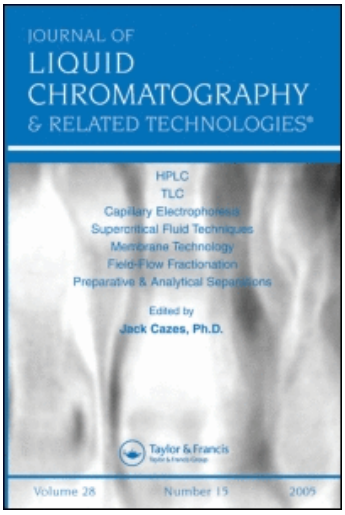

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DETERMINATION OF OCHRATOXIN A IN CEREALS AND FEED BY SAX-SPE CLEAN UP AND LC FLUORIMETRIC DETECTION

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ABSTRACT

We have developed a sensitive, reliable and highly specific method for the determination of ochratoxin A (OA) in cereals and animal feed. The samples were extracted in acidified acetonitrile and, they were then thoroughly purified by a new procedure combining a pH-controlled liquid-liquid partition with a strong anionic exchange solid-phase extraction. Both RP-TLC and RP-HPLC methods for semi-quantitative and quantitative determination have been described respectively, together with a RP-HPLC confirmation procedure, via conversion to OA-methyl ester. The TLC and HPLC determination limits were 8.0 ng/g and 0.10 ng/g respectively. The mean recovery from spiked samples was 95%.

INTRODUCTION

Mycotoxins are secondary fungal metabolites, i.e. end-products of fungal cellular metabolism and acting either in fungal cell differentiation or as antibiotics against microbial competitors and predators.

A large number of cereals and other crops are often contaminated with mycotoxins, produced by different fungi, either in the field or during storage. The ochratoxin A (OA), chemically (R)-N- [(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl) carbonyl]-L-phenylalanine, is produced by Penicillium and Aspergillus genera (i.e. A. ochraceus, P. viridicatum).[1] OA is one of the most important reasons accounting for mycotoxic nephropathy, mainly in swine species.[2] This mycotoxin has been found to contaminate a variety of agricultural commodities, particularly cereals, different kinds of beans (soya, cocoa, coffee), red and black peppers, cottonseed, citrus fruits, and peanuts and tobacco.[3] OA has carcinogenic, nephrotoxic, teratogenic and immunotoxic properties for a wide number of animals.[4-5] For example, it causes a proteic synthesis inhibition at cellular level and, in the kidney, it induces periglomerular and interstitial fibrosis leading to glomerular atrophy. Furthermore OA, as a dihydro-hydroxycumarin derivative, acts as an anti-vitamin K xenobiotic, leading to the haemorrhage syndrome. Swine are the most sensitive species to nephrotoxic effect. The minimal amount associated with swine nephropathy seems to range from 100 to 200 ppb.[6-7]

Many analytical methods have been developed,[8] including thin layer chromatography (TLC),[9-13] high performance liquid chromatography (HPLC),[13-19] gas chromatography (GC)[20] and enzyme-linked immunosorbent assay (ELISA).[21-22] Often, the procedures of extraction and clean up of the samples are too time-consuming, and the final extract is frequently matrix-dependent in regard to interfering substances. This results in unsatisfactory and insufficient clean up levels, thus strongly affecting its detection limit. Some published clean up methods mainly employ a pH-controlled liquid-liquid partition, frequently combined with a solid-phase extraction on silica or C_{18} sorbents.[13,16,18,19,23,24] This widespread approach, though easily applicable, is not so specific as to provide high recoveries, together with free interferences extracts. A GPC method has been used,[25] which needs special requirements, such as a dedicated GPC system, when most laboratories do not have such systems. More recently, immunoaffinity columns (IAC) have been successfully used,[26] but unfortunately, they are too expensive for routine use.

The aim of our work was to develop a highly sensitive and readily reliable TLC/HPLC method for the determination of low OA concentrations indipendent of the characteristics of an analyzed matrix. Several kinds of cereals and mixed feed were analyzed by the procedure described. We thus stressed the clean up procedure. For the first time, we have described a very effective, and yet rapid, clean up method based upon a combination of a liquid-liquid partition and a solid-phase extraction. Our method has given us successful and high recovery rates, as well as very well clean extracts. These extracts were then suitable, either for TLC screening, HPLC analysis or HPLC confirmation as methyl ester, without any additional purification.

EXPERIMENTAL

Reagents

Ochratoxin A, in a crystalline form, was purchased from Sigma. A 500 ng/μL stock solution in acetonitrile was then prepared. For sample spiking and TLC/HPLC analysis, 1 ng/μL standard solution was prepared in the HPLC mobile phase, acetonitrile-0.2 % orthophosphoric acid solution (55:45, v/v). Stock and standard solutions were protected from light in screw-cap amber tubes, and were stored frozen at -20° C. Methyl ester of OA was prepared by esterification of corresponding OA standards. All chemicals and solvents were purchased from Analyticals Carlo Erba (RPE reagent). The solvents employed for standards and mobile phase preparation were of HPLC grade (Lichrosolv Merck). All water used was bi-distilled and de-ionized by a Milli-Q Water System. A typical 100 mL tris.HCl buffer solution (pH=7.20), was prepared by mixing 25 mL of 0.2 M tris solution with 4.5 mL of 1 M HCl, and diluting it to 100 mL with de-ionized bi-distilled water. Isolute XL-SAX columns (500 mg sorbent, 10 mL volume), were supplied by IST-StepBio. The KC 18 RP-TLC 10x10 cm plates were purchased from Whatman.

Apparatus

The flask shaker was universal table shaker (mod. 709) from Chimica Omnia. The centrifuge was a Beckman J6-MC model. The SPE columns were connected to an SPE vacuum manifold apparatus, supplied from Supelco. The HPLC equipment was obtained from Perkin-Elmer and consisted of a series 200 LC quaternary pump, equipped with a Rheodyne injector (50 μl loop). The detector was an LC-240 fluorescence spectrophotofluorimeter. The chromato-integrator was a PE Nelson 1022 LC plus model. Separations were made at room temperature, on a 5 μm Supelcosil LC-18 250x4.6 mm column (Supelco).

Extraction of Cereals and Mixed Feeds

20 g of representative finely ground commodities were mixed with 80 mL of 3% acetic acid in acetonitrile in a 250 mL jar. It was closed and automatically shaken for 30 min. The raw extract was clarified by filtration through a folded filter. A 60 mL aliquot of the filtered mixture was transferred into a 100 mL round bottomed flask and evaporated until dry at 45° C. The residue was then dried under a helium flow for 1 min, in order to remove any acetic acid traces. The residue was quantitatively and accurately transferred to a 40 mL capped Nalgene centrifuge tube by using two 2.5 mL portions of n-

hexane, alternating with another two 5 mL aliquots of tris.HCl buffer (pH=7.20). A pH control of the aqueous phase was made and, if necessary, was corrected by adding 10 µl of 45 % NaOH solution. Afterwards, the bi-phase system was vigorously shaken on a vortex for 5 min, and then centriguged for 20 min at 4000 rpm, at 20° C. The upper organic layer was discharged off. Whenever a gelification of the aqueous phase occurred, further n-hexane washing was thoroughly made without affecting the recovery. A 5 mL aliquot of the aqueous buffered phase was collected and submitted to SAX-SPE clean-up.

SAX Column Clean-Up

The buffered aqueous extract was subjected to a SPE-SAX clean up procedure. The SAX column, connected to a vacuum manifold, was rinsed with 5 mL of methanol and conditioned with 5 mL of tris.HCl buffer solution (pH=7.20), at a flow rate of 1 drop/sec. It was recommended that the column was not allowed to run dry. The aqueous extract (5 mL) was passed through the SAX column at a flow rate of ca 0.5 mL/min to be purified. The column was then washed with 5 mL of buffer solution, in sequence, followed by 5 mL of deionized water, 5 mL of methanol and finally 4 mL of 1 % acetic acid in methanol.

The flow rate was not important at these washing stages. Ochratoxin A was then eluted off the SAX column by 7 mL of 5% acetic acid in methanol, at a flow rate of 1 mL/min. The eluate, collected in a 10 mL test tube, was then dried under a gentle stream of nitrogen at 45° C, reconstituted in 250 µl of HPLC mobile phase, and stored at - 20 ° C until analyzed by TLC and/or HPLC.

TLC Screening

A 10x10 cm RP-TLC plate was marked (maximum 6 mm diameter) with 20 µl aliquots of sample and spiked extract, together with 2, 5, 10, 15, 20 µl of OA standard solution (1 ng/µl). The spots were completely dried before they were developed. The plate was developed in a equilibrated tank with the mobile phase, acetonitrile-0.5 M sodium chloride in 0.2 % orthophosphoric acid solution (55:45, v/v). The solvent was kept to a minimum. It should wet no more than the bottom 3 mm of the plate, once it is placed in the tank.

After about 15 min developing time, the plate was air-dried and observed under long wave (365 nm) UV light. The OA appears as a blue-green spot with a retention factor of 0.5. Sample spots were compared with spike and standards.

HPLC Analysis

High performance liquid chromatograph, with fluorescence detector, was employed for the quantification of ochratoxin A and, then, its presence was confirmed as a methyl ester, after proper derivatization. Acetonitrile-0.2 % orthophosphoric acid (55:45, v/v) was used as a mobile phase, at a flow rate of 1 mL/min. A typical injection volume was 50 µl. The excitation and emission wavelenghts were set at 340 nm and 460 nm respectively. The concentration of OA was calculated by means of a linear, six concentration level, calibration graph in a range of 0.15-25 ng injected, based upon the peak area.

HPLC Confirmation by OA Derivatization to Methyl Ester

The eluate from the SAX column was placed in an amber screw-cap tube and dried under a gentle nitrogen stream at 45° C. Accordingly to the Zimmerli's method,[26] the residue was dissolved in 2.5 mL of methanol and 100 µl of 37 % HCl solution. The tube was closed and kept at room temperature for at least 20 h, protected from the light. The reaction mixture was evaporated until dry, under a nitrogen flow at 45° C. The residue was finally reconstituted in 250 µl of HPLC mobile phase, 50 µl of it were then analyzed for OA methyl ester content by HPLC.

RESULTS AND DISCUSSION

Acidified organic solutions turned out to be effective extraction media for ochratoxin A from cereals and mixed feed (13,19,27). OA is endowed of a common carboxylic moiety, whose estimated pKa value is 3.5 (on the basis of phenylalanine-containing dipeptides). It, therefore, appeared that an acidic extraction solvent ensures not only the complete conversion of the ionized to the non-ionized form and, consequently, the disrupting of strong ionic interactions with positively charged functional groups of the matrix components, but also, the analyte transfer to the bulk solution. In our study, the choice of acetic acid arose because it is readily soluble in organic solvents and yet sufficiently volatile to be easily removed from the medium. In our opinion, the use of any other inorganic acid was not advisable. Indeed, a neutralization step is needed before subjecting the sample to SAX clean up. This could provide a remarkable ionic strenght increase and, consequently, a reduced isolate retention on the anionic exchanger, by establishing competitive equilibria. Moreover, the extraction organic solvent, used in this work (acetonitrile), played a fundamental role in promoting the uptake of the ochratoxin A. This was either by denaturating the proteic matrix, responsible for *in vivo* interacting with this mycotoxin, or by acting as a hydrogen bond acceptor towards ochratoxin A.[28]

For the clean up of raw extracts, a new combined procedure of liquid-liquid partition and solid-phase extraction was used. A typical pH-controlled liquid-liquid partition, as reported elsewhere,[18,24] is based upon an unselective distribution between a non-polar organic solvent, such as chloroform, and a strongly alkaline aqueous solution, such as sodium hydroxide. This is incapable of distinguishing the analyte from many other acid interferences, and thus, uptaking all of them without any discrimination of their relative acidities. We greatly reduced the pH of the aqueous receiving phase at a value just enough for complete ionization of OA and a few other acid compounds, which mainly had pKa values lower than the ochratoxin A. We applied the pH-controlled liquid-liquid partition between a very apolar solvent such as n-hexane and a buffer solution of tris.HCl, with appropriate pH (7.2) and ionic strenght (0.045 M). This allowed not only an effective defatting of the raw extract, but also a quantitative partition of the analyte in the neutral aqueous phase. Indeed, the pH value of 7.20 was sufficient for a complete conversion of the ochratoxin A to water-soluble ionized form. It was fortunately unable to ionize other acidic interferences weaker than OA, hence partitioning on behalf of the organic phase. In this way, the OA transfer into the neutral aqueous solution was more specific and, above all, more selective. Nevertheless, the choice of tris.HCl buffer solution, rather than of other inorganic buffers, greatly contributed to this recognition selectivity. Such an organic buffer could favourably interact as quaternary ammonium salt, with the ochratoxin A, by a reasonable ion pair mechanism,[29] in order to competitively displace it from the interactions with some matrix components, thus, stabilizing it in the aqueous medium. Furthermore, a remarkable share in this stabilization was brought by the hydroxymethyl groups of the buffer molecule, acting as hydrogen bond donors towards OA. Indeed, we recovered quantitatively the ochratoxin A, together with more polar interferences by HPLC analysis of such buffered solution.

A subsequent step of solid-phase extraction was necessary not only to remove these interfering substances but also mainly for selectively preconcentrating the analyte in an organic phase. Instead of the usual and aspecific silica or C_{18} SPE,[18,24] we successfully exploited, for the first time, the carboxylic moiety. This makes ochratoxin A suitable for specifically and selectively interacting with the trimethylaminopropyl group of the strong anionic exchanger sorbent.

Figure 1 (right). Chromatogram of (a) blank maize sample containing no OA, (b) the same sample spiked to contain 5 ng/g OA and (c) a maize flour sample naturally contaminated with 1.70 ng/g OA.Conditions: mobile phase, acetonitrile-0,2 % orthophosphoric acid (55:45, v/v), column Supelcosil LC-18 (250x4.6 mm), flow rate 1 mL/min, excitation at 340 nm, emission at 460 nm.

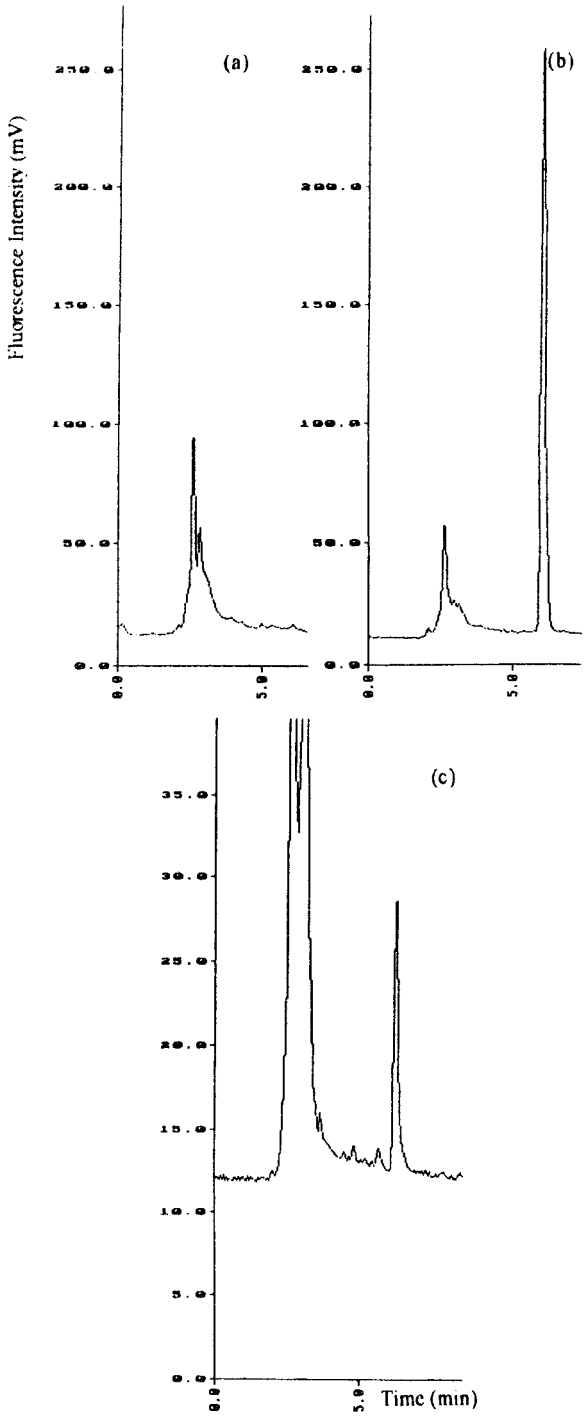

Table 1

Recovery of Ochratoxin from Spiked Samples of Blank Control Maize

Concentration Range of the Sample (ng/g)	Number of Samples	Mean Recovery (%)	CV (%)
1 - 5	5	94	7
10 - 50	6	96	7
100	4	95	4

Conditioning the ion exchange sorbent, before applying sample solutions, with 5 mL of tris.HCl buffer (pH=7.20; I=0.045 M), resulted in a pre-equilibration step with a low selectivity chloride counter-ion. This gave the OA anionic form the best chance of displacing the counter-ion and remaining on the sorbent. Nevertheless, the low ionic strenght (0.045 M) of the sample provided the strongest isolate retention. Indeed, no loss in the OA amount during the loading step occurred, as confirmed by HPLC. Likewise, all three subsequent washing steps allowed the removal of most interferences, without any loss of the mycotoxin. In particular, washing with methanol eluted off a number of matrix interfering substances, presumably polar non-ionic molecules, retained even on SAX by non-functionalized silanolic group fractions.

We further improved the cleaning by modulating the acidity percentage in methanol. Acidity strongly affects the OA retention behavior on the SAX sorbent. Indeed, we observed that a 1% amount of acetic acid in methanol eluted nearly all remaining interferents, together with a variable amount of OA, depending upon volume used. Washing with 6 mL of 1% acetic acid in methanol yielded a loss in OA content from 1 to 7%. Evidently, the elution volume of OA with this low acidity solvent is far higher. Thus, a reduction to 4 mL provided no loss of OA and, meanwhile, there was a complete or almost complete elimination of interferences. OA recovery was finally achieved by increasing the acetic acid from 1 to 5%, 7 mL of this elution mixture were sufficient. In Figure 1, typical chromatograms of (a) a blank maize sample, (b) a sample spiked containing 5 ng/g ochratoxin A and (c) a naturally contamined maize flour sample with a concentration of 1.70 ng/g are shown, all showing the high cleaning level achieved.

Using OA standard solution, the absolute detection limit was 40 pg, at a signal-to-noise ratio of 3:1. We evaluated the limit of determination in maize sample at 0.10 ng/g. At this spiking level, the mean recovery of five triplicates was 97% with a CV of 12%. In Table 1, are listed the recovery and the CV value of maize samples spiked in the range 1-100 ng/g.

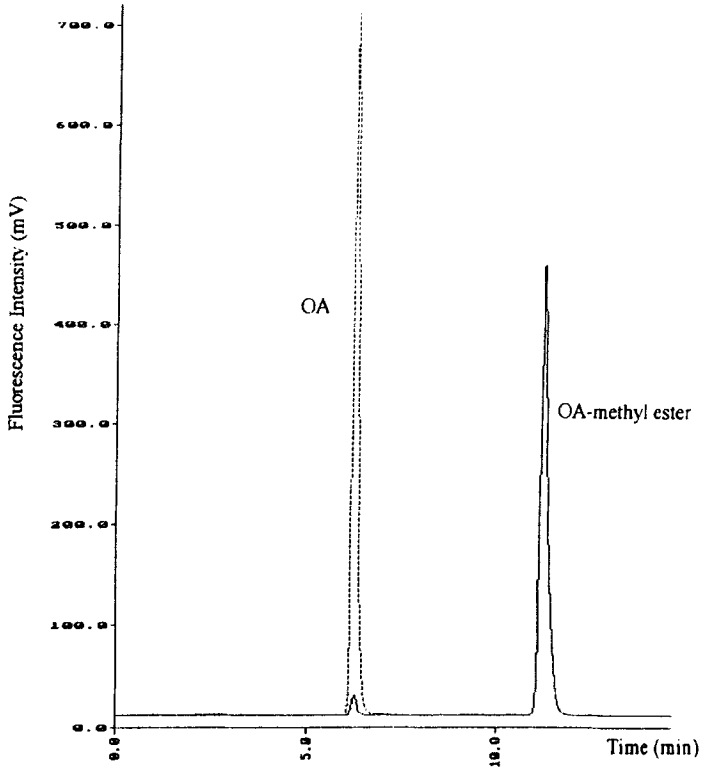

Figure 2. Overlapped chromatograms of OA (20 ng) and its methyl ester derivative.Conditions: mobile phase, acetonitrile-0,2 % orthophosphoric acid (55:45, v/v), column Supelcosil LC-18 (250x4.6 mm), flow rate 1 mL/min, excitation at 340 nm. emission at 460 nm.

Low variation coefficients, indicating a narrow data dispersion, confirmed that recovery rates were independent of spiking level, as well as the good repeatibility of the method. In the range 1-100 ng/g, the overall recovery percentage was 95% with CV 6%.

Wherever high HPLC sensitivity is not important, a reverse phase, thin-layer chromatography procedure was developed, in order to recognize rapidly an OA contamination in analyzed samples. The spotted extracts were the same, alternatively submitted to HPLC for quantitative determination and confirmation. The almost complete absence of interferences allowed an unambigous identification of the ochratoxin A spot (Rf=0.5).

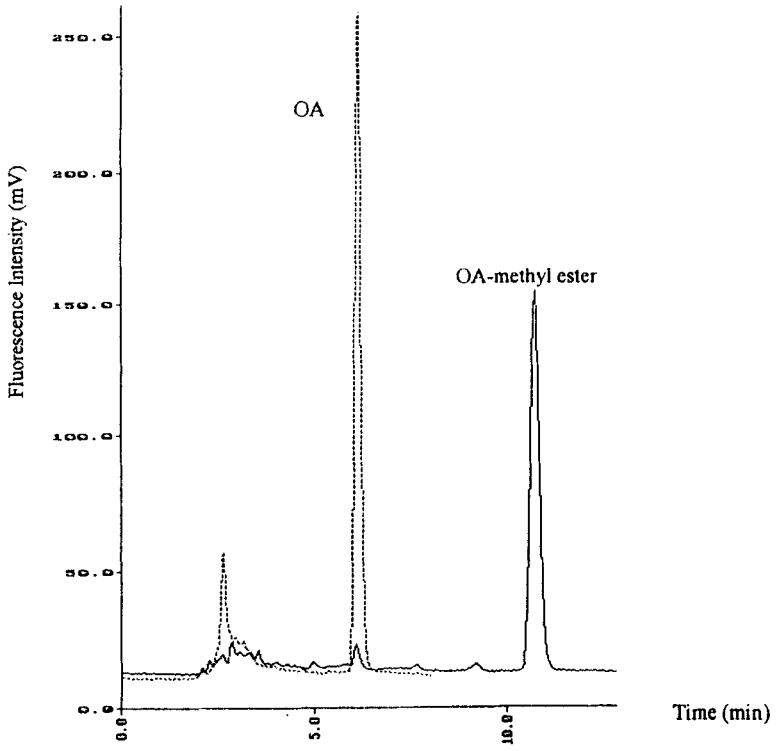

Figure 3. Overlapped chromatograms of OA and its methyl ester in extracts of 10 ng/g spiked maize sample. Conditions: mobile phase, acetonitrile-0,2 % orthophosphoric acid (55:45, v/v), column Supelcosil LC-18 (250x4.6 mm), flow rate 1 mL/min, excitation at 340 nm, emission at 460 nm.

Moreover, we observed a remarkable increase in the OA fluorescence intensity on reverse phase plates rather than direct phase.[9-13] This was presumably due to a reduction of the quenching process.

The combination of such factors provided a detectable limit of 8.0 ng/g, which was very low from a TLC point of view. The mobile phase used for plates development was the same as for HPLC analysis, except for the presence of 0.5 M NaCl in order to avoid the disruption of binding properties of the layer. This ensured the stability of the stationary phase without affecting the separation.

Besides TLC monitoring and HPLC quantitative determination, we exploited a confirmation method by conversion to methyl ester of ochratoxin A and sequent HPLC analysis. Following the derivatization protocol of Zimmerli,[26] OA was converted into the methyl ester. By lengthening the reaction time to at least 20 hours, there was an improvement of mean yield to 97%, which was calculated on unreacted ochratoxin A. Figure 2 displays the overlapped chromatograms with reference to 20 ng of OA, before and after derivatization. The ochratoxin A peak decreased and a new peak appeared five minutes afterwards, due to a less polar molecule than OA, almost definately the corresponding OA-methyl ester. Figure 3, shows the typical overlapping of OA and OA-methyl ester chromatograms, relatively to a 10 ng/g spiked maize sample. We are currently applying this matrix-independent method on other types of animal feed, which has also confirmed good reproducibility.

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