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SIMULTANEOUS DETERMINATION OF MYCOTOXINS (OCHRATOXIN A AND DEOXYNIVALENOL) IN BIOLOGICAL SAMPLES

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Abstract

Approximately (about) 300 to 400 substances are recognized as mycotoxins, comprising a broad variety of chemical structures produced by various mould species on many agricultural commodities and processed food and feed. Various mycotoxins may occur simultaneously, depending on environmental and substrate conditions, so that humans and animals are exposed to mixtures rather than to individual compounds. The aim of this study was to develop a simple method for simultaneous determination of OTA and DON in biological samples. A very important part of the method is the extraction, the clean-up procedure and the preconcentration.
***Keywords**: ochratoxin A, deoxynivalenol, HPLC*

Introduction

Mycotoxins are toxic metabolites produced by various fungal species, mainly of the genera *Aspergillus*, *Fusarium* and *Penicillium*, that under favorable environmental conditions can infest crops in the field or foodstuffs during processing, transportation and storage.

The analysis of mycotoxins has become an issue of global interest, in particular because most countries already set up regulative limits or guideline levels for the tolerance of such contaminants in agricultural commodities and products. Approximately 300 to 400 substances are recognized as mycotoxins, comprising a broad variety of chemical structures produced by various mould species on many agricultural commodities and processed food and feed.

Most important target analytes are aflatoxins, trichothecenes, zearalenone and its derivatives, fumonisins, ochratoxins, ergot alkaloids, and patulin. Various mycotoxins may occur simultaneously, depending on environmental and substrate conditions. Considering this coincident production, it is very likely that humans and animals are exposed to mixtures rather than to individual compounds.

The ochratoxins (OTA) constitute a group of closely-related derivatives of isocoumarin linked to L-phenylalanine, and classified according to biosynthetic origin as pentaketides within the group of polyketides (Turner, 1971).

Ochratoxins are produced by several species of the fungal genera *Aspergillus* and *Penicillium.* These fungi are ubiquitous and the potential for the contamination of foodstuffs and animal feed is widespread. Ochratoxin A, the major compound, has been found in a number of countries in Australasia, Europe, and North America. Ochratoxin formation by *Aspergillus* species appears to be limited to conditions of high humidity and temperature, whereas at least some *Penicillium* species may produce ochratoxin at temperatures as low as 5 °C.

The highest incidences of ochratoxin A contamination have been found in cereals, and to a lesser extent in some beans (coffee, soya, cocoa). Ochratoxin B occurs extremely rarely.

4-Deoxynivalenol (DON, vomitoxin, dehydronivalenol, RD-toxin) is a type B trichothecene, an epoxy-sesquiter-penoid. Deoxynivalenol has a 12,13-epoxy group, three OH functions, and an *alpha*,*beta*-unsaturated keto group. Its chemical name is therefore 12,13-epoxy-3,4,15-trihydroxytrichothec-9-en-8-one. Its molecular formula is $C_{15}H_{20}O_6$ and its relative molecular mass is 296. The sesquiterpenoid trichothecenes possess the tetracyclic 12,13-epoxytrichothecene skeleton. To date, 148 trichothecenes, characterized chemically as having the same basic tetracyclic scirpenol ring system, are known. These compounds are produced primarily by moulds belonging to the genus *Fusarium,* though other genera, including *Trichoderma, Trichothecium, Myrothecium,* and *Stachybotrys,* are also known to produce metabolites now characterized as trichothecenes. Only a few of the known trichothecenes have been found to contaminate food or animal feed including: deoxynivalenol (DON), nivalenol (NIV), diacetoxyscirpenol (DAS), and T-2 toxin. Of these, by far the most

commonly encountered in food and animal feed is DON. DON inhibits the synthesis of DNA and RNA and protein synthesis at the ribosomal level.

Current methods used for mycotoxin determination include thin layer chromatography (TLC), liquid chromatography (LC), gas chromatography (GC) with electron capture detection (ECD) or mass spectrometry (MS), RIA and ELISA methods (Kotal, 2002). TLC is a fast and low cost method, but selectivity is not sufficient for quantitative determination. GC/ECD needs derivatisation procedure prior to the injection into the chromatograph. HPLC is a quick and simple method for mycotoxins determination. For determination UV detection in the wavelength range of 214-219 nm is used.

Regardless of the fact that DON belongs to the group of the least toxic trichotecenes, knowledge of its content is of great importance for food quality control, because of its exceptional prevalence and its use as a main indicator of possible presence of other more toxic trichotecenes (Lombaert, 2002).

The aim of this study was to develop a simple method for determination of OTA and DON in biological samples (chicken bile).

Experimental

Samples were extracted, filtered, cleaned up by charcoal alumina column, evaporated, redissolved, cleaned-up, then analyzed by HPLC with UV detection.

The HPLC system used in this work was a Jasco PU-1580 solvent delivery system and a MD-1510 UV/VIS detector, with a 10 μl flow cell. A reversed-phase Nucleosil C18 column (25 cm x 0.4 mm, μparticle size) was used for separation. A Reodyne 7725 injector with a 10 μL external loop was used for sample introduction. A Borwin chromatography workstation (system control version 1.5) was used to control the operation of HPLC, obtain the chromatogram, and perform data calculation.

The working conditions were: Debit: 0.5 mil/min, Detector UV/VIS 218 nm, eluent: water/ acetonitrile 1:1.

The mycotoxin standards were supplied by Sigma-Aldrich.

Results and Discussions

A very important part of the method is the extraction, the clean-up procedure and the preconcentration.

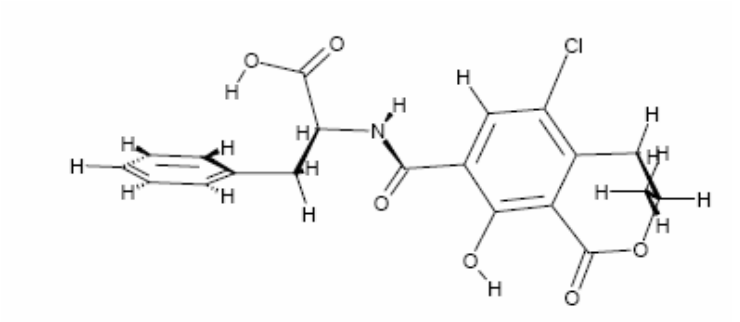

Figure 1. Chemical formula of OTA

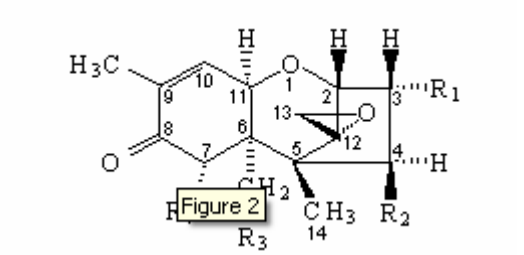

Figure 2. Chemical formula of trichothecenes

Ochratoxin A (Figure 1) in acidified commodities is readily soluble in many organic solvents, and this characteristic has been used as the principle of extraction in several methods.

As it is a type B trichothecene (Figure 2), deoxynivalenol is soluble even in water and in polar solvents such as aqueous methanol, aqueous acetonitrile, and ethyl acetate.

The relatively polar deoxynivalenol and other type B trichothecenes are usually extracted by mechanical shaking or blending with aqueous acetonitrile or aqueous methanol.

In our experiment the two mycotoxins were extracted simultaneously by a method based on solvent partition. 25 g of ground

sample were weighed out into a 250 mL Berzelius glass and 50 mL chloroform:methanol 9:1 were added. The mycotoxins were extracted for several hours, but only the first our by mechanical shaking.

The extracted mycotoxins were filtered through fast qualitative filter paper. The method used for clean-up was liquid-liquid partitioning. Interfering lipids were removed by extracting the sample with n-hexane before further clean-up. Liquid-liquid partitioning was performed by shaking the sample extract with a nonmiscible solvent in a separation funnel.

The samples were preconcentrated by evaporating the solvent and then resolved in 0.5 mL acetonitrile: water 1:1.

Figure 3 presents the chromatogram of the standards. The retention time of OTA is 179 sec (A = 497) and of DON 293 sec (A = 3626)

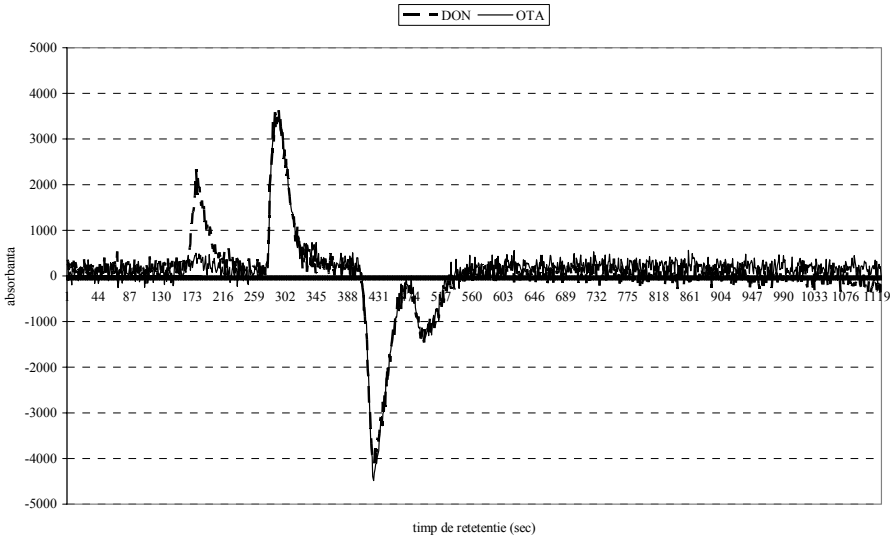

Figure 3. HPLC chromatogram of OTA and DON standard solutions

Figure 4 presents the chromatogram of the bile sample. OTA and DON are present, revealing an efficient extraction of both mycotoxins. The chromatogram shows also that the sample was not well cleaned-up; other substances interfered with the detection of the mycotoxins.

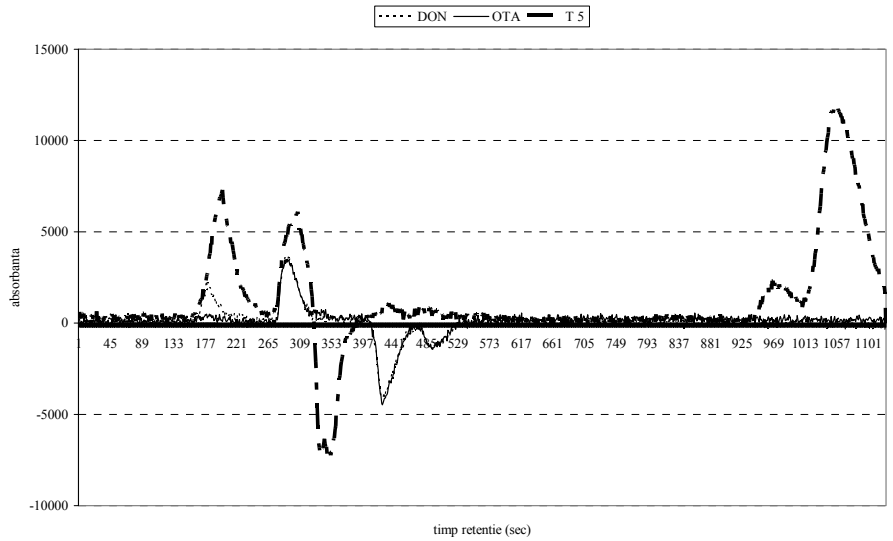

Figure 4. HPLC chromatogram of the standards and the extract sample

Conclusions

OTA and DON in biological samples can be extracted in a chloroform:methanol (9:1) mixture and determined simultaneous by high performance liquid chromatography.

The bile samples must be cleaned-up by an alternative technique, as Solid Phase Extraction (SPE).

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