The pH and Mobile Phase Composition Effects Ochratoxin A Fluorescence at Liquid Chromatography

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Abstract

Changes in the fluorescence behavior of fungal toxic metabolite ochratoxin were studied. The influence of ratio aqueous/organic solvent, the ionic composition of solution, and pH were investigated. The chromatographic separation under conditions yielding the highest fluorescence intensity were tested and compared with results obtained under commonly used chromatographic methods. The developed method was applied on analysis of spiked and naturally contaminated cereal samples.

Introduction

Ochratoxin A (OTA, Figure 1) belongs to a family of toxic products of secondary metabolism produced by various fungi of *Aspergillus* and *Penicillium* species. It is one of the most widely occurring mycotoxins contaminating feed and food, such as cereals, spices, wine grapes, etc. The entrance of OTA into the food chain is possible in all its parts. Under proper conditions for fungi growth, the feed or food of plant origin can be contaminated by OTA in each step of its production: on the field, during harvest, transportation, and storage. The optimal temperature for OTA production is 24°C (1), but there were reported cases that OTA was produced at temperatures as low as 5°C (2). In addition, meat and meat products made from animals fed with OTA-containing feed are other sources of OTA and its metabolites. Similarly to other mycotoxins, the appearance of OTA in food can be earlier than the visual evidence of fungal infection. OTA has a toxic effect on animal and human organisms. Toxicity is related mainly to proteosynthesis inhibition. OTA (derivative of isocumarin and L-β-phenylalanine) is competitive substrate to amino acid phenylalanine in phenylalanine-t-RNA catalyzed reactions due to its similarity with phenylalanine.

Acute toxicity of OTA is relatively low [LD$_{50}$ (mouse) = 46–58 mg/kg of body weight]. Intoxication is accompanied by internal bleeding and in certain cases liver and kidney necrosis. From dietary point of view, the chronic toxicity is more significant where nephrotoxic, hepatotoxic, teratogenic, and immunotoxic effect is registered (3). Chronic exposition of human to OTA is

probably one of the causes of Balkan endemic nephropathy disease (4). The suggestions about the possibility of provocation of Parkinson's disease symptoms also appeared (5). The toxicity of OTA was illustrated in literature (3,6). Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommended provisory tolerable daily intake to 100 ng/kg per week (7). Long-term exposition of rats to OTA caused kidney and liver tumor induction. Based on these facts, International Agency for Research on Cancer (IARC) classified OTA as "potentially carcinogenic" (Class 2B). Moreover, in vivo and in vitro experiments demonstrated its ability to cause DNA damage (8).

Figure 1. Chemical structure of ochratoxin A.
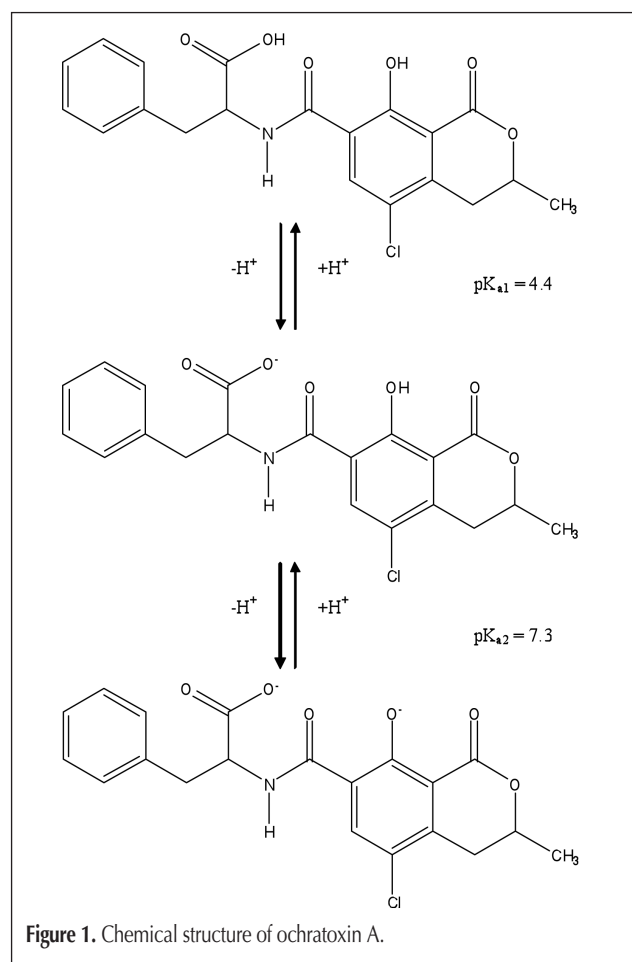
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With regards to cases of chronic exposition of OTA from contaminated food, there is a need to develop new faster and more sensitive methods for its determination. Chromatographic methods, such as high-performance liquid chromatography (9–14) or thin-layer chromatography (15,16), are the most used in OTA determination. Fluorescence detection is still preferred beyond the more expensive mass spectrometry (17).

Fluorescence intensity and sensitivity of analytical analysis strongly depends on solution composition, such as concentration and type of ions, pH, aqueous-to-organic phase ratio, cyclodextrin addition (10), terbium (III) ion presence (13), etc. In this work the influence of commonly used buffers, phosphate and acetate, was investigated with relation to fluorescence intensity of OTA.

Experimental

Chemicals

The standard OTA solution in acetonitrile with concentration 10.04 ± 0.14 µg/mL and acetonitrile (LC-gradient grade) were obtained from Sigma Aldrich (Prague, Czech Republic). Phosphoric acid, p.a. (85%), was supplied by Fluka; acetic acid, p.a. (99–100%), and potassium hydroxide, p.a. was supplied by Riedel-de Haën (Seelze, Germany). Aqueous solution of 33% ammonia, p.a. was obtained from Lachema (Zagreb, Croatia). Demineralized water was prepared using Demiwa 10 ros (Watek, s.r.o., Ledec nad Sázavou, Czech Republic). Standard solution of OTA was stored at –12°C. The working solutions were prepared freshly before experiments.

Apparatus

pH meter pH526 (WTW, Weilheim, Germany) was used for control of mobile phase pH. Calibration buffers with pH 4, 7, and 10 were supplied by Radiometer Analytical (Lyon, France). Fluorescence spectra were recorded on HPLC chromatograph Agilent 1100 series (Agilent, Palo Alto, CA) consisting of vacuum degasser (model G1322A), quaternary pump (G1311A), autosampler unit (G1313A), variable-wavelength detector (G1314A), fluorescence detector (G1321A), and mass spectrometer (G1946VL). Nitrogen generator model NM18LA was delivered by Peak Scientific Ltd. (Renfrewshire, Scotland). Chromatographic columns Zorbax Extend C_{18} (3.0×100 mm, 3.5 µm) (Agilent) and ODS-Hypersil C_{18} (250×4.6 mm, 5 µm) (Supelco, Bellefonte, PA) were used for chromatographic separations.

Preparation of solutions

Acetate and phosphate buffer solutions were prepared with concentration 83 mmol. pH value was adjusted by titration with potassium hydroxide or ammonium solution to obtain pH 3.0, 5.0, and 9.0, respectively. All buffers were degassed by nitrogen stream.

The measured solutions were mixed directly in the chromatograph using a programmed autosampler unit and stop-flow method. Volume of 1 µL of standard OTA solution was mixed with an appropriate amount of acetonitrile and buffer to give 50 µL. Final OTA concentration in measured samples was $200.8 \pm$

2.8 ng/mL. The mixture was injected into flow of water (flow rate 0.2 mL/min) and delivered to fluorescence detector. Then fluorescence spectra were collected in the range $\lambda_{ex} = 200$–420 nm (step 5 nm) and $\lambda_{em} = 300$–500 nm (step 5 nm). The bandwidth of monochromator was 20 nm. Fluorescence spectra were evaluated, and excitation and emission wavelength yielding maximum fluorescence intensity were recorded.

Calibration solutions

Calibration solutions were prepared fresh from stock solution of OTA by dilution with an appropriate amount of mobile phase. All solutions were stored at 4°C in dark place until the analysis. Spiked samples were prepared by addition of pure mycotoxin standard into wheat flour samples and stored for one day. After this period, spiked samples were analyzed.

Samples of wheat flour

Twenty samples of milled wheat were collected during harvest in 2008 in various parts of the Czech Republic.

OTA extraction

The extraction of OTA and subsequent extract purification procedure described (21) was applied. Briefly, 20 g of sample was extracted by mixture of 144 mL of acetonitrile and 16 mL of 4% aqueous solution of potassium chloride acidified by 0.32 mL of concentrated sulphuric acid. Next, the suspension was filtrated through paper filter Whatman no. 4 and extracted for 1 min by 100 mL of hexane. This step was repeated two times. Then, to the lower (aqueous) phase was added 20 mL of chloroform and shaken for 20 min. The chloroform phase was removed, and the upper phase re-extracted two times.

Joined chloroform extracts were alkalinized and extracted three times with 50 mL of 5% sodium bicarbonate and shaken for 10 min. Subsequent aqueous phases were combined and acidified to pH 1.5 with concentrated hydrochloric acid and allowed to stand 20 min. This solution was three times extracted by chloroform (100, 50, and 50 mL). Chloroform extracts were pooled and evaporated near to dryness under vacuum on rotatory evaporator at 40°C. Two milliliters of methanol were added, solution transferred to 1.8-mL vial, and evaporated to the dryness under nitrogen flow. Prior the analysis, the sample was dissolved in 500 µL of methanol.

Results

The combinations of two cations (potassium and ammonia), two anions (phosphate and acetate), three pH values (3.0, 5.0, and 9.0), and five acetonitrile-buffer ratios were prepared and measured. Prior measurement the solutions were degassed by bubbling with nitrogen to minimize molecular oxygen content that is a well-known fluorescence quencher.

Excitation/emission wavelength

Two excitation wavelengths yielding maximum fluorescence were found. The first one showed excitation maximum at 230 nm, and it was independent on pH, buffer composition, and ace-

tonitrile-buffer ratio. The second excitation maximum was pH-dependent and observed at either 335 nm or 380 nm. Otherwise, the maximum emission wavelength was pH-dependent. While solutions with pH 3.0 showed fluorescence maximum emission at 460 nm, the samples with higher pH (5.0 and 9.0) did it at 445 nm.

OTA is presented in two forms at pH 3.0, where the non-dissociated one is predominant (96%) and dissociated at carboxylic group of phenylalanine moiety is in minority (4%) (Figure 2).

Selection of pH and composition of buffer

There were not significant differences between OTA fluorescence intensity in buffers with pH 3.0. No influence of phosphate, acetate, potassium, ammonium, and oxonium ions were observed.

On the contrary, a very important role in fluorescence intensity plays an ionic composition of measured solutions adjusted to pH 5.0 and higher. From a chemical point of view OTA is a derivative of cumarine moiety amide linked to L-β-phenylalanine. It is weak acid with dissociation constant pK_a approximately equal to 4 for carboxyl group of phenylalanine and 7 for phenolic groups. The fluorescence emitted at pH 5.0 is a sum of fluorescence of 80% OTA^{1-} and 20% of OTA^{2-}. Generally, it was observed that in the case of phosphate buffers the fluorescence intensity is higher for ammonium ions in comparison with potassium ones. The fluorescence emissions of acetate buffers were more complex. When higher excitation energy is used (230 nm), the cation influence is unremarkable instead acetate-acetonitrile ratio equal to 14:86, where fluorescence of potassium containing buffer is about 80% higher in comparison with ammonium one. Lower excitation energy (335 nm) did not show differences in solutions with near to 100% of aqueous portion. With increasing content of organic modifier (acetonitrile), the fluorescence of ammonium containing buffers decreased, which is not correlated with potassium ones, where the influence was independent with exception of near 100% acetonitrile solutions.

At alkaline pH 9.0 are carboxylic and phenolic groups of OTA near to complete dissociated, and OTA undergoes a phototautomerization that results in an excitation and emission wavelength shift. Fluorescence intensity of both acetate and phosphate buffers had similar tendency and values at excitation at 230 nm with the exception of phosphate-acetonitrile ratio 14:86. Acetates had significantly higher fluorescence emissions for acetate adjusted with potassium hydroxide than ammonium at 380 nm. Phosphates buffers adjusted with potassium hydroxide had 50% higher fluorescence intensity than those adjusted with ammonium in solutions with high aqueous portion content. This behavior is changing in solution with higher acetonitrile content.

Influence of acetonitrile

The fluorescence intensity of most of measurements performed was constant or decreased with increasing acetonitrile content. Also, this rule is applicable for water-acetonitrile mixtures. There were some abnormalities registered at buffer-acetonitrile ratio 50:50. At pH 5.0 and 9.0, the fluorescence significantly increased for ammonium phosphate when λ_{ex} was 230 nm. The same phenomenon was observed for this buffer also at pH 9.0 and λ_{ex} 380 nm. The highest fluorescence emission from all tested solutions was obtained for ammonium phosphate adjusted to pH 5.0 with 50% of acetonitrile and λ_{ex} was 230 nm, respectively, with 2% of acetonitrile and λ_{ex} 380 nm.

HPLC measurement

There were performed HPLC measurements under conditions that yield the highest fluorescence intensity. Chromatographic column with stability extended to alkaline pH range was used, and flow rate was set to 0.4 mL/min. Generally, all measurements at λ_{ex} 230 nm were very noisy, probably due to traces of impurities in solvents and OTA standard solution. For measurement, there were selected excitation wavelengths 335 (pH 5.0) respective 380 nm (pH 9.0) and emission 460 respective 445 nm. The results showed that the retention time of OTA in phosphate ammonium buffer increases with increasing aqueous portion in mobile phase. For example, 83% of phosphate buffer content in mobile phase resulted in retention time greater than 56 min. With decreasing amount of buffer portion, the fluorescence intensity of OTA was lowered significantly. The retention time of OTA was lower at pH 9.0 then at pH 5.0. With 83% of potassium acetate in mobile phase, its retention time was 7.2 min (Figure 3). Higher aqueous solution-acetonitrile ratio in extremely long retention times.
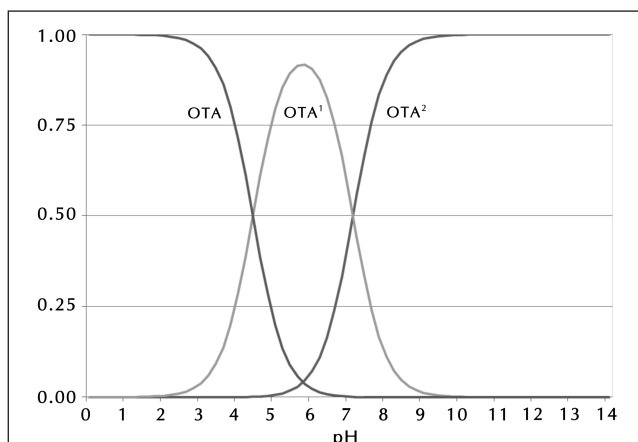

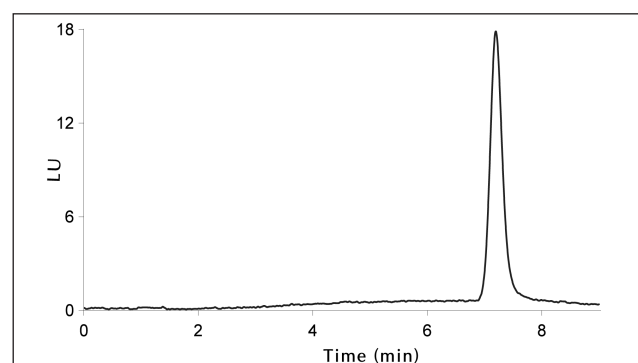**Figure 2.** Distribution diagram of ochratoxin A.

Figure 3. Chromatogram of OTA obtained under the conditions yielding the highest fluorescence: 83 mM potassium acetate pH 9.0–acetonitrile (83:17, v/v) at a flow rate of 0.4 mL/min.

Discussion

For buffer solutions, a concentration of 83 mM anion (acetate respective phosphate) was selected, which is the most often used in OTA determination by high-performance liquid chromatography (18). OTA shows excitation maximum at 335 nm in acidic solutions (pH 3.0 and 5.0) and 380 nm in alkaline buffers. All these observations can be explained by phototautomerization (19, 20). OTA is a weak acid with dissociation constant pK_a equal approximately to 4.4 for carboxyl group of phenylalanine and 7.1 for phenolic group (20). The measured fluorescence spectra at pH 3.0 are mostly of non-dissociated OTA. The dissociated OTA undergoes a structural change in the excited state (phototautomerization) that increases conjugation to generate the red-shifted emission spectrum. Phenolic system of cumaric moiety can form intramolecular hydrogen bonds and undergo an excited state intramolecular proton transfer (ESIPT) (19).

Table I. Fluorescence Emission of Ochratoxin A*

pH	Buffer composition anion	Buffer composition cation	2:98 LU	13:87 LU	50:50 LU	86:14 LU	100:0 LU
3.0	phosphate	NH_4^+	18.5	18	16.5	20.8	
3.0	phosphate	K^+	19.8	16.5	14.6	24.6	
3.0	acetate	H^+	10.9	16.9	15.1	19.6	
5.0	phosphate	NH_4^+	23.1	19.7	50.5	34.6	
5.0	phosphate	K^+	16	11.8	16.9	20	
5.0	acetate	NH_4^+	17.7	16.6	11.3	7.2	35
5.0	acetate	K^+	18.1	18.1	19	7.6	
9.0	phosphate	NH_4^+	25.4	23.6	34.8	13.9	
9.0	phosphate	K^+	26.6	26.8	24	11.2	
9.0	acetate	NH_4^+	23.9	23.5	21.6	8.1	
9.0	acetate	K^+	30	27.4	19.9	11	
5.5	water	H^+	32.1	28.9	25.3	5.8	

Header spanning "Acetonitrile-83 mM buffer ratio" over the five LU columns.

* Excitation 230 nm, emmision 460 (pH 3.0) respective 445 nm (pH 5.0; 9.0 and 100% acetonitrile).

Table II. Fluorescence Emission of Ochratoxin A*

pH	Buffer composition anion	Buffer composition cation	2:98 LU	13:87 LU	50:50 LU	86:14 LU	100:0 LU
3.0	phosphate	NH_4^+	10.5	10.3	9.3	11.6	
3.0	phosphate	K^+	11.1	9.2	8.3	12.5	
3.0	acetate	H^+	9	9	8	10.1	
5.0	phosphate	NH_4^+	42.9	10.6	18.6	13.7	
5.0	phosphate	K^+	9.4	5.8	9.7	10.7	
5.0	acetate	NH_4^+	10.2	9.7	6.3	3.8	22.9
5.0	acetate	K^+	10.5	11	11.3	3.7	
9.0	phosphate	NH_4^+	22.9	21.9	32.5	5.5	
9.0	phosphate	K^+	33.2	31.9	26.7	4.8	
9.0	acetate	NH_4^+	23.3	19.5	14.7	4.4	
9.0	acetate	K^+	35.6	27.1	20.2	10.5	
5.5	water	H^+	42.4	36.2	27.7	4.5	

Header spanning "Acetonitrile-83 mM buffer ratio" over the five LU columns.

* Excitation 335 (pH 3.0 and 5.0) respective 380 nm (pH 9.0), emmision 460 nm (pH 3.0 and 5.0), or 445 nm (pH 9.0 and 100% acetonitrile).

The detection limit reached for samples with pure OTA was 0.3 ng/mL. This is comparable with results obtained by other authors, which used pH 9.8 and 20 mM NH_4Cl/NH_3 buffer (20).

Finally, the developed method was applied for analysis of spiked cereal samples, and obtained results were compared with commonly used method (22). Reference method uses ODS-Hypersil C_{18} reversed-phase column (Supelco; 250 × 4.6 mm, 5 µm). The mobile phase flow rate was 1 mL/min, and isocratic elution with a mixture of acetonitrile–water–acetic acid (99:99:2; v/v/v) was applied. The sample injection volume was 50 µL. OTA was detected by fluorescence detector with excitation wavelength of 333 nm and emission wavelength of 477 nm.

While the standard method has the detection limit for OTA determination in real samples 2.3 µg/mL, the newly developed one was four times lower (0.6 µg/mL).

Conclusion

The influence of buffer composition and pH used as mobile chromatographic phase on OTA fluorescence was investigated. Two excitation maximums were observed. While the excitation at wavelength 230 nm was pH-independent, the second one at 335 nm changed in alkaline pH to 380 nm due to the structural changes in the excited state of OTA molecule. There was a trend of decreasing intensity of most tested solutions with increasing acetonitrile content with certain exceptions. The buffers with the highest fluorescence emission were evaluated. The best chromatographic parameters showed 83 mM potassium acetate solution at pH 9.0, where the detection limit 0.3 ng/mL was reached. Developed method was compared with standard method, and the sensitivity was demonstrated. The method can be applied for sensitive determination of OTA in samples of food or environmental origin.

Acknowledgment

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