aspergillus* and *Penicillium* genera. Its chemical structure is (*R*)-*N*-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl)carbonyl] phenylalanine as shown in Figure 1.

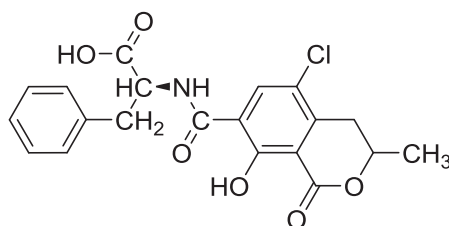


Figure 1. The chemical structure of OTA.

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The biological behavior or effect of OTA on an organism is well documented. There have been reports on its immunosuppressive nature, and teratogenic, fertility inhibitory, mutagenic, and carcinogenic effects.^{1,2} It is involved in endemic Balkan nephropathy that often is accompanied by upper urinary tract urothelial cancer.³ OTA has been detected in both human and animal fluids, wines and beers, coffee, spices, dried fruits,⁴ food and feeds including meat, cereal, and nuts.^{5–9} Since the intake of OTA leads to its build-up in the circulatory system, liver, and other tissues, such as adipose and muscles, its presence in animal feed and tissue can lead to OTA intake in humans, as it passes up the food chain.¹

Some guidance values were published for cereal, cereal products intended for animal feed, and complete and complementary pig and poultry feed.¹⁰ Chickens are a sensitive species and it is assumed that OTA is the leading cause of poultry nephropathy. Herbivores rely on cecal rather than ruminal fermentation and may absorb intact OTA in the small intestine since they are probably more sensitive than ruminants, but quantitative data are lacking. Contamination of animal feeds with OTA may result in the presence of residues in edible offal and blood products, whereas OTA contamination in meat, milk, and eggs is negligible. However, higher concentrations of OTA may occur in certain local specialties such as black puddings and sausages prepared with pig blood serum. A maximum level for OTA for edible offal and blood products is currently under discussion in Europe.¹¹

Thus, there is a need for compliance with guidelines and for monitoring and surveying of OTA levels in food and animal feed. For the determination of OTA, the most common technique used in most studies is HPLC with fluorescence detection (HPLC-FL) because of its natural fluorescence.^{1,2,6–9} Among these HPLC-FL methods, there is one method where internal standard (IS) has been used.⁹ Apart from the HPLC method, thin layer chromatography,¹ gas chromatography–mass spectrophotometry,¹² liquid chromatography–mass spectrophotometry,¹³ and capillary electrophoresis¹⁴ without IS usage have been used for the determination of OTA in different foods.

The aim of the present study was to develop a validated HPLC method using double internal standards because the IS employed during the analysis can be interfered with by some compounds depending on the behavior of a certain matrix. To prevent possible interference effects, double IS was utilized in this study and the first application of the double IS usage was realized for the determination of OTA in poultry feed in the scientific field.

2. Results and discussion

2.1. Optimization of the method

Different flow rates in the range of 0.6–1.4 mL min⁻¹ were applied for the determination of the OTA by using this mobile phase (ACN:water:formic acid, 50:50:1.25 as volume). The best flow rate was 1.0 mL min⁻¹ from the viewpoint of peak morphologies.

Different acids (such as acetic, phosphoric, and formic) were tried, keeping the ratio of acetonitrile and water ratio constant (ACN:water, 50:50), to determine the optimum mobile phase composition at first. The most convenient was formic acid in the mobile phase regarding peak sharpness, area, and migration time. The effect of the formic acid variation was tested by adding different volumes between 0.25 and 1.50 mL.

Certain chemicals were examined to achieve the available double IS in the optimum conditions to increase the repeatability and sensitivity of the method. Parabens (–methyl, –ethyl, –propyl, and –butyl) were tried. Since methyl paraben and butyl paraben peaks interfere with the matrix peaks, ethyl paraben (IS1) and propyl paraben (IS2) (Figure 2) were the best internal standards for this system.



Figure 2. Chemical structures of (a) ethyl paraben (IS1) and (b) propyl paraben (IS2).

The maximum and the same peak normalization ratios were obtained in the mobile phase including all those between 1.0 and 1.5 mL of formic acid for IS1. However, the maximum peak normalization ratios were observed in the 1.25 mL formic acid medium for IS2. Thus, the composition having 1.25 mL of formic acid was accepted as an optimum (ACN:water:formic acid, 50:50:1.25 as volume) mobile phase.

The variation of injection volume was also tested in the HPLC system between 6 and 14 μL . Peak normalization ratios are at the greatest value and the same for both IS1 and IS2 at 12 μL and 14 μL ; 12 μL was selected as an optimum injection volume.

In the chromatogram, the OTA, IS1, and IS2 appeared at 9.86, 4.09, and 5.78 min, respectively.

It was observed that the duration of the analysis is very reasonable from an analytical point of view and no interference effect was encountered for the studied feedstuff samples. The typical chromatogram of the final concentrations of standard OTA (8.20×10^{-8} M), IS1 (3.00×10^{-6} M), and IS2 (3.46×10^{-6} M) under the optimum conditions is shown in Figure 3.

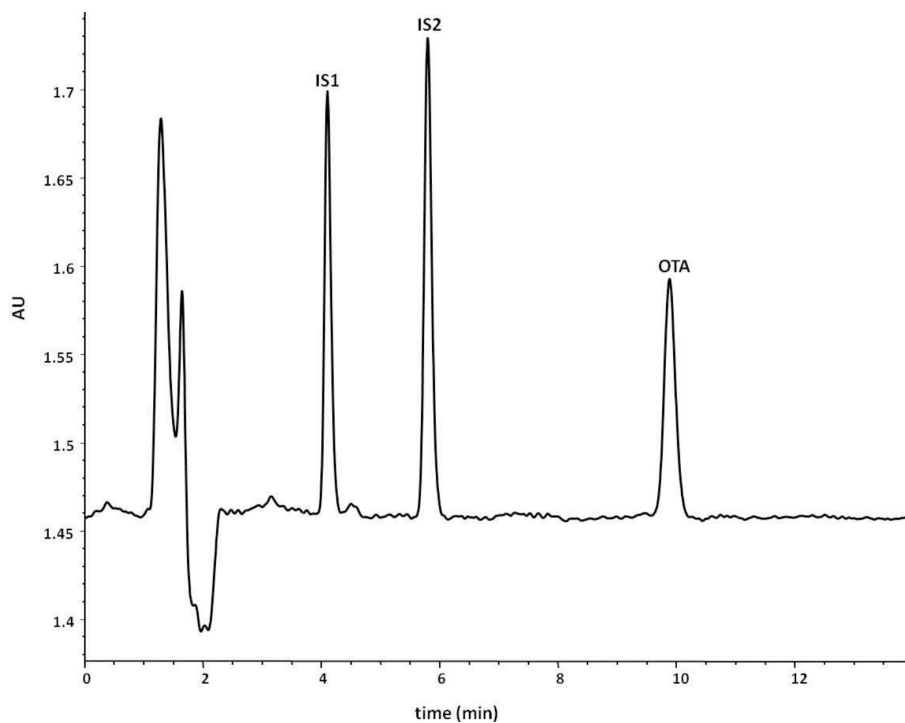


Figure 3. A typical chromatogram of OTA, IS1, and IS2. The mobile phase consisting of ACN:water:formic acid (50:50:1.25, v/v/v); flow rate: 1.0 mL min⁻¹; injection volume: 12 μL ; fluorescence detection [278(λ_{ex})/315(λ_{em}) nm at 0–8 min, 330 (λ_{ex})/450(λ_{em}) nm at 8.01–20.0 min].

2.2. Validation studies

The International Conference on Harmonization (ICH) of Technical Requirements (CPMP/ICH/281/95) guidelines¹⁵ were used for the validation of the method with respect to precision, linearity, accuracy, limit of detection (LOD), and limit of quantification (LOQ).

2.3. Precision

Standard solutions of OTA at three different final concentrations (8.25×10^{-8} , 1.65×10^{-7} , 2.48×10^{-7} M) and constant IS mixtures (1.50×10^{-5} M IS1 + 1.73×10^{-5} M IS2) were prepared. Each concentration of OTA was injected on three successive days ($l = 3$) with six repetitions ($n = 6$). Then the outputs of the data were obtained and they were processed as follows: the peak normalization (PN) values were calculated by dividing the area values by their retention times. Next, the rates of peak normalization were found by dividing those of peak normalization values of OTA by both IS1 and IS2, separately. The intraday and interday results of rates of peak normalization and their statistical parameters are shown in Table 1.

Table 1. The results of repeatability obtained from OTA determination, expressed on an intraday and interday basis, utilizing ratios of peak-normalization values ($R1 = PN_{OTA}/PN_{IS1}$ and $R2 = PN_{OTA}/PN_{IS2}$).

| | | | | |
|---|--------------------------------------|------------|-----------|--|
| OTA solution I 8.25×10^{-8} M | Repeatability for IS1 (R1, n=6 each) | | | Intermediate precision for IS1 (R1, n=18) |
| | First day | Second day | Third day | |
| \bar{X} | 0.42 | 0.41 | 0.44 | 0.42 |
| RSD% | 0.47 | 1.96 | 1.60 | 3.21 |
| OTA solution I 8.25×10^{-8} M | Repeatability for IS2 (R2, n=6 each) | | | Intermediate precision for IS2 (R2, n=18) |
| | First day | Second day | Third day | |
| \bar{X} | 0.45 | 0.44 | 0.48 | 0.46 |
| RSD% | 1.11 | 1.47 | 1.39 | 4.02 |
| OTA solution II 1.65×10^{-7} M | Repeatability for IS1 (R1, n=6 each) | | | Intermediate precision for IS1 (R1, n=18) |
| | First day | Second day | Third day | |
| \bar{X} | 0.86 | 0.88 | 0.92 | 0.88 |
| RSD% | 1.30 | 1.11 | 0.97 | 3.02 |
| OTA solution II 1.65×10^{-7} M | Repeatability for IS2 (R2, n=6 each) | | | Intermediate precision for IS2 (R2, n=18) |
| | First day | Second day | Third day | |
| \bar{X} | 0.90 | 0.95 | 1.00 | 0.95 |
| RSD% | 1.89 | 1.11 | 1.16 | 4.73 |
| OTA solution III 2.48×10^{-7} M | Repeatability for IS1 (R1, n=6 each) | | | Intermediate precision for IS1 (R1, n=18) |
| | First day | Second day | Third day | |
| \bar{X} | 1.29 | 1.31 | 1.28 | 1.29 |
| RSD% | 0.80 | 0.69 | 1.80 | 1.62 |
| OTA solution III 2.48×10^{-7} M | Repeatability for IS2 (R2, n=6 each) | | | Intermediate precision for IS2 (R2, n=18) |
| | First day | Second day | Third day | |
| \bar{X} | 1.39 | 1.42 | 1.38 | 1.39 |
| RSD% | 0.63 | 0.42 | 1.06 | 1.49 |

Abbreviations: \bar{X} mean; RSD%: percent of relative standard deviation

The results of the precision tests belonging to the three different concentration of the OTA levels are within the limits of 1.62–3.21 (RSD%) and 1.49–4.73 (RSD%) for IS1 and IS2, respectively, as intermediate precision. These indicate that the method is highly repeatable.

2.4. Linearity and sensitivity

For further validation, linearity was investigated for the OTA. It was observed that the relationship of the OTA concentration versus the rate of peak normalization value is linear, at least within the concentration range (1.60×10^{-8} M to 6.40×10^{-6} M).

For the procedure, three sets (intraday: $l = 3$) and five concentrations of the standard OTA solutions (number of experiments: $n = 6$ each), within the range from 1.60×10^{-8} M to 6.40×10^{-6} M, each having the same concentration IS mixture as in the precision tests were used. Evaluations of the linearity were also performed by employing the ratio of peak normalization. An individual calibration equation was constructed by linear regression analysis based on the least square method for each IS. The statistical evaluations were always realized at a 95% probability level.

Certain analytical parameters, such as the LOD and LOQ values, were found by computing the processed integrated peak from the HPLC chromatogram. The LOD and LOQ values were estimated [(standard deviation of regression equation)/(slope of regression equation)] by multiplying by 3.3 and 10, respectively (Table 2).

Table 2. Linearity obtained in the 1.60×10^{-8} M to 6.40×10^{-6} M OTA concentration range under the optimum HPLC conditions.

| | Intraday for internal standard 1 (IS1), n = 6 each | | | Interday for internal standard 1 (IS1), n = 18 |
|------------------|--|---------------------------------|---------------------------------|--|
| | First day | Second day | Third day | All days |
| a \pm SD | $(4.97 \pm 0.02) \times 10^6$ | $(4.97 \pm 0.02) \times 10^6$ | $(4.93 \pm 0.05) \times 10^6$ | $(4.96 \pm 0.03) \times 10^6$ |
| b \pm SD | $(1.4 \pm 0.9) \times 10^{-3}$ | $(1.4 \pm 0.9) \times 10^{-3}$ | $(2.7 \pm 1.7) \times 10^{-3}$ | $(1.9 \pm 1.2) \times 10^{-3}$ |
| r ² | 0.9999 | 0.9999 | 0.9999 | 0.9999 |
| S _{y,x} | 0.0012 | 0.0012 | 0.0024 | 0.0028 |
| LOD (M) | | | | 7.83×10^{-10} |
| LOQ (M) | | | | 2.37×10^{-9} |
| | Intraday for internal standard 2 (IS2), n = 6 each | | | Interday for internal standard 2 (IS2), n = 18 |
| | First day | Second day | Third day | All days (n = 18) |
| a \pm SD | $(5.36 \pm 0.04) \times 10^6$ | $(5.42 \pm 0.04) \times 10^6$ | $(5.42 \pm 0.04) \times 10^6$ | $(5.39 \pm 0.09) \times 10^6$ |
| b \pm SD | $(2.3 \pm 1.4) \times 10^{-3}$ | $(-5.9 \pm 1.3) \times 10^{-3}$ | $(-6.0 \pm 1.3) \times 10^{-3}$ | $(-2.7 \pm 3.3) \times 10^{-3}$ |
| r ² | 0.9998 | 0.9999 | 0.9999 | 0.9998 |
| S _{y,x} | 0.0020 | 0.0019 | 0.0019 | 0.0078 |
| LOD (M) | | | | 2.01×10^{-9} |
| LOQ (M) | | | | 6.10×10^{-9} |

Abbreviations: a: slope; b: intercept; r: correlation coefficient; S_{y,x}: standard deviation of calibration equation

As seen, the calibration equations are linear ($R1 = (4.96 \pm 0.03) \times 10^6 C_{OTA} + (1.9 \pm 1.2) \times 10^{-3}$ for interday, IS1 and $R2 = (5.39 \pm 0.09) \times 10^6 C_{OTA} + (-2.7 \pm 3.3) \times 10^{-3}$ for interday IS2) with good correlation coefficients and the intercepts of the curves are not significantly different from zero.

The LOD and LOQ values were calculated to be 7.83×10^{-10} M and 2.37×10^{-9} M for IS1 and 2.01×10^{-9} M and 6.10×10^{-9} M for IS2, respectively, on an interday basis. The results are reasonably low for LOD and LOQ by determining the OTA in poultry feed samples.

The values found are higher than those of the research that utilized immunoactive chromatography,^{4–6,14,16} but almost equivalent to those of other HPLC studies.^{7,17,18}

2.5. Accuracy

The accuracy of the OTA was determined using standard addition. The OTA standards at three different levels between 9.90×10^{-8} M and 3.96×10^{-7} M were spiked into the poultry feed sample that does not contain OTA. Two parallel experiments and six replicates at each concentration were realized. The SPE extraction procedure was applied as presented in the Preparation of the extracts part of the Experimental section. The results were calculated using the calibration equation. The recovery, SD, and RSD% values were then computed and they are presented in Table 3.

Table 3. The results of accuracy of OTA.

| Recovery data for IS1, n = 6 each | | |
|-----------------------------------|--------------------------------|------------------|
| Added OTA, M | Found OTA, M, \bar{X} (RSD%) | Recovery% (RSD%) |
| 9.90×10^{-8} | 8.47×10^{-8} (1.47) | 85.57 (1.36) |
| 1.98×10^{-7} | 1.57×10^{-7} (6.70) | 79.10 (6.70) |
| 3.96×10^{-7} | 3.35×10^{-7} (1.80) | 84.67 (11.70) |
| Recovery data for IS2, n = 6 each | | |
| Added OTA, M | Found OTA, M, \bar{X} (RSD%) | Recovery% (RSD%) |
| 9.90×10^{-8} | 7.59×10^{-8} (2.09) | 76.66 (2.33) |
| 1.98×10^{-7} | 1.43×10^{-7} (2.34) | 71.98 (2.34) |
| 3.96×10^{-7} | 2.92×10^{-7} (2.07) | 73.79 (2.07) |

Abbreviations: \bar{X} : mean; RSD%: percentage of relative standard deviation

The recovery values are between 79.10% and 85.57% and between 71.98% and 76.66%, and the RSD% values are in the range of 1.36–11.70 and 2.07–2.34 for IS1 and IS2, respectively. European Commission performance limits are 50–120 for recovery% and ≤ 60 for RSD%.¹⁹ The recovery data show that the extraction method is highly efficient for the determination of the OTA in poultry feed samples.

2.6. Robustness

The robustness of the method can be assessed by examining the eventual effects of different sets of conditions placed on the method. In this study, three parameters were considered: the composition of the mobile phase, the flow rate of the solvent, and the injection time. The impact of the changing mobile phase composition was tested by comparing the results obtained from analyzing the OTA solution with the IS mixture in the optimum conditions using two different mobile phases (1) 49:51:1.25 (v/v/v) and (2) 51:49:1.25 (v/v/v) ACN:water:formic acid.

Two different flow rates (0.9 and 1.1 mL min⁻¹) were tested for comparison with those in the optimum condition as to the effect of the flow rate of the solvent.

The results of the injection volume of 11 and 13 μ L were also tried. The RSD% values concerning all three robustness parameters were smaller than 2.5 for both the usage of the IS1 and IS2 (Table 4), showing that the developed method allowed highly reliable determination of OTA during usage.

Table 4. The results of robustness of OTA.

| Parameters for IS1 | % RSD | SE |
|---|-------|-------|
| The composition of mobile phase (ACN:water:formic acid, 49:51:1.25) | 1.45 | 0.01 |
| The composition of mobile phase (ACN:water:formic acid, 51:49:1.25) | 0.67 | 0.003 |
| Flow rate of solvent (0.9 mL min ⁻¹) | 1.83 | 0.01 |
| Flow rate of solvent (1.1 mL min ⁻¹) | 1.72 | 0.01 |
| Injecting volume (11 μ L) | 2.21 | 0.01 |
| Injecting volume (13 μ L) | 0.88 | 0.01 |
| Parameters for IS 2 | % RSD | SE |
| The composition of mobile phase (ACN:water:formic acid, 49:51:1.25) | 2.45 | 0.01 |
| The composition of mobile phase (ACN:water:formic acid, 51:49:1.25) | 0.18 | 0.001 |
| Flow rate of solvent (0.9 mL min ⁻¹) | 2.04 | 0.01 |
| Flow rate of solvent (1.1 mL min ⁻¹) | 0.61 | 0.003 |
| Injecting volume (11 μ L) | 2.04 | 0.01 |
| Injecting volume (13 μ L) | 0.91 | 0.005 |

Abbreviation:SE (standard error of mean) = $\frac{SD}{\sqrt{n}}$; SD:standard deviation

2.7. Stability

A standard solution of OTA (3.2×10^{-8} M) in methanol was prepared as in the Experimental section. The solution was kept in the laboratory at an ambient temperature in darkness for 1 week. Prior to the analysis, the solution was diluted, the IS mixture was added, and then it was injected each successive day for a week. As a result, a meaningful change was observed for the OTA concentration for 1 week regarding the results of both the IS1 and the IS2.

2.8. Application of the method to the feedstuff samples for poultry

The developed method was applied to the ten feedstuff samples for poultry. For the procedure, sample preparations were made as described in the Experimental section. The determination of OTA in the mentioned samples was achieved by employing optimum conditions. The OTA was detected in only one feed stuff sample for poultry among the ten samples. The chromatogram of this sample is shown in Figure 4.

As seen from the chromatogram, three peaks for OTA, IS1, and IS2 clearly appeared. The average uncorrected OTA was found to be 1.81×10^{-8} M (RSD%: 3.19) and 1.52×10^{-8} M (RSD%: 3.29) for IS1 and IS2, respectively (n = 6). These values correspond to 7.31×10^{-3} and 6.14×10^{-3} mg kg⁻¹ sample for IS1 and IS2, respectively. The OTA values determined by this method are below the EU limit (0.1 mg kg⁻¹ for complementary and complete poultry feedstuff).¹⁰

In conclusion, the results of the study show that the method is highly applicable for the determination of OTA for feedstuffs and also for cereals.

3. Experimental

3.1. Apparatus

An Agilent 1100 series auto sampler system from Agilent, GL Sciences Inc (Waldbronn, Germany) equipped with a system controller, a RF detector (G 1321A), a quaternary LC pump (G1311A), and a C18 column (150

mm \times 4.6 mm \times 3 μ m particles) from GL Sciences Inc. (Tokyo, Japan) was used for the HPLC analysis. A solid phase extraction (SPE) column, a Supelclean LC-18 from Supelco Inc. (Bellefonte, PA, USA), was used for the extraction procedures. A Sonorex Ultrasonic Bath from Bandelin (Berlin, Germany) was used as a degasser for all of the solutions. A Buchi-Rotavapor R-200 with Buchi heating Bath B-490 from Buchi Labortechnik Co. (Flawil, Switzerland) was utilized for the evaporation procedures.

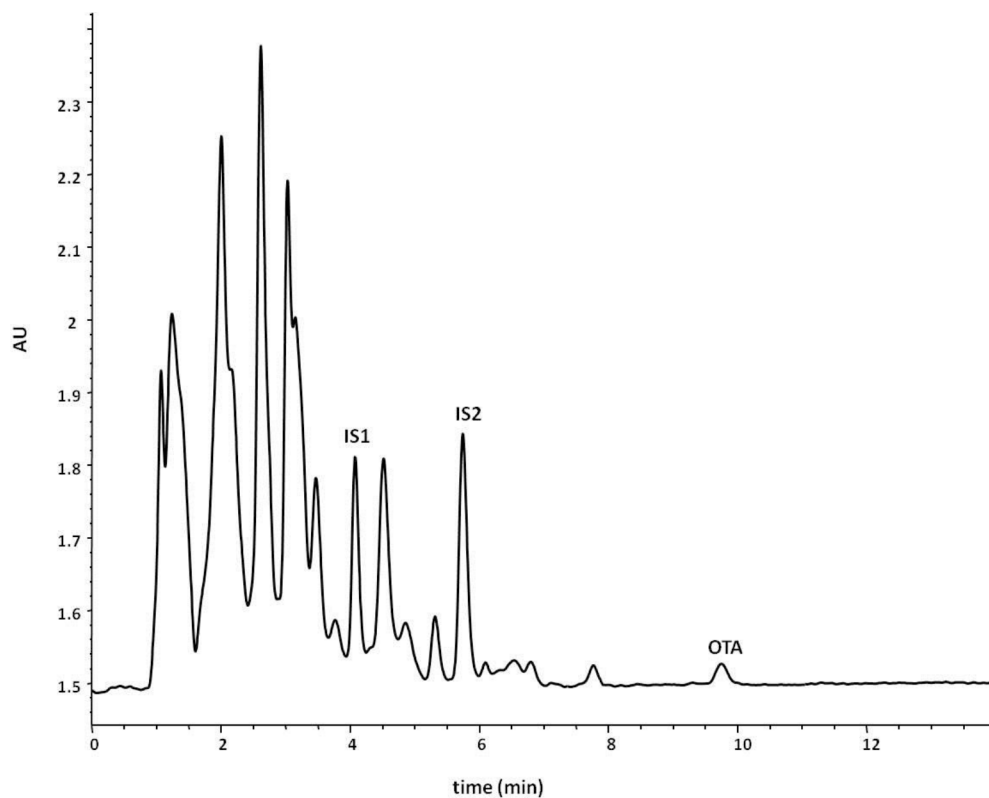


Figure 4. The chromatogram of feeding stuff sample 7 for poultry, IS1 (3.00×10^{-6} M) and IS2 (3.46×10^{-6} M) in the optimum conditions.

A pH 301 pH/ion meter was used with a Hanna HI 1131 glass electrode (Sarmeola di Rubano, Italy). All the buffers and the sample solutions were filtered through 0.45 μ m membrane filters (La-Pha-Pack, USA) before analysis. The ultrapure deionized water (specific resistivity of 18 M Ω cm) was purified by Millipore, Synergy Water Purification System (Rotterdam, Netherlands).

3.2. Chemicals and samples

The OTA and internal standards (IS), such as ethyl paraben (IS1) and propyl paraben (IS2), were purchased from Sigma (St. Louis, MO, USA), and the methanol, ethanol, acetonitrile (ACN), and formic acid were provided by Merck (Darmstadt, Germany). All of the other chemicals used were of analytical grade. They were all employed without further purification. Ten poultry feeds were obtained from a local market. These samples were processed before applying the extraction procedure in the following manner: the feedstuffs were transferred to containers where they were irrigated and then left capped at room temperature for a week. The sample preparation procedures were conducted under the same conditions as detailed in the Experimental section.

3.3. Preparation of the solutions

Double distilled water was prepared in our laboratory using an all-glass apparatus.

OTA standard (1 mg) was dissolved in methanol in a 25-mL volumetric flask (9.9×10^{-5} M) and was stored at -32 °C. All dilutions were made from this stock solution using the same solvent prior to the analysis for the calibration and optimization studies. The diluted solutions were also kept at -4 °C in darkness. Each solvent was filtered through a $0.45\text{-}\mu\text{m}$ membrane and degassed by ultrasonification.

A buffer phosphate saline solution (PBS) was prepared with a minor modification as described by Scott et al.²⁰ For the preparation of PBS, a mixture of 19 mL of 0.2 M sodium dihydrogen phosphate, 81 mL of 0.2 M disodium hydrogen phosphate, 14.04 g of sodium chloride, 0.402 g of potassium chloride, and 1.0 g of sodium azide was utilized and was adjusted to pH 7.4, and made up to 200 mL. Then a 10-mL aliquot was diluted 10-fold.

Next 25.0 mg of ethyl paraben (IS1) and 31.2 mg of propyl paraben (IS2) were weighed and they were transferred to a 100-mL flask. They were dissolved in 30 mL of methanol. Then about 10 mg of sodium bicarbonate was added to this solution followed by dilution with double distilled water to make it up 100 mL. Next 1 mL of IS mixture including IS1 and IS2 was always used for 4 mL of standard or sample solution.

3.4. HPLC conditions

During the analysis, the flow rate and injection volume were 1 mL min^{-1} and $12\ \mu\text{L}$, respectively. Signals were detected at $278(\lambda_{ex})/315(\lambda_{em})$ nm at 0–8 min and $330(\lambda_{ex})/450(\lambda_{em})$ nm at 8.01–20.0 min by fluorescence detection taking into account the excitation and emission wavelengths of the IS1, IS2, and OTA, respectively. Chromatographic separation was carried out by an isocratic elution using a solvent system [ACN:water:formic acid (50:50:1.25, v/v/v)].

3.5. Preparation of the extracts

Various extraction procedures were tried in order to find the optimum extraction conditions for the poultry feeds. The most successful extraction method was found to be that applied cereal products.⁷ However, we made some minor modifications to the method. As a result, it was applied as follows: the samples under investigation were first crushed and then ground. The fatty material was extracted from the sample using hexane in a Soxhlet apparatus and the hexane was then removed (3.6%, w/w, fat in the sample). After that, 10 mL of ethanol was added to 1.0 g of the fat-free sample and stirred by magnetic stirrer for 30 min. The mixture was centrifuged at 5000 rpm and then the ethanol was evaporated off dryness using a rotavapor (at 40 °C and 175 mbar).

Next 10 mL of NaHCO_3 (1%, w/v) was added to this residue and it was stirred using a magnetic stirrer for 30 min. Then 10 mL of a diluted buffer phosphate saline (PBS) solution (1/10, v/v) was added to the resulting solution. The sample was passed through a SPE cartridge that was previously conditioned with 10 mL of PBS. The cartridge was washed with 1 mL of double distilled water. The retained compounds were eluted by 4 mL of methanol. Then 1 mL of IS mixture including 1.50×10^{-5} M IS1 + 1.73×10^{-5} M IS2 (final concentrations of 3.00×10^{-6} M IS1 and 3.46×10^{-6} M IS2) was added to this eluent and it was injected through the HPLC column.

To conclude, an HPLC method with fluorescence detection using double IS, which prevents matrix effects, was proposed for the determination of OTA in feed samples for poultry. The determination of OTA was achieved by gradient elution in a 10 min analysis time without losing peak efficiency. After validation, the method was

successfully applied for the determination of OTA in different real samples subjected to a suitable extraction procedure that provided good recovery values. The obtained results prove the applicability of the HPLC technique using double IS in the analysis of such complex matrices as feedstuff samples, which contain many different components.

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