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Retention data for five ketotrichothecenes in reversed-phase high-performance liquid chromatography with different eluent systems[a]

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ABSTRACT

The effect of the eluent composition (nature of the modifier and its mole fraction in the mobile phase) on the retention of trichothecene mycotoxins of group B (derivatives of 12,13-epoxytrichothecene-9), *i.e.*, desoxynivalenol (DON), nivalenol, DON-3-acetate, DON-15-acetate and 7-desoxy-DON was studied. Ethanol, acetonitrile or tetrahydrofuran were used as the organic component (modifier) of the binary water–organic mobile phases. The retention mechanism and separation selectivity of the trichothecene mycotoxins in reversed-phase high-performance liquid chromatography (RP-HPLC) on Nucleosil C-18 is discussed. Optimum conditions were determined for the separation of the five trichothecene mycotoxins with the use of RP-HPLC under isocratic conditions.

INTRODUCTION

Trichothecene mycotoxins are a group of chemically similar secondary metabolites of the *Fusarium* mould fungus. Structurally they belong to derivatives of 12,13-epoxytrichothecene-9 and can be classed into two groups: group B with the carbonyl oxygen atom in position 8 and group A without this atom (Fig. 1) [1].

The purpose of this work was to optimize the conditions for determining trichothecene mycotoxins of group B in grain samples by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Milichrome microcolumn chromatograph (USSR).

EXPERIMENTAL

Instruments and reagents

A liquid microcolumn chromatograph (Milichrome) equipped with a syringe pump (eluent flow-rate 2–600 μl/min) and a spectrophotometric detector (spectral

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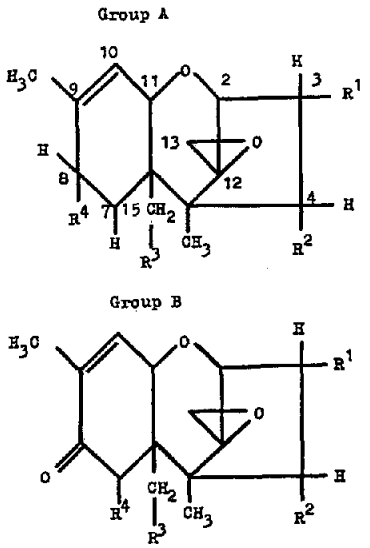

Fig. 1. Structures of trichothecene mycotoxins. Group A: T-2 tetraol, $R^1 = R^2 = R^3 = R^4 = OH$; DAS, $R^1 = OH$, $R^2 = R^3 = OAc$, $R^4 = H$; HT-2 toxin, $R^1 = R^2 = OH$, $R^3 = OAc$, $R^4 = -OC(O)C_4H_9$; T-2 toxin, $R^1 = OH$, $R^2 = R^3 = OAc$, $R^4 = -OC(O)C_4H_9$. Group B: nivalenol, $R^1 = R^2 = R^3 = R^4 = OH$; DON, $R^1 = R^3 = R^4 = OH$, $R^2 = H$; 7-desoxy-DON, $R^1 = R^3 = OH$, $R^2 = R^4 = H$; DON-3-acetate, $R^1 = OAc$, $R^2 = H$, $R^3 = R^4 = OH$; DON-15-acetate, $R^1 = R^4 = OH$, $R^2 = H$, $R^3 = OAc$. OAc = acetate.

range 190–360 nm) was used. The steel column (62 × 2 mm I.D.) was packed with Nucleosil C_{18} (specific surface area 300 m^2/g, mean particle diameter 5 μm) using the suspension technique. The retention volume of a non-sorbed substance (V_0) was measured by using sodium nitrite and was 89 μl; the column efficiency with respect to desoxynivalenol was 2500–3000 theoretical plates. The UV spectra of the substances were recorded in the range 190–360 nm with a 2-nm spacing in the detector cell where the flow of the mobile phase was preliminarily stopped. The reference cell was filled with the eluent.

The following retention parameters were determined using the chromatograms: the adsorbate capacity factor $k' = (V_R - V_0)/V_0$, where V_R is the adsorbate retention volume, and the relative retention volume (selectivity) for pairs of components, $\alpha_{i/j} = k'_i/k'_j$, where k'_j and k'_i are the capacity factors of substances j and i.

Ethanol, acetonitrile (ACN) and tetrahydrofuran (THF) were purified by distillation; acetonitrile was preboiled with potassium permanganate and THF was distilled over potassium hydroxide. Solutions of desoxynivalenol (DON), nivalenol, DON-3-acetate, DON-15-acetate and mixtures of DON with 7-desoxy-DON in methanol were used. In addition, samples of grain extracts purified and evaporated to dryness were used. The extraction mixture was water–acetonitrile (1:5) and adsorption purification of the extracts was performed on glass columns packed with AGN-type active carbon and Celite-545. The solutions and purified extracts were provided by the Laboratory of Mycotoxicology, All-Union Research Institute of Veterinary Sanitation.

Chromatographic measurements

DON solutions with concentrations of 1, 5, 10, 20, 40 and 200 μg/ml were prepared by sequential dilution of the initial solution (10 mg/ml) with water–THF (76:24). Evaporated samples of grain extracts were also dissolved in 1 ml of this mixture, which was then used as the eluent for the determination of DON. A test sample of the solution investigated (5 μl) was taken with the needle of the eluent feeder of the chromatograph (0.5–20 μl range with 0.1-μl spacing). The eluent flow-rate was 50 μl/min. The detection wavelength was 224 nm.

RESULTS AND DISCUSSION

The most difficult pairs to separate are DON–7-desoxy-DON and DON-3-acetate–DON-15-acetate. Separation of the latter using HPLC has not been described in literature. Separation of the former has been attained by using much more polar eluents (containing 10% of methanol [2]). In this case, k' increases to 15 and higher [3], which makes the analysis longer and more complicated and the simultaneous determination with DON in mixtures with more hydrophobic trichothecenes (such as acetates) under isocratic conditions impossible. For this reason, we investigated less polar organic modifiers for the optimization of the eluent composition in order to determine simultaneously all five mycotoxins investigated.

The following requirements were established for choosing the modifier: unlimited solubility in water, low viscosity, ready availability, low absorption threshold in the UV range and classification with various groups of selectivity according to Snyder and Kirkland [4,5]. Ethanol, acetonitrile and tetrahydrofuran were chosen. We employed the strategy of De Galan and co-workers [6–8] to find the optimum composition of the eluent. The investigation of the dependence of the retention of trichothecene mycotoxins on the composition of binary aqueous eluents was carried out first. The data (capacity factors, k', and relative retention volumes, α) for three binary systems are given in Table I.

Fig. 2 and Table I show that the retention of trichothecene mycotoxins increases with increasing hydrophobity. Fig. 2 shows that dependence of $1/k'$ on N_b is linear. For this reason, the dependence of the capacity factor on the eluent composition (the nature of the modifier and its mole fraction in the mobile phase) for DON-3-acetate, the most retained sorbate of those studied, was considered for the optimization of the duration of analysis. Fig. 3 shows that large amounts of polar modifier compared with the less polar modifier should be introduced into the eluent in order to attain the same retention value for mycotoxins, *e.g.*, $CH_3CN > C_2H_5OH > C_4H_8O$. The dependence of the mole fraction of the mobile phase modifier (N_b) corresponding to the retention of DON-3-acetate ($k' = 10$; see Fig. 3) on the polarity, P', of the modifier is linear (Fig. 4).

When ethanol is employed as a modifier, the separation selectivity is low: DON and 7-desoxy-DON are not separated at all ($\alpha_{3/2} = 1.0$) and DON-3- and -15-acetate are poorly separated ($\alpha_{5/4} = 1.05$) (see Fig. 5 and Table I).

The selectivity of the trichothecene separation with the use of acetonitrile-containing mobile phases is higher than that with ethanol-containing mobile phases, although it remains insufficient for complete separation of DON and 7-desoxy-DON and particularly of DON-3- and -15-acetate. The separation selectivity of the pair

TABLE I

DEPENDENCE OF CHROMATOGRAPHIC PARAMETERS OF TRICHOTHECENE SEPARATION ON THE MOBILE PHASE COMPOSITION

Mobile phase	Composition (%, v/v, of organic component)	Capacity factor (k')					Relative retention volume (α)					
		Nivalenol (1)	DON (2)	7-desoxy-DON (3)	DON-3-acetate (4)	DON-15-acetate (5)	2/1	3/1	3/2	2/3	5/4	4/5
Ethanol–water	10	2.16	4.92	4.92	21.47	21.47	2.27	2.27	1.00	1.00	—	—
	15	1.57	3.16	3.16	14.40	15.20	2.01	2.01	1.00	1.00	1.06	0.95
	20	1.15	2.17	2.17	7.31	7.66	1.89	1.89	1.00	1.00	1.05	0.95
	25	0.85	1.58	1.58	5.07	5.29	1.85	1.85	1.00	1.00	1.04	0.96
	30	0.66	1.16	1.16	3.93	3.93	1.75	1.75	1.00	1.00	1.00	1.00
Acetonitrile–water	18	2.46	7.46	8.38	21.47	21.47	3.03	3.40	1.12	0.89	—	—
	25	1.76	5.68	6.45	21.47	21.47	3.23	3.66	1.14	0.88	—	—
	33	1.40	2.83	3.09	16.29	17.98	2.02	2.20	1.09	0.92	1.10	0.91
	36	1.36	2.40	2.61	13.72	13.72	1.76	1.92	1.09	0.92	1.00	1.00
	40	1.25	1.92	2.08	9.39	8.86	1.54	1.66	1.08	0.92	0.94	1.06
	50	1.06	1.48	1.62	5.52	5.06	1.40	1.53	1.09	0.91	0.92	1.09
Tetrahydrofuran–water	15	1.63	3.00	2.52	13.56	13.35	1.84	1.55	0.84	1.19	0.98	1.02
	18	1.30	2.22	1.85	8.11	7.49	1.71	1.42	0.83	1.20	0.92	1.08
	20	1.26	2.16	1.81	7.79	7.17	1.71	1.43	0.84	1.19	0.92	1.09
	25	1.08	1.88	1.54	6.08	5.37	1.74	1.42	0.82	1.22	0.88	1.13
	30	1.01	1.70	1.43	4.52	3.85	1.68	1.42	0.84	1.19	0.85	1.17

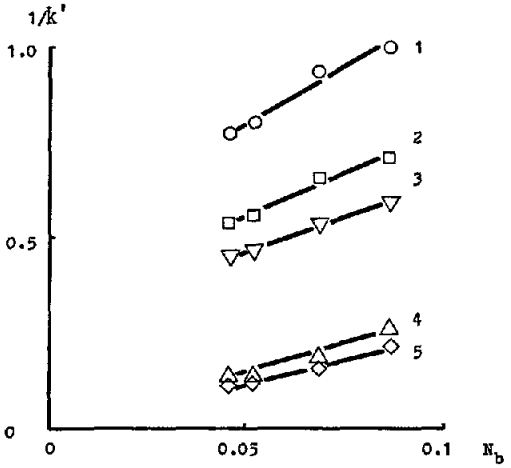

Fig. 2. Dependence of $1/k'$ for trichothecene mycotoxins on the composition of the mobile phase (N_b) modified with THF. 1 = Nivalenol; 2 = 7-desoxy-DON; 3 = DON; 4 = DON-15-acetate; 5 = DON-3-acetate.

7-desoxy-DON–DON is virtually constant ($\alpha_{3/2} \approx 1.10$) over the entire concentration range of acetonitrile (18–50%). The highest selectivity for the pair DON-3-acetate–DON-15-acetate ($\alpha_{5/4} = 1.10$) was observed at an acetonitrile concentration of 33% when the retention of acetates (and, consequently, the separation of peaks and the duration of analysis) are too high ($k' > 15$; see Fig. 3). A concentration of acetonitrile of 40% is considered to be the optimum, as the retention of all the components remains

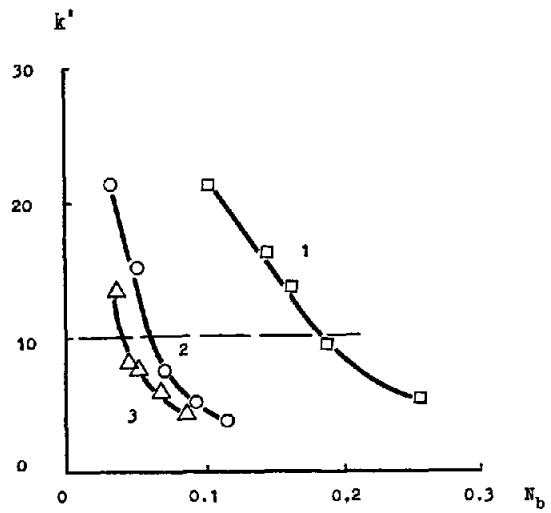

Fig. 3. Dependence of the capacity factor k' of DON-3-acetate on the mole fraction (N_b) of the organic component in the eluent. 1 = Acetonitrile; 2 = ethanol; 3 = tetrahydrofuran.

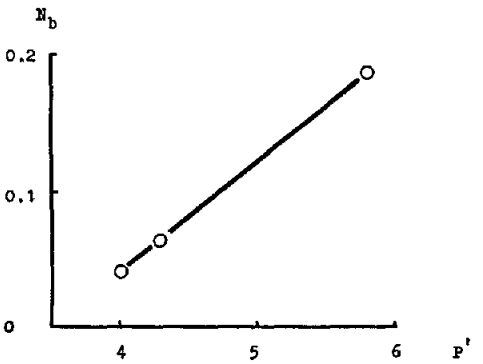

Fig. 4. Dependence of the mole fraction of the mobile phase modifier (N_b) corresponding to the retention of DON-3-acetate ($k' = 10$) on the polarity (P') of the modifier.

in the k' range 1.25–8.86 and the selectivity for the 7-desoxy-DON–DON pair is $\alpha_{3/2} = 1.08$ and for the DON acetates $\alpha_{4/5} = 1.06$.

The best separation selectivity among the binary mobile phases investigated was observed with the THF–water system. As a less polar modifier capable of forming hydrogen bonding with C–OH groups, THF provides for separations due both to the difference in the number of hydroxyl groups in the toxin molecule and to the different positions of the acetyl groups. The increase in selectivity of the separation DON-3- and -15-acetate on increasing the THF content, the selectivity of the pair 7-desoxy-DON–DON remaining constant, indicates that the effect of THF on the separation of more hydrophobic adsorbates is stronger.

Fig. 5 shows that an increasing amount of THF in the eluent increases the separation selectivity of the components of the mixture and decreases the duration of

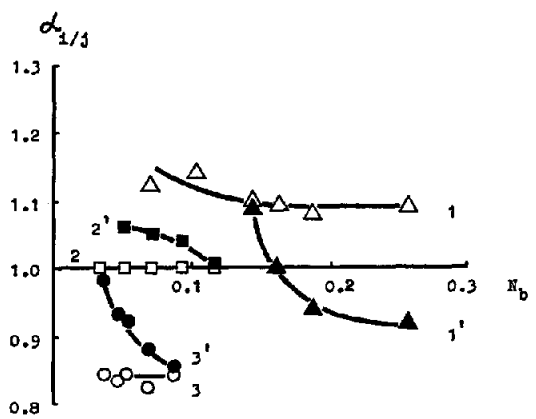

Fig. 5. Dependence of the relative retention volume ($\alpha_{i/j}$) on the mole fraction (N_b) of the organic component in the eluent for poorly separated pairs of substances: open symbols, 7-desoxy-DON–DON; closed symbols, DON-15-acetate–DON-3-acetate. 1,1′ = Acetonitrile; 2,2′ = ethanol; 3,3′ = tetrahydrofuran.

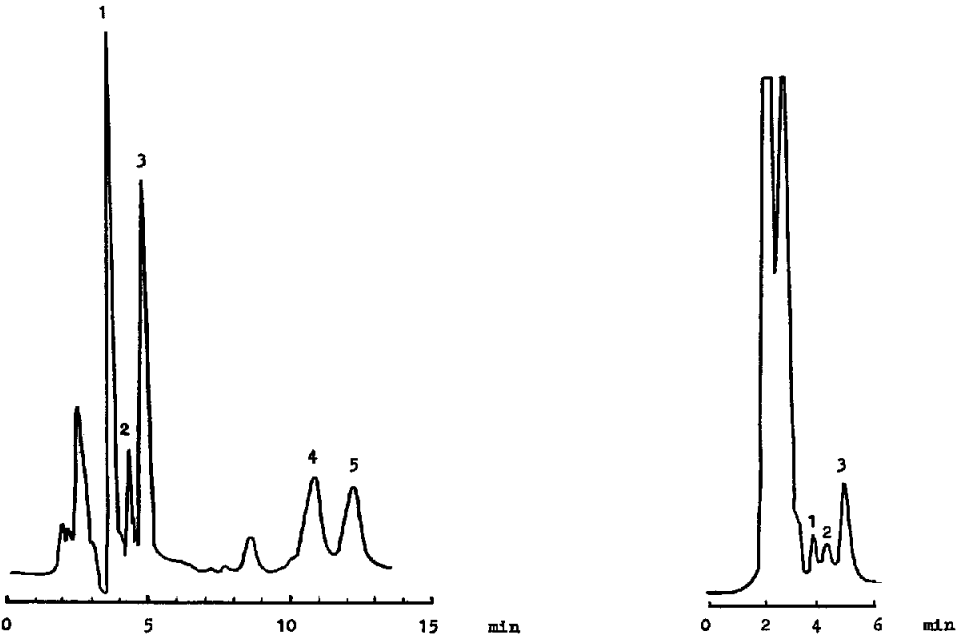

Fig. 6. Chromatogram of trichothecene separation with the optimized composition of the eluent, water–tetrahydrofuran (76:24). Nucleosil C_{18} column (62 × 2 mm I.D.); flow-rate, 50 μl/min; λ, 224 nm. 1 = Nivalenol; 2 = 7-desoxy-DON; 3 = DON; 4 = DON-15-acetate; 5 = DON-3-acetate.

Fig. 7. Chromatogram of an extract from wheat meal. 1 = Nivalenol; 2 = 7-desoxy-DON; 3 = DON. Conditions as in Fig. 6.

analysis (see Fig. 3). However, when the THF content is 30% (N_b = 0.087), the retention of nivalenol and 7-desoxy-DON decreases to such an extent that their peaks begin to overlap with those of polar impurities present in the samples. However, when the THF content decreases, the separation of the DON acetates deteriorates. A compromise (optimum) composition of mobile phase with 24% of THF provides both a good separation of all the components of the mixture (Fig. 6) and an acceptable duration of analysis (14 min). The substances elute from column in the sequence nivalenol < 7-desoxy-DON < DON < DON-15-acetate < DON-3-acetate. The selectivity for the pair DON–7-desoxy-DON was 1.18 and for the pair DON-3-acetate–DON-15-acetate it was 1.13. The separation of DON acetates has not been reported previously.

Fig. 7 shows the chromatographic analysis of an extract from wheat meal.

CONCLUSIONS

Optimum conditions are determined for the separation of five trichothecene mycotoxins (nivalenol, 7-desoxy-DON, DON, DON-3-acetate and DON-15-acetate) using RP-HPLC under isocratic conditions. The chromatographic system was shown to exhibit high selectivity when using an eluent containing THF.

REFERENCES

1 R. J. Cole and R. H. Cox (Editors), *Handbook of Toxic Fungal Metabolites*, Academic Press, New York, 1981.
2 G. A. Bennet, *J. Am. Oil Chem. Soc.*, 58 (1981) 1002A.
3 M. W. Witt, L. P. Hart and J. J. Pestka, *J. Agric. Food Chem.*, 33 (1985) 745.
4 L. R. Snyder, *J. Chromatogr. Sci.*, 16 (1978) 223.
5 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979.
6 P. J. Schoenmakers, H. A. Billet and L. de Galan, *J. Chromatogr.*, 205 (1981) 13.
7 P. J. Schoenmakers, H. A. Billet and L. de Galan, *J. Chromatogr.*, 218 (1981) 261.
8 A. C. Drouen, H. A. Billet and L. de Galan, *J. Chromatogr.*, 352 (1986) 127.