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Journal of Chromatography A, 862 (1999) 147–159

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Reversed-phase liquid chromatographic behavior of the mycotoxins citrinin and ochratoxin A

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Received 7 May 1999; received in revised form 17 August 1999; accepted 26 August 1999

Abstract

The reversed-phase (RP) chromatographic behavior of citrinin (CT) and ochratoxin A (OA), the latter introduced as reference substance, were studied as a function of hydrophobicity and silanophilic activities of the stationary phase, pH, type of acid in the eluent, its composition as well as of the column temperature. While OA's affinity to RP materials was not influenced by phase material properties, CT showed a high affinity to hydrophobic phase materials, and its elution order, compared to OA, depended strongly on the phase material chosen. In practice, all octadecyl stationary phases under investigation allowed proper conditions for CT and OA chromatography if judicious selection of influencing parameters, especially a low pH and applying an acid with a $pK_a < 2.3$, were chosen.

Keywords: Stationary phases, LC; Mycotoxins; Citrinin; Ochratoxin A

1. Introduction

Mycotoxins have long been recognized as the major cause of feed toxicosis in animals, and may also constitute a serious hazard to human health, especially in developing tropical countries. Citrinin (CT; IUPAC: (3*R*,4*S*)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid; CAS: 518-75-2) is a toxic fungal secondary metabolite produced by different strains of fungi of the genera *Penicillium*, *Aspergillus* and *Monascus*. Since CT is found commonly in field samples along with ochratoxin A (OA; IUPAC: (*R*)-*N*-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl)carbonyl]-L-phenylalanine; CAS: 303-47-9), OA was also included in the investigation. *P. verrucosum* [1], *P. purpurescens* [2], *P. palitans* [2], *P. cyclopium* [2] and *P. viridicatum* [2,3] are known to produce both CT and OA. The latter two are ubiquitous fungi in temperate and cool climates and are found in agricultural commodities and even in treated or processed food [2]. Both mycotoxins are nephrotoxic and might occur together in foodstuffs such as cereals, fruits and meat [4]. In addition, OA is carcinogenic in laboratory animals, whereas CT apparently is not. However, it appears that in mice CT acts synergistically with OA and additive effects for renal carcinogenesis were noted [5]. Structures of both mycotoxins are given in Fig. 1.

While the exposure of humans to OA is relatively well known [6], data on CT are limited. Typically, Swiss cereal flour samples contained 0.2–1 ng/g CT [7,8]. The reason for this lack of data might be either analytical problems [9], as also mentioned in several

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00929-2

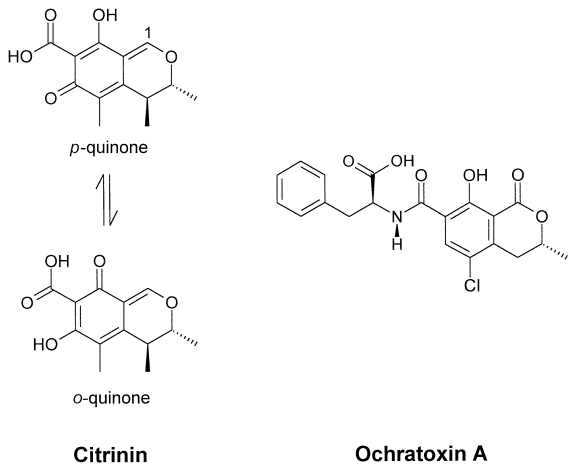

Fig. 1. Structure of the mycotoxins under investigation. CT exists as mixture of tautomers in aqueous solution at room temperature.

AOAC general referee reports [10–13], its non-occurrence, or its instability in foods. From the chemical-analytical point of view it is recommended that the chelating ability, the effect of pH and the effect of temperature on CT properties be considered [14]. CT exists in two tautomeric forms, namely the *p*- and *o*-quinone form (interconversion rate $>10^6$ s^{-1}), in aqueous solution at room temperature and is susceptible to Michael-type nucleophilic addition reactions at carbon 1 [15], marked in Fig. 1. In addition CT is known to form the citrinin H1 toxin, built up of two CT molecules, at temperatures above 100°C [16].

Common methods used to analyze CT (and OA) are thin-layer [17–20] and high-performance liquid chromatography (HPLC) with UV [21–25] or fluorescence detection (FD) [8,22,26–35] as well as enzyme immunoassays [35,36]. Mostly reversed-phase (RP) materials have been applied. Regarding the chromatography on reversed phases, usually eluents were acidified with orthophosphoric acid [25,28–30,32,33,35] or ion pair chromatography [21,22,26,27] was applied. HPLC in normal phase on a buffered silica gel column was also proposed [8]. Detection limits for CT from the above-cited literature were in the range 0.01–22.5 ng/g for FD and 2–20 ng/g for UV detection.

Since we had not been able to adopt suggested RP-HPLC methods in the past at first attempt [8] and because the choice of stationary phase material seems to be crucial and has to be made in time consuming tests as reported by others [35], it was the goal of this work to study the liquid chromatographic properties of CT in detail in order to develop an accurate and sensitive method to determine CT and OA together in foodstuffs.

2. Experimental

2.1. Reagents

CT and OA were from Sigma (Buchs, Switzerland), while trifluoroacetic (TFA), maleic (MaleicA), citric (CitrA) and orthophosphoric acid (H_3PO_4) were delivered by Fluka (Buchs, Switzerland). Oxalic acid (OxalicA) was from Siegfried (Zofingen, Switzerland). Gradient-grade methanol (MeOH) and acetonitrile (ACN) were from Biosolve (Brunschwig, Basel, Switzerland) and the deionized water was purified using a Barnstead EASYpure UV system (Bioblock Scientific, Frenkendorf, Switzerland). All chemicals were of analysis grade and were used without further purification.

Stock solutions of CT and OA were prepared by dissolving 1.3415 mg CT and 1.3220 mg OA, respectively, in 50 ml methanol. These were stored at –20°C in the dark. Unless otherwise noted, dilutions of 1:200 in methanol were normally used and 10 μl injected into the HPLC system, corresponding to an amount of about 130 pg each of CT and OA.

2.2. Instrumentation

The chromatographic system consisted of up to three LC-10AD high-pressure gradient LC pumps (Shimadzu, Kyoto, Japan), a dynamic mixer (Portmann, Biel-Benken, Switzerland) an SIL-10A autoinjector (Shimadzu), a SPD-M10AVp diode array detector (Shimadzu) and a RF-10A fluorescence detector (Shimadzu) in series. For data acquisition a PC board in combination with the Class LC-10 software V1.63 was used (Shimadzu). A Gastorr 104 degasser (Omnilab, Mettmenstetten, Switzerland), was used and the temperature of the column was kept, unless otherwise mentioned, at 25°C by a Pelcooler column oven (Portmann). Reversed-phase materials used were custom fills (ChromCart) from

Macherey-Nagel (Oensingen, Switzerland) and included the stationary phases Hypersil ODS, Inertsil ODS2, Kromasil, Nucleosil and Spherisorb ODS1 which were all in columns of 250×4 mm, except for Hypersil (250×4.6 mm). To determine the post-column apparent pH of the eluents, a MP225 pH meter equipped with a LE410 combined glass electrode (Mettler, Nänikon, Switzerland) was used. Indicated pH and pK_a values are, unless otherwise noted, as derived in the water–methanol mixtures and marked by an asterisk. Non-marked pH and pK_a values are as for pure aqueous solutions.

2.3. Chromatographic conditions

Both gradient and isocratic elution were used, according to the experimental requirements. General conditions are listed below and apply unless otherwise noted.

For isocratic elution the mobile phase consisted of methanol–0.25 *M* orthophosphoric acid (80:20, v/v) with a flow-rate of 0.5 ml/min.

Gradient elution was achieved as follows: eluent composition changed from (80:20) to (10:90) inorganic–organic eluent. Solvent program: linear gradient from (80:20) to (10:90) in 9 min, 15 min isocratic at (10:90), linear gradient from (10:90) to (80:20) in 2 min, 4 min conditioning at (80:20). Typical flow-rate was 0.5 ml/min. As for the composition of the inorganic and organic eluent, see Section 3. To determine the retention time for an unretained compound (t_0), thiourea was used [37]. These data agreed within ±5% with calculated t_0 values [38]. In this context, the capacity factor k' of an analyte was defined to be equal to the distance between t_0 and the analyte's band center, divided by the distance from injection to t_0 [38].

OA and CT exhibit UV absorption (OA: λ_{max}^{MeOH} (nm; ϵ)=331(6325) [this work], 333(6400) [39], 333(6640) [40]; CT: λ_{max}^{MeOH} (nm; ϵ)=321(5490) [this work]) and native fluorescence. Fluorescence detection (FD) was carried out at an excitation wavelength of 331 nm and the emission wavelength of 500 nm for CT and OA, optimized for CT detection. The UV diode array detection (DAD) range was set from 200 to 400 nm.

The detection limit by FD for CT and OA on the analytical column was about 100 and 30 pg, respectively, for a signal-to-noise ratio of 3 (on Spherisorb column, isocratic conditions, eluent 0.25 *M* H_3PO_4–MeOH (20:80), 10 μl injection, flow-rate 0.5 ml/min).

Precision was evaluated by injecting 10-μl aliquots of CT and OA standard solutions containing 1.3 ng each of CT and OA in a gradient run. Reproducibility of retention time and peak area over a several-week period resulted in relative standard deviations (RSD) for CT of 1% (n=7) and 2.7% (n=8), respectively, and for OA of 1% (n=9) and 2% (n=4), respectively. Ten consecutive injections revealed no variation in retention time and an RSD of 1% for the peak area of CT. All precision and reproducibility data were from runs on the Spherisorb column.

3. Results and discussion

3.1. Reversed-phase materials

The wide range of achievable selectivity with reversed-phase materials is a great advantage for optimizing HPLC separations. On the other hand it is sometimes difficult to find a second column of identical selectivity. Therefore five RP materials have been tested in this study according to Engelhardt's test [37]. Tracers used were toluene to explore hydrophobic interactions and isomeric (*o*-, *p*-) toluidines to check for silanophilic interactions. Isocratic runs with methanol–water (60:40, v/v) were executed, with UV detection at 250 nm. Analytes were identified by DAD.

Hydrophobic properties were measured by the capacity factor k' of toluene in the methanol–water system. Increasing k' of toluene indicates increasing hydrophobicity. As predicted from literature [41], the following order of increasing hydrophobicity resulted (k' of toluene given in parenthesis): Spherisorb (2.8), Nucleosil (4.1), Hypersil (4.3), Inertsil (6.9) and Kromasil (7.7). As expected, the capacity factor of toluene is proportional to the carbon content of the phase materials. Silanophilic properties were studied through the interactions of basic compounds (*N*,*N*-dimethylaniline, toluidines) with silanol groups [37]. The two isomeric (*o*-, *p*-) toluidines, which have the

same hydrophobicity but a different pK_b value, should elute as single peak on a phase material with low silanol activity. This was only the case for Kromasil. The bases *N*,*N*-dimethylaniline and the toluidines were eluted only on Kromasil and Inertsil as narrow peaks, whereas they had a severe tailing on Nucleosil and Hypersil. All three bases were adsorbed on Spherisorb.

The results together with the physical and chemical properties of the stationary phases as delivered by the producers are given in Table 1.

According to the Engelhardt tests, the reversed-phase materials were provisionally classified as follows: due to their high hydrophobicity and low silanol activity, Kromasil and Inertsil were termed columns of type I, whereas Nucleosil, Hypersil and Spherisorb in this context were termed columns of type II.

The metal content of a stationary phase is important when separating *polar* analytes like CT and OA, which according to their structure may have also excellent complexing properties. Unfortunately, the manufacturers gave insufficient or incomplete data on this subject (Table 1) to be able to correlate the chromatographic behavior of CT and OA to these values. Only a tentative estimation was possible on this purpose: both RP materials of type I, Inertsil and Kromasil, have low metal content, whereas the older generation phase materials Hypersil, Nucleosil and Spherisorb are expected to have much higher metal contents.

To chromatograph the analytes CT and OA, elution at low pH was chosen, since CT is known to be a strong acid [42]. However, we failed to find a corresponding pK_a value in the literature. Under ion suppression conditions the silanophilic interactions are suppressed partially since silanolic groups are protonated [37]. The silanophilic activity of the stationary phases may, therefore, be of minor importance. The chromatographic behavior of the analytes

Table 1
Physical and chemical properties of the octadecyl-derived stationary phases

Material[a] (batch)	Particle size (μm)	Carbon (%)	Pore size (nm)	End-capped	Metal content (μg/g)	Σ (μg/g)	Hydrophobic activity[b]	Silanophilic activity[c]	Number of plates, N[d] (×1000)		Manufacturer
									N_{CT}	N_{OA}	
Inertsil ODS2 (1204-2)	5	18.5	15	Yes	Al (5), Ca (10), Cr (nd), Fe (1.5), Mg (1), Mn (nd), Na (5), Ni (nd), Ti (nd), Zn (1)	23.5	+++	++	8.7	4.5	G.L. Science (Japan)
Kromasil C_{18} (0123)	6.1	19.3	10	Yes	Al (8.5), Ba (0.1–0.2), Ca (4.3), Cd (<0.1), Cu (0.3), Fe (8.9), Mg (1.9), Mn (0.1–0.2), Na (12.3), Ni (0.7), Pb (<0.1), Sr (<0.1), Ti (8.9), Zn (0.1–0.2)	46.3	+++	+	1.5	5.3	Akzo Nobel (Sweden)
Hypersil ODS (4003)	4.5–5.5	9.5	12	Yes	Al, Ca, Fe (200–300), Na	>300	++	+++	2.7	6.8	Shandon (UK)
Nucleosil C_{18} (4064)	3	11	12	Yes	Fe (<20)	≫20	++	+++	1.5	5.9	Macherey-Nagel (Germany)
Spherisorb ODS1 (94/108)	3–7	7	8	No		?	+	+++	1.4	4.2	Waters (USA)

[a] All columns were of formerly unused quality.

[b] According to the Engelhardt test [37]: hydrophobic properties studied through retention of toluene in the system methanol–water (60:40). Qualitatively, the higher the hydrophobicity the more '+' were assigned.

[c] According to the Engelhardt test [37]: silanophilic properties studied through retention and resolution of (*o*-, *p*-) toluidine isomers and *N*,*N*-dimethylaniline in the system methanol–water (60:40). Qualitatively, the higher the activity of rest silanol groups, the more '+' were assigned.

[d] Calculated as 5.54×(retention time/width at half height)^2 in the isocratic system 0.1 *M* H_3PO_4–MeOH (20:80).

Particle size, carbon content, pore size, endcapping and metal content indication are as given by the manufacturer.

nd, not detectable.

under these conditions was studied systematically. Under ion suppression conditions the investigated RP materials could be subdivided into two groups. With the first group, CT eluted before OA, while in the second group CT eluted after OA. This classification of the stationary phases coincides with that according to Engelhardt's test. It is supposed that hydrophobic and/or metal interactions are the cause of this different selectivity of the tested phase materials. The differences in surface coverage can be envisaged to produce the differences in steric requirement and hydrophilic–hydrophobic properties, which determine the chromatographic behavior of the analytes. CT is expected to be more planar than OA, which increases its ability to be retained more efficiently in the ordered high density hydrocarbon surface of the type I phase materials. The bonded phase coverage for both columns of type I is ~3.2 $\mu mol/m^2$ and decreases in the order Hypersil (~2.8)>Nucleosil (~2.1)>Spherisorb (~1.5) [43].

The interaction of CT with type II stationary phase material is low, while for OA there is no significant difference of the affinity against stationary phase materials of type I or II. It might be that OA is not able to penetrate as deep into the stationary phase chains as CT and, therefore, the stationary phase coverage appears to be of minor importance in OA retention.

In order to understand the parameters influencing the selectivity of the phase materials more precisely, eluent composition, pH range, the influences of the acid used and of temperature were further studied.

3.2. Influence of eluents

Selective separations are generally optimized by the use of ternary or even quaternary eluent systems. Water, methanol and acetonitrile are mostly used as a ternary system. The retention of the analytes is given by the water content, whereas the composition of the organic modifier (ratio methanol–acetonitrile) determines the elution order and resolution of the analytes.

In a gradient system consisting of the eluent acidified water–organic modifier the composition of organic modifier was varied from 0 to 100% (v/v) MeOH in ACN in steps of 20, 33.33, 50, 66.66 and 80% (Fig. 2). The analytes were chromatographed

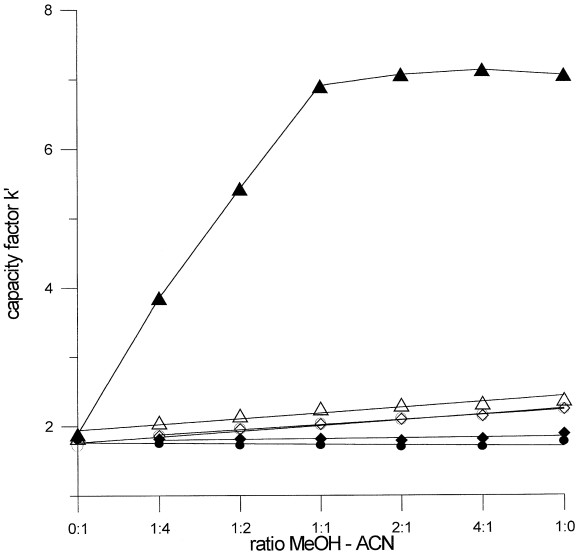

Fig. 2. The capacity factor k' is plotted versus the composition of organic modifier, expressed as ratio MeOH–ACN. The organic modifier is changed over time according to the gradient program described in the text in the system 0.25 *M* H_3PO_4–organic modifier (80:20→10:90). The composition of the organic modifier is varied from 0 to 100% (v/v) MeOH in ACN in steps of 20, 33.33, 50, 66.66 and 80%. (♦) CT, Spherisorb, H_3PO_4; (●) CT, Spherisorb, OxalicA; (▲) CT, Inertsil, OxalicA; (◇) OA, Spherisorb, H_3PO_4; (○) OA, Spherisorb, OxalicA; (△) OA, Inertsil, OxalicA.

on both an example of a type I (Inertsil) and a type II (Spherisorb) column. Analytes were detected by FD. The acids used were either 0.25 *M* orthophosphoric acid (H_3PO_4) or 10 m*M* oxalic acid (OxalicA). OxalicA was chosen since due to its chelating properties it is susceptible to interact with metal ions, also present on the surface of the solid-phase material.

As shown in Fig. 2, k' of OA does not much depend on either composition of the organic modifier or the type of acid used. On Inertsil, OA is slightly more retarded. The interaction of OA and the phase material increases slowly with increasing MeOH content, independent of the tested phase material.

For the elution of CT the stationary phase material, but not the acid, is of major importance. On Inertsil it seems that the polar character of CT rapidly decreases with increasing hydrophilic activity of the eluent, as shown by increasing k' values in Fig. 2. The higher the MeOH content of the organic

modifier, the stronger the interaction of CT with the Inertsil stationary phase. Strongest interaction is reached for an organic modifier composition of MeOH–ACN (1:1), while at higher MeOH content k' does not increase any further and a plateau establishes. This points to a different chemical behavior of CT on the two types of stationary phase materials tested. The higher the ACN content of the organic modifier, the poorer the resolution between CT and OA becomes, finally resulting in coelution of CT and OA if the organic modifier consists of pure ACN.

We decided to continue our inquiry with a simple, binary system with MeOH as organic modifier. Using pure ACN was not appropriate due to the low selectivity of the system, although CT seems to be much more strongly solvated in MeOH than in ACN and sensitivity loss in FD occurs (see Section 3.6). The fact that MeOH is less toxic and less expensive supported our decision.

3.3. Influence of pH

In order to study the influence of pH* on the elution of the analytes CT and OA, in isocratic runs the acid concentration in the eluent (H_3PO_4–MeOH) was changed from 0.005 to 0.5 *M* H_3PO_4 in seven steps and the MeOH content was varied. No buffer was used in order to avoid the effect of the association of the acid anions with buffer cations. For Inertsil and Kromasil the ratios H_3PO_4–MeOH were either (10:90) or (20:80), while for Spherisorb it was either (20:80) or (30:70) and for Nucleosil and Hypersil (20:80). The pH* was measured post-column and FD was applied to detect the analytes. As expected, k' augmented with increasing water content of the eluent used.

Over the resulting pH* range of 2.3–3.6, the k' of OA shows little variation, independent of stationary phase material chosen. The retention of CT on the other hand is strongly influenced in this range of pH*. Apparently, under these conditions OA is in its protonated apolar form (pK_a=4.4 [44]), while CT's dissociation degree strongly depends on the acidity of the eluent and the stationary phase material.

Ion suppression conditions in this pH* range seems not to be ideal for CT, since small variations in pH result in marked changes of k'. A lower pH* would be required to ensure a protonated apolar CT but lowering pH below 2 risks irreversible damage of the octadecyl columns. Ion pair chromatography could be a constructive alternative to the approach of chromatographing CT and has been applied in several attempts [21,22,26,27].

On RP materials of type I (Inertsil and Kromasil) k' of CT increases strongly with increasing pH*, while on the contrary, on type II materials (Hypersil, Nucleosil, Spherisorb), k' increases with decreasing pH* of the eluent. This is very surprising since the acid CT would be expected to behave as seen on type II columns but not as on stationary phase materials of type I, where it appears to act as a base.

In a RP material comparison study by others [45], bases at varying pH have been chromatographed. The authors found that under certain circumstances, e.g., using a stationary phase material with high metallic impurities or residual silanol groups having very low pK_a values, some of the bases under investigation acted as acids, i.e., their k' decreased with increasing pH, instead of vice versa as expected. The same study states that some stationary phase materials may contain small quantities of nitrogen, in the way that unusual high retention of aldehydes and carboxylic acids have been found. In a study dealing with the chromatographic behavior of pyridine- and indolecarboxylic acids it was shown that for some of the acids retention decreased with increasing pH on alkyl columns, while on alkylamide columns they were longer retained at the higher pH values [46].

That there is coherence of the carbon load of the stationary phase material and CT retention in our study is shown by the fact that k' of CT augments with increasing bonded phase coverage. For a pH* 3.6 (2.5) k' is 12.2 (1.6) for Inertsil, 2.8 (1.0) for Kromasil, −0.1 (0.3) for Nucleosil, −0.2 (0.2) for Spherisorb and −0.3 (0.2) for Hypersil. It seems that, in addition to the hydrophobic interactions, at elevated pH very strong ionic interactions on type I columns arise, while on type II columns on the contrary negative k' values result. Negative k' values are known to be due to ionic exclusion effects [47]. The low metal content, which otherwise enhances the acidity of surface silanol groups, could also contribute to this effect [48].

Indeed, the chemical modifications of RP materi-

als of newer generation improve analysis of basic substances but induce non-ideal interactions for the analysis of carboxylic acids. Especially the type of end-capping used by the manufacturers is likely to be responsible for this behavior. Recently hydrophobic stationary phases containing hydrophilic groups near the surface or in the alkyl chain itself or in the end-capping have become available for polar analytes like acids [49].

The low pK_a value of CT (see below), its greater charge effects and ability to form intermolecular hydrogen bonding compared to OA might contribute to its unexpected retention behavior on type I columns in the investigated pH range.

Independent from the chosen stationary phase material, the structure of CT detected by DAD was dependent on pH in the same way, showing a neutral CT spectrum at pH* 2.3 (λ_{max} (nm): 213, 222sh, 246sh, 327) and CT^- at pH* 3.3 (λ_{max} (nm): 213, 252, 317) in the acidic methanol eluent.

In the chosen range of pH*, where the dissociation of CT is strongly dependent on the proton concentration, the pK_a^* was determined [47]. In Fig. 3 normalized log k' values of CT are plotted against pH*. For clarity only data of the retention on Inertsil and Spherisorb are shown, however retention behavior of CT on Kromasil was analog to Inertsil and all other type II columns were in analogy to Spherisorb. Sigmoid curves were fitted through the experimental data. The inflection point of the curves where pH*=pK_a^* is marked through the intersection of the sigmoid curves with the dashed line in the graph. The determined apparent acidity constants are functions of the solvent used. At higher water content, dissociation of acids is favored and the apparent pH for the same nominal content of acids is lower, while for bases the apparent acidity is higher with increasing water content [50]. Indeed, CT on Spherisorb shows an increasing acidity (lower pK_a^*) with increasing water content of the eluent, while on Inertsil the acidity decreases with increasing water content (Fig. 3). Note that the data shown are not corrected for the difference in eluent composition.

In order to estimate the pH and pK_a values of CT in pure aqueous solution, data points at pH*=pK_a^* were read out of Fig. 3 and corrected according to Refs. [51] or [52] resulting in a pK_a of CT in pure water of 2.3±0.2 or 2.2±0.2 (n=10), respectively.

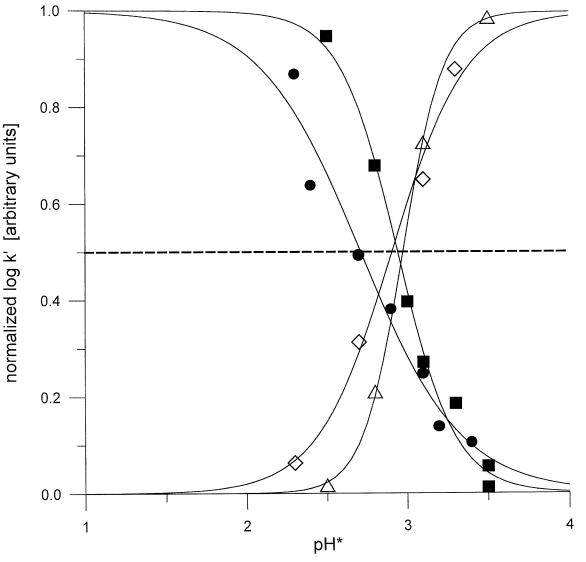

Fig. 3. Determination of pK_a^* for CT on Inertsil and Spherisorb phase materials. Logarithmic k' values were normalized and sigmoid curves fitted through the data points. The inflection point of the curves, i.e., the locus where pH*=pK_a^*, is indicated by the intercept of the dashed line with the sigmoid curves. In parenthesis the ratios inorganic–organic modifier are given. (◇) Inertsil (10:90); (△) Inertsil (20:80); (■) Spherisorb (20:80); (●) Spherisorb (30:70).

To our knowledge this is the first rough estimate of the pK_a for CT.

3.4. Influence of acids

Five different acids were used to investigate their influence on the chromatographic behavior of CT and OA. In a gradient system, mineral and organic acids with different structures and chelation potentials (mono-, di-, tridentate) were used. Citric (CitrA), orthophosphoric (H_3PO_4), maleic (MaleicA), oxalic (OxalicA) and trifluoroacetic acid (TFA) were part of the aqueous eluent in concentrations of 10 mM (Table 2), while the other parameters were as described in Section 2 for a gradient system. The analytes were detected by fluorescence.

The resulting pH values of the aqueous eluents were in the range 1.7–2.6 and the measured pH* at CT elution was in the range 2.8–3.6. Within this range, in accordance with Fig. 3, it should be

Table 2
Properties of acids used and shape of eluted peaks in the gradient system 10 m*M* acid–methanol (80:20→10:90)[a]

Acid	pK_a	Inertsil CT/OA	Kromasil CT/OA	Hypersil CT/OA	Nucleosil CT/OA	Spherisorb CT/OA
CitrA	3.14, 4.77, 6.39	-/n	-/n	-/n	n/n	n/n
MaleicA	1.83, 6.07	b/n	b/n	-/n	t/n	t/n
H_3PO_4	2.12, 7.21, 12.67	b/n	b/n	t/n	n/n	n/n
OxalicA	1.23, 4.19	b/n	b/n	t/n	n/n	n/n
TFA	0.23	b/n	b/n	-/n	b/n	t/n

[a] -, no peak detected; n, narrow peak (tailing <2, ratio area to height <20); b, broad peak (tailing <2, ratio area to height≥20); t, tailing peak (tailing≥2). Peak tailing was calculated according to Ref. [38].

possible to detect CT on either octadecyl phase material of type I or II.

As already stated above, on stationary phases of type I the capacity factor k' for CT is much higher than for OA. Contrarily on stationary phases of type II k' of CT is lower than for OA, i.e., the elution order of CT and OA is reversed. While there are only slight effects on the k' of OA independent of acid used (k' increases in the order Hypersil<Nucleosil< Spherisorb<Kromasil≤Inertsil from 1.5 to 2.4), k' of CT increases considerably in the order Hypersil, Nucleosil, Spherisorb<Kromasil <Inertsil from 1 to 6. The retention of both the mycotoxins depends apparently much more on the stationary phase material chosen than on the type of acid used. This might be related to the properties of the stationary phase materials, i.e., bonded phase coverage and silanol content.

Still, it is interesting to note that the acids seem to have an influence on solvation of CT depending on their properties in the eluent's pH* range of 2.8–3.6. So, CT is not eluted with some acids and stationary phases used, while OA under the given conditions elutes with any tested acid on any phase material as narrow peak (Table 2). For CT, this might be due to its partial dissociation in the chosen pH range. Further, the properties of the acids, i.e., pK_a and/or chelating properties influence chromatographic behavior and CT's degree of dissociation will depend on eluent's pH and on pK_a of the acid used. A high degree of dissociation for CT is apparently reached with CitrA as acidifier, since CT was only detected on Spherisorb and Nucleosil with very poor intensity (see Section 3.6) but fully adsorbed on the others (Table 2). Since CitrA is a weaker acid than CT, it is likely that CT under these conditions exists as deprotonated polar CT^-.

On Inertsil a variation of k' for CT was noted depending on the acid chosen. While CT was not eluted with CitrA as mentioned above, its k' value (given in parenthesis) decreased in the order H_3PO_4, MaleicA (~6)>OxalicA (~5)>TFA (~3.7). It can be assumed that protonation degree of CT^- increases in the same order, since the pK_a values of the acids (Table 2) decrease in the order given above. Contrary to expected, k' augments with increasing deprotonation degree of CT, as stated already in Section 3.3. for columns of type I. This strong influence of the acid could not be seen on the other columns, which might be due to enhanced phase coverage and a higher ordered structure of the Inertsil stationary phase material compared to the others.

The mono- and polydentate acids and also CT and OA, are expected to interact with the metal ions of the stationary phase materials. Especially the type II columns are likely to have high metal contents. Indeed, CT, which under the given pH conditions is partially dissociated and, therefore, susceptible to ionic interactions, is either eluted as tailing peak or not at all, especially on Hypersil. On the other hand CT is eluted as narrow peak on Nucleosil and Spherisorb with CitrA, H_3PO_4 or OxalicA. If only the strength of acid would be responsible for peak shape, an increased tendency for tailing peaks in the order TFA <OxalicA <MaleicA <H_3PO_4<CitrA would be expected. Since this was not the case, we conclude that not only ion suppression of silanols and of CT, but also ionic interactions of CT with the metal ions occur. That the acidity of surface silanol

groups is strongly influenced by the metal content of the stationary phase material is also worth mentioning [48].

The broad CT peaks on the columns of type I are mainly due to their late elution in the isocratic part of the gradient run, i.e., caused by the chosen program and not by interactions. Peak broadening decreases with decreasing k'. That peaks are always symmetric correlates well with the low metal content of the stationary phase materials.

3.5. Temperature effects

To investigate the affinity of the analytes for the stationary phase materials, chromatograms at a temperature range of 25–55°C were registered under isocratic conditions (acidified water–MeOH (10:90)) and fluorescence detection. Stationary phase materials (and acids) used were Inertsil (10 m*M* TFA or 0.25 *M* H_3PO_4), Kromasil (10 m*M* OxalicA), Hypersil (0.25 *M* H_3PO_4), Nucleosil (10 m*M* TFA or 0.25 *M* H_3PO_4) and Spherisorb (10 m*M* OxalicA). The results are shown as Van't Hoff plot in Fig. 4, where the natural logarithms of the capacity factors of CT and OA are plotted against the inverse of absolute temperature. Since straight lines resulted over the chosen temperature range, the sorption enthalpies for CT and OA were calculated from the slope of corresponding regression lines and are listed in Table 3.

On stationary phase materials of type I k' of CT and OA decrease with increasing temperature, while on type II columns k' of OA decreases, whereas k' of CT increases with increasing temperature. Therefore on type I columns and for OA on type II columns exothermic sorption enthalpies result, while positive enthalpies result for CT on type II columns. It seems to be energetically more favorable for CT to be in the mobile phase when stationary phase materials of type II are used, and consequently CT elutes always earlier than OA. On Spherisorb and Hypersil even negative k' values resulted for CT and, therefore, no reasonable sorption enthalpy could be determined.

From the intercept of the Van't Hoff plot the entropy of the system may be calculated unless the phase ratio of the column (the volume of the stationary phase divided by the volume of the mobile

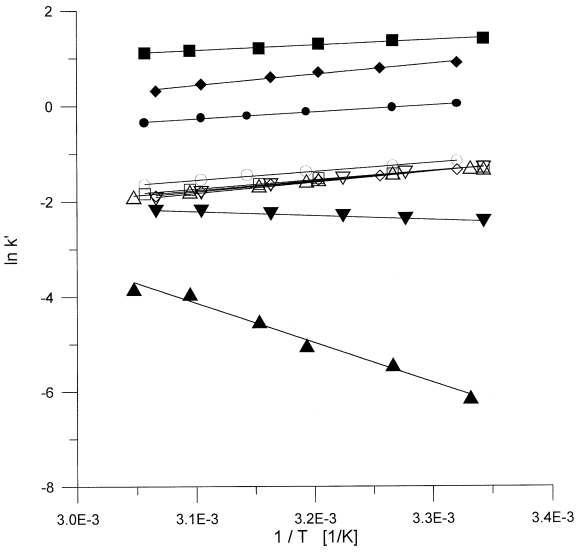

Fig. 4. Influence of temperature on the elution of CT and OA on Inertsil, Kromasil and Nucleosil stationary phase materials. In isocratic runs (acidified water–MeOH (10:90)), the aqueous eluent was acidified either with 10 m*M* TFA or OxalicA or with 0.25 *M* H_3PO_4 (indicated in brackets below). (■) CT, Inertsil (H_3PO_4); (♦) CT, Inertsil (TFA); (●) CT, Kromasil (OxalicA); (▼) CT, Nucleosil (TFA); (▲) CT, Nucleosil (H_3PO_4); (□) OA, Inertsil (H_3PO_4); (◇) OA, Inertsil (TFA); (○) OA, Kromasil (OxalicA); (▽) OA, Nucleosil (TFA); (△) OA, Nucleosil (H_3PO_4).

phase) is known [53]. Since the phase ratio relies on physical data of the column it may be assumed that its value remains constant for different eluent compositions, i.e., acids used. Therefore the values of the intercepts for Inertsil (10 m*M* TFA or 0.25 *M* H_3PO_4) and Nucleosil (10 m*M* TFA or 0.25 *M* H_3PO_4) were compared. The intercept for both stationary phase materials and either of the acids is around −8.5 for OA, while for CT the intercept is always higher when the eluent is acidified with H_3PO_4 instead of TFA, on Inertsil it was −2.6 or −7.0, respectively, and on Nucleosil 21.3 or 0.41, respectively. This difference in entropy change might be attributed to different degrees of solvation of CT in the mobile phase depending on the acid used, as already seen in the previous section. CT seems to be less solvated when H_3PO_4 instead of TFA is used and indeed in our study CT was retained longer in case H_3PO_4 instead of TFA was used, especially on Inertsil.

Table 3
Experimental enthalpies of sorption for CT and OA on the stationary phase materials under investigation[a]

RP material	Column type[b]	Acid	ΔH_{CT} (kJ/mol)	n	ΔH_{OA} (kJ/mol)	n
Inertsil ODS2	I	0.25 *M* H_3PO_4	−10.09±0.02	6	−16.25±0.02	6
Inertsil ODS2		0.01 *M* TFA	−19.92±0.03	6	−18.16±0.02	6
Kromasil		0.01 *M* OxalicA	−12.62±0.01	6	−15.56±0.02	6
Hypersil ODS	II	0.25 *M* H_3PO_4	–[c]	6	−16.82±0.08	6
Nucleosil		0.25 *M* H_3PO_4	68.16±0.03	6	−17.27±0.02	8
Nucleosil		0.01 *M* TFA	7.01±0.01	6	−19.47±0.03	6
Spherisorb ODS1		0.01 *M* OxalicA	–[c]	12	−22.00±0.02	12

[a] The number of data pairs is indicated by *n*. The eluent consisted of acidified water–MeOH (10:90), pH* was in the range 2.9–3.0 and temperature in the range 25–55°C.
[b] See Section 3.1.
[c] Not determined due to negative k' values.

In this way, the entropy term over-compensates the higher interaction affinity with the stationary phase material of CT in TFA-acidified eluent, since although the enthalpy term is more negative for TFA-acidified eluent (Table 3), CT is retained longer for the eluent acidified with H_3PO_4. Note that the concentration of hydrogen ions for all three acidifiers were pH* 2.9–3.0.

For OA almost the same intercept values and enthalpies result independent of acid or stationary phase material chosen (Fig. 4). It seems that the bonded phase coverage and the solvolytic property of OA in the range pH* 2.9–3.0 is of minor importance for its retention.

3.6. Detection

As long as native fluorescence of the analytes is maintained, FD is a factor of 10–100 more sensitive than DAD. In our study, fluorescence of CT and OA depended on the eluent chosen, its pH and the acid used for ion suppression.

3.6.1. Eluent

Varying the composition of the organic modifier from pure MeOH to pure ACN as described in the previous Section 3.1 led to an almost linear increase of the fluorescence response by a factor of ~4 for CT, while an approximately constant one resulted for OA. Since absorption and emission maxima in acidic MeOH and ACN were comparable, it is expected that, apart of radiationless deactivation in MeOH [54], CT is more solvated in MeOH than in ACN and the anion is non-fluorescent. Therefore the more MeOH is present in the organic modifier, the less CT is susceptible of fluorescence. The noted increasing detection sensitivity of CT with increasing ACN content of the organic modifier is in agreement with others [54].

No sensitivity difference between Inertsil (type I) and Spherisorb (type II) could be seen, but the pH of the eluent caused a sensitivity increase of a factor of 1.1 when 0.25 *M* H_3PO_4 (pH 1.4) instead of 10 m*M* OxalicA (pH 2.0) was used. No effect of pH was noted on detection sensitivity of OA since it is assumed that due to its higher pK_a it is fully in its apolar protonated form.

A higher ACN content offers increased sensitivity in our ternary system but decreases resolution. With increasing protic eluent, k' of CT increases markedly on Inertsil. Increasing the MeOH content increases the resolution of CT and OA but the more protic the organic modifier chosen is, the more important it is to keep the eluent pH low.

*3.6.2. pH**

While OA's detection sensitivity by fluorescence is not susceptible to pH in the chosen range in methanolic eluent, the sensitivity for CT detection increases with decreasing pH*, with a slope of 2.8±0.2 per pH* unit. At pH=pK_a the dissociation degree α (ratio of dissociated to total concentration of acid) is 0.5, i.e., only 50% of CT is in its protonated neutral fluorescent form. In order to reach high detection sensitivity for CT in a protic eluent like 0.25 *M* H_3PO_4–MeOH, α has to be as low as

possible. This means for a detection of 99% CT ($\alpha=0.01$), that the pH at CT detection should be adjusted to zero ($pH=pK_a+\log(\alpha/(1-\alpha))$, $pK_a=2.3$).

3.6.3. Acid

The influence of acids on CT and OA fluorescence detection is shown in Fig. 5. CitrA with a higher pK_a than CT leads to very low or no CT detection at all (Table 2). On Hypersil no CT could be detected in case CitrA, MaleicA or TFA were used as ion-suppressing acids and low sensitivity resulted with OxalicA. This could be due to CitrA being too weak an acid to protonate CT and of increased interactions with metal impurities from the stationary phase material compared to the other columns under investigation.

From the selected acids, H_3PO_4 appears to be of universal applicability since it allows detection of CT and OA at about comparable sensitivities independent on chosen RP materials (Fig. 5). The otherwise unaffected OA shows a surprising intensity increase when MaleicA is used to acidify the eluent on Inertsil, Nucleosil and Spherisorb columns. This effect seems to be independent of metal content and hydrophobicity. If interactions of the acid with either CT or OA would be the cause of this effect, one would expect marked changes in k' of the analytes on different phase materials. However, no such effects could be observed.

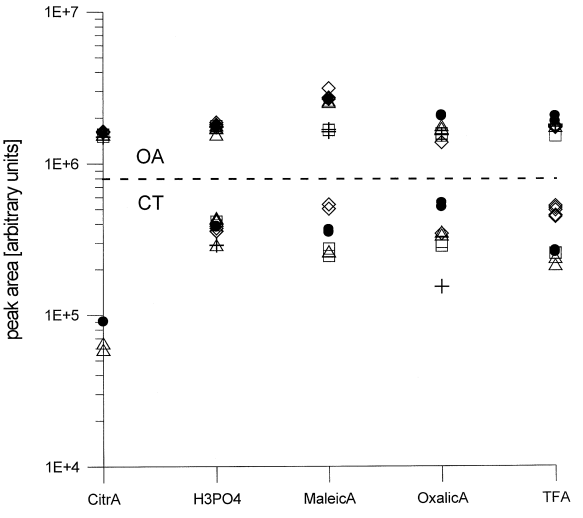

Fig. 5. Peak area of fluorescence detected CT (below dashed line) and OA (above dashed line). Conditions: gradient 10 m*M* acidifier–methanol (80:20→10:90, solvent program described in Section 2), pH* 2.8–3.6. (+) Hypersil; (◇) Inertsil; (□) Kromasil; (●) Nucleosil; (△) Spherisorb.

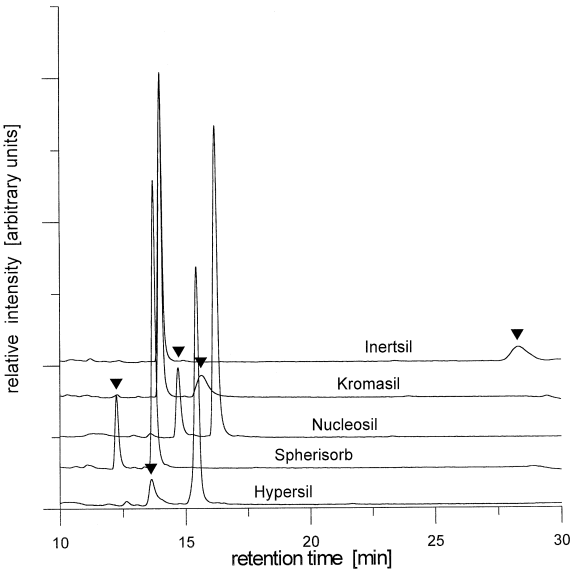

Fig. 6. Chromatograms of CT (marked by a triangle) and OA on the five stationary phase materials under investigation. Conditions: gradient 0.25 *M* H_3PO_4–methanol (80:20→10:90, see text for solvent program).

Fig. 6 clearly shows that reasonable chromatograms of CT and OA (injected amount for both ~0.13 ng) on all five stationary phase materials under investigation are gained, e.g., in the gradient system 0.25 *M* H_3PO_4–methanol (time program described above). Note that the solvent program was not optimized for runs where CT eluted after OA.

To ensure an apolar fluorescent CT in the eluent, the acid used should have a pK_a value of <2.3 and in an eluent containing a protic modifier like MeOH a $pH \leq 0$ is expected to provide optimal sensitivity. This could be realized through post-column addition of a strong acid instead of lowering the eluent's precolumn pH, in order to protect the octadecyl stationary phase material from degradation.

4. Conclusions

The octadecyl reversed-phase materials under

investigation were classified into two groups, according to their elution behavior against apolar and basic molecules. The RP materials with high hydrophobicity and low silanol activity were termed type I columns and the phase materials with low hydrophobicity and high silanol activity were termed columns of type II. This classification matched well the chromatographic influence of the stationary phase materials on CT and OA chromatography, since two different behaviors were noted: on stationary phase materials of type I CT eluted after OA and on columns of type II the contrary applied. RP materials chosen or selected acids used did not significantly influence the chromatographic behavior of OA.

However, the major finding that can be drawn is: there is no recommendable column type for CT chromatography, i.e., a successful chromatography is strongly dependent on the interaction of stationary phase material and chosen chromatographic parameters, such as pH, type of acid in the eluent, eluent composition and temperature. Especially the combination of the acid selected to establish ion suppression conditions and the stationary phase material had a strong influence on peak shape and retention behavior. Using ion suppression overcomes the problem of reproducibility of stationary phases, because the slight differences in silanol concentrations that cause large changes in chromatographic retention are masked.

While CT on stationary phase materials of type I eluted as a symmetric but broad peak with high capacity factor, it was eluted as narrow or tailing peak with low capacity factor or not at all on stationary phase materials of type II. According to the low pK_a of CT of 2.3 ± 0.2 a low eluent pH is required, and the acid selected should thus have a pK_a of <2.3. However, it should be noted that the required pH is at the lower limit of the normally recommended working range of octadecyl reversed-phase materials. If fluorescence detection is applied, the pH within the flow cell should be pH$\leq$0 since only the undissociated form of CT is susceptible of fluorescence. This could be accomplished, e.g., through post column addition of a strong acid in order to reach appropriate pH values. Also, using an organic modifier less protic than MeOH, e.g., ACN, would help to overcome the dissociation problem of CT.

As a practical application, the high affinity of CT on type I and low affinity on type II stationary phase materials, respectively, could be exploited in such a way that using both types of columns on two systems in parallel could help to confirm the presence of CT in a sample. Further, the retarded elution of CT on columns of type I could be helpful if important amounts of interfering peaks from real samples are present in the beginning of a separation, or if using a high affinity RP material in a pre-column as cleanup procedure.

Still, due to the required low pH value of the eluent, ion pair chromatography should be envisaged as a possible alternative method, although under these conditions the native fluorescence of CT is lost. In addition, in ion pair chromatography increased attention has to be paid to eluent compositions and their stability since they may induce sensitive changes in retention time [55].

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