Journal of Chromatography, 627 (1992) 247–254
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 627

Supercritical fluid chromatography of *Fusarium* mycotoxins

J. Christopher Young

Plant Research Centre, Agriculture Canada, Ottawa, Ontario K1A 0C6 (Canada)

David E. Games

Mass Spectrometry Research Unit, Department of Chemistry, University College of Swansea, Singleton Park, Swansea SA2 8PP, Wales (UK)

(First received May 22nd, 1992; revised manuscript received September 16th, 1992)

ABSTRACT

Capillary- and packed-column supercritical fluid chromatography has been used for the separation of *Fusarium* mycotoxins of various structure types such as the trichothecenes including deoxynivalenol and its acetylated derivatives and T-2 toxin, as well as butenolide, culmorin, sambucinol and zearalenone. The effect of modifier concentration and column temperature and pressure was also studied. Retention indices based on alkylphenones were determined for these mycotoxins on two of the capillary columns employed.

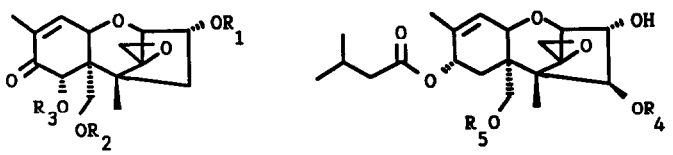
INTRODUCTION

The fungi of the genus *Fusarium* are capable of producing an impressive diversity of secondary metabolites that cause a variety of toxic effects on humans, animals and plants [1,2]. These metabolites (mycotoxins) include (see Fig. 1): simple monocyclic compounds such as butenolide, sesquiterpenes such as the trichothecenes 4-deoxynivalenol and T-2, the modified trichothecenes such as sambucinol and longifolene-like culmorin, and aromatic lactones such as zearalenone.

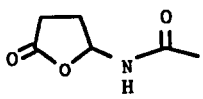
The analysis of these compounds typically involves extraction from the sample matrix, some preliminary cleanup, chromatographic separation [thin-layer (TLC), gas (GC) or high-performance liquid (HPLC)], and detection [3]. GC requires that the compounds be volatile and relatively non-polar. Many of the above compounds are sufficiently non-volatile to require reaction, such as silylation or polyfluoroacylation, to afford a volatile derivative. Such steps add to the complexity of the analysis. Typical universal GC detection methods include flame ionization (FID) and mass spectrometry (MS), while electron-capture detection (ECD) require an element such as fluorine to be present in the molecule. Liquid chromatography generally does not require derivatization to aid in the chromatography, however the commonly used ultraviolet (UV) or fluorescence detectors require the presence of UV or fluorescence chromophores in the molecule. HPLC–MS is more universally applicable although the type of interface employed may place limits on the chromatographic solvents that can be used.

Supercritical fluid chromatography (SFC) adds a new dimension to the analysis of mycotoxins. SFC can be conducted with heavily cross-linked bonded fused silica columns of 50 μm or 100 μm I.D. or with columns packed with conventional HPLC

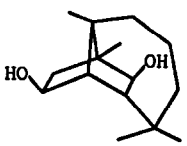
Correspondence to: J. C. Young, Plant Research Centre, Agriculture Canada, Ottawa, Ontario K1A 0C6, Canada.



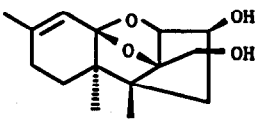
	R_1	R_2	R_3	R_4	R_5
3-Acetyldeoxynivalenol	Ac	H	H	-	-
15-Acetyldeoxynivalenol	H	Ac	H	-	-
3,7,15-Triacetyldeoxynivalenol	Ac	Ac	Ac	-	-
Deoxynivalenol	H	H	H	-	-
HT-2 Toxin	-	-	-	H	Ac
T-2 Toxin	-	-	-	Ac	Ac
T-2 Triol	-	-	-	H	H



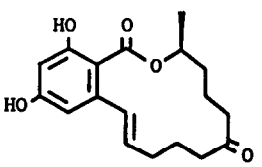
Butenolide



Culmorin



Sambucinol



Zearalenone

Fig. 1. Structures of some *Fusarium* mycotoxins.

packings or those designed for SFC. In addition to temperature as a variable to aid in eluting compounds from a capillary column, SFC also permits pressure/density programming and addition of modifying solvents. This enables temperatures to be kept low for thermally unstable substances. Detection requirements are the same for both SFC and conventional GC/HPLC. However, SFC is more amenable to interfacing with a mass spectrometer than HPLC because of the lower mobile phase gas volumes generated.

Retention indices (*I*), such as the Kováts scale [4], have been used for many years for standardization of GC data [5]. Such indices have only recently been used for HPLC data [6]. Homologous series employed for generation of HPLC *I* values include alkan-2-ones [7,8], aryl ketones [9] and 1-nitroalkanes [8,10,11]. The alkylphenones [8,12–15] and the related 1-[*p*-(2,3-dihydroxypropoxy)phenyl]-1-alkanones [16] have been investigated the most extensively. HPLC *I* values for some *Fusarium* and other mycotoxins have been determined [13,14,16]. The alkylphenones have also been used to determine *I* under packed-column SFC conditions [17].

This study was initiated to determine the efficacy of SFC for the resolution and possible analysis of some *Fusarium* mycotoxins that exhibit a wide variety of structure types. The SFC–MS analysis of only a few trichothecene mycotoxins on capillary columns has been previously published [18–20],

however to our knowledge, SFC analyses of *Fusarium* mycotoxins on packed columns have not been reported. In this study, the SFC of selected *Fusarium* mycotoxins on three capillary and six packed columns is reported along with a comparison of retention indices.

EXPERIMENTAL

Mycotoxin standards

HT-2 toxin and T-2 triol were from Sigma, St. Louis, MO, USA. The remainder were obtained from liquid cultures of various *Fusarium* spp. (prepared in the Plant Research Centre laboratories, Ottawa, Canada). Standard solutions were prepared with dichloromethane as solvent. A solution of zearalenone in a clear glass vial was allowed to stand at room temperature exposed to light for several weeks to give a mixture of starting material and a photo isomer.

Reagents

All reagents and solvents were of analytical-reagent grade. The series of C_8–C_{24} alkylphenone standards was obtained in kit form (catalogue No. 29 858-1) from Aldrich, Milwaukee, WI, USA. Approximately 15 mg/ml solutions of the alkylphenones in methanol and dichloromethane were prepared for analysis on the capillary and packed columns, respectively. Instrument-grade carbon dioxide supplied in cylinders with a dip tube (BOC, London, UK) and glass-distilled methanol were used as eluents.

Chromatographic columns

The SB-Methyl-100, SB-Biphenyl-30 and Carbowax capillary columns (each 10 m × 50 μm I.D. with adsorbent in a 0.25 μm film) were obtained from Lee Scientific (Division of Dionex, Salt Lake City, UT, USA). The packed columns (each 250 × 4.6 mm I.D.) were filled with one of Apex II Diol, Apex Phenyl S5B, Partisil 10 ODS3, Spherisorb Amino 5 μm, Spherisorb Cyano 3 μm or Spherisorb Silica 5 μm.

Chromatographic equipment

A Carlo Erba Model SFC 3000 series SFC LC fitted with a flame ionization detector (Carlo Erba, Milan, Italy) was used for the capillary column analyses. Injections were made into a pneumatic-activated VICI valve containing a 200-nl injection loop with a 150 to 200 ms injection time. Pure carbon dioxide was used as eluent.

Packed column SFC analyses were conducted on either (1) a Hewlett-Packard 1084B liquid chromatograph (Hewlett-Packard, Avondale, PA, USA) modified for SFC [21] with a Hewlett-Packard VWD-79875 variable-wavelength detector set at 224 nm or (2) a system consisting of a Gilson 302-303 pump system (Gilson, Villiers-Le-Bel, France), a Carlo Erba oven and an ABI Analytical Kratos Division Spectroflow 757 UV detector set at 224 nm.

The liquid carbon dioxide and the pump heads of the various chromatographs were cooled to −25°C using a Neslab RTE-4Z refrigerated bath (Neslab Instruments, Newington, NH, USA).

LC analyses

Capillary column analyses were typically made at 100°C using a 0.2 to 0.7 g/ml density program with a 0.01 g/ml per min linear gradient. Packed column analyses were made at an eluent flow-rate of 4 ml/min.

Retention indices

A mixture of the alkylphenones was coinjected with the mycotoxins. Retention indices were determined by a comparison of capacity factors (k').

RESULTS AND DISCUSSION

Retention indices

The relationship between carbon number and k' for the alkylphenones and capillary columns employed in this study is shown in Fig. 2. The plots were curvilinear and the data could be fitted by linear regression analyses to the equation

$$k' = A(1 - e^{-bN}) + c$$

where A, b and c are constants and N is the equivalent carbon number.

The calculated I values ($N \times 100$) for selected *Fusarium* mycotoxins on two of the capillary columns are listed in Table I. The alkylphenones were readily resolved on Carbowax, however they eluted before any of the mycotoxins, so I values could not be determined for this column. These data show

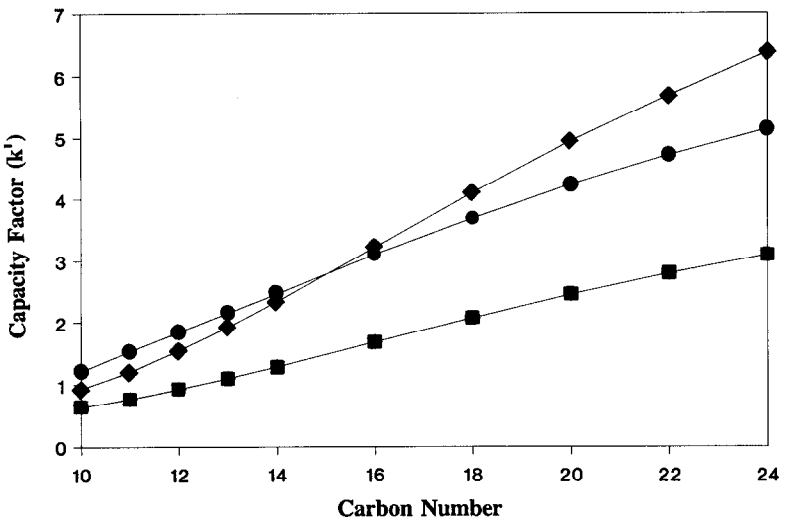


Fig. 2. Plot of capacity factors *vs.* alkylphenone carbon number for SFC at 100°C on 10 m × 50 μm I.D. capillary columns with 0.25 μm film of (◆) SB-Methyl-100, (●) SB-Biphenyl-30 and (■) Carbowax. Eluent, supercritical CO_2, density programmed from 0.2 to 0.7 g/ml at 0.01 g/ml per min.

TABLE I

RETENTION INDICES OF SELECTED *FUSARIUM* MYCOTOXINS ON CAPILLARY COLUMNS UNDER SUPERCRITICAL FLUID CHROMATOGRAPHIC CONDITIONS

Columns 10 m × 50 μm I.D. with 0.25 μm adsorbent coating. Eluted with supercritical CO_2 with a linear density program from 0.2 to 0.7 g/ml at 0.01 g/ml per min at 100°C. Indices based on homologous *n*-alkylphenones.

Compounds	SFC capillary columns		Literature for conventional reversed-phase HPLC
	Methyl	Biphenyl	
3-Acetyldeoxynivalenol	2046	2414	–
15-Acetyldeoxynivalenol	1990	2341	–
Triacetyldeoxynivalenol	1984	2107	–
Butenolide	1170	1657	–
Culmorin	1575	1701	–
Diacetoxyscirpenol	1956	2264	866, 1326[b]
Deoxynivalenol	2060	2646[c]	689, 1037[b]
HT-2 toxin	2249	2555[c]	1434[b]
Sambucinol	1837	2212	–
T-2 toxin	2198	2300	1016, 1513[b]
T-2 triol	2339	2797[c]	–
Zearalenone	2589[c]	3765[c]	1069[a], 1024[d]
Zearalenone isomer	2482[c]	2420[c]	–

[a] Ref. 14. Indices based on homologous *n*-alkylphenones.
[b] Ref. 16. Indices based on homologous 1-[*p*-(2,3-dihydroxypropoxy)phenyl]-1-alkanols.
[c] Estimated by extrapolation.
[d] Ref. 13.

that I values are column dependent. It is not surprising that Frisvad and Thrane [14] noted some differences between their results and those of Hill *et al.* [13] under conventional HPLC conditions.

The alkylphenones were not retained on any of the six packed HPLC columns investigated under SFC conditions. They eluted as one broad unresolved band immediately after the solvent peak and well before any of the mycotoxins. Thus if I values for *Fusarium* mycotoxins on packed SFC columns are to be developed, either a homologous series other than simple alkylphenones, perhaps such as that

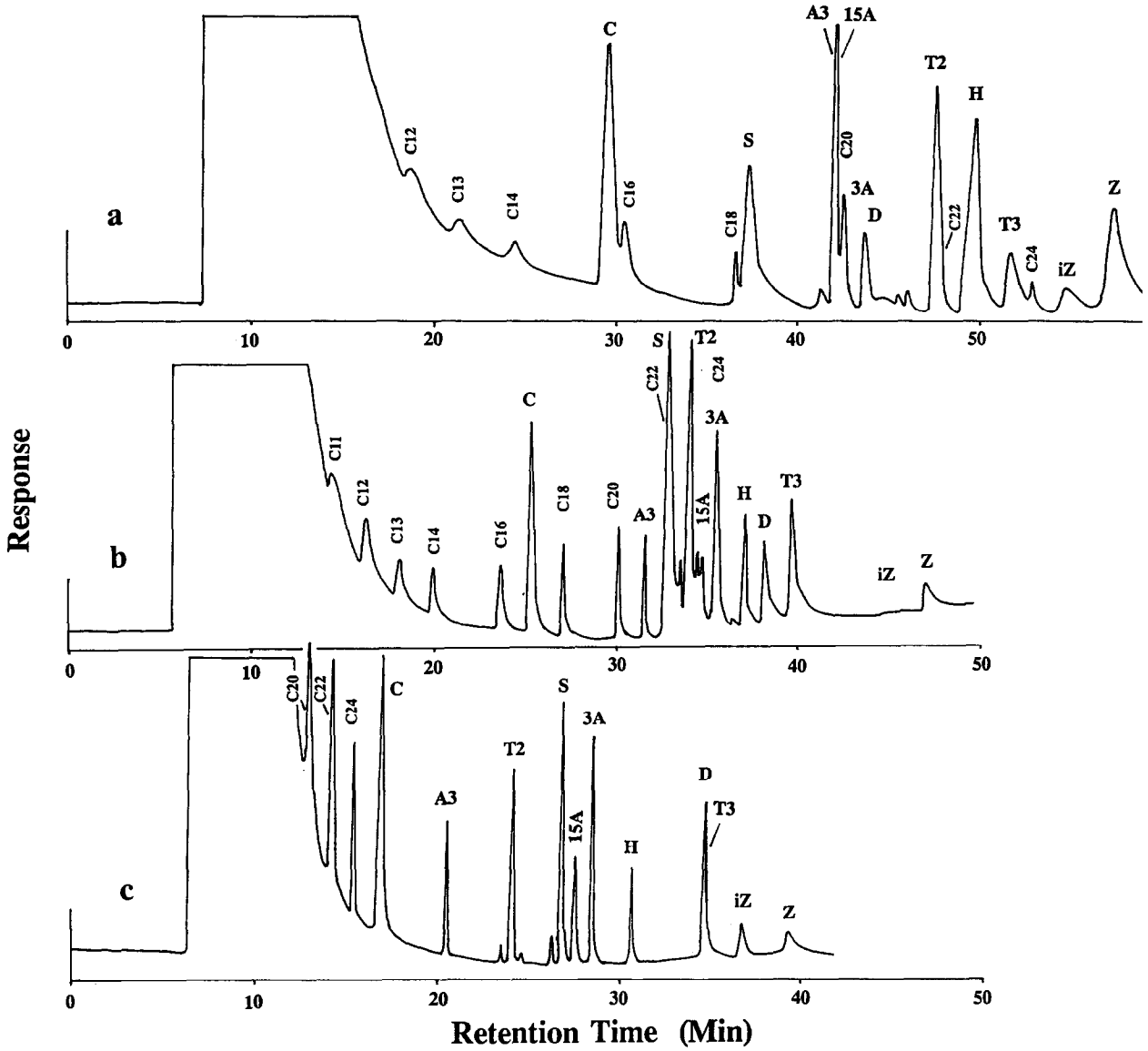


Fig. 3. Chromatograms of a mixture of *Fusarium* mycotoxins (A3) triacetyldeoxynivalenol, (3A) 3-acetyldeoxynivalenol, (15A) 15-acetyldeoxynivalenol, (C) culmorin, (D) deoxynivalenol, (H) HT-2 toxin, (S) sambucinol, (T2) T-2 toxin, (T3) T-2 triol, (Z) zearalenone and (iZ) zearalenone isomer separated by SFC with eluent supercritical CO_2 at 100°C on 10 m × 50 μm I.D. capillary columns with 0.25 μm film and detected by FID. (a) SB-Methyl-100, density programmed from 0.2 to 0.7 g/ml at 0.01 g/ml per min. (b) SB-Biphenyl-30, density programmed from 0.2 to 0.7 g/ml at 0.01 g/ml per min. (c) Carbowax, density programmed from 0.2 to 0.7 g/ml at 0.015 g/ml per min.

investigated by Kostiainen and Kuronen [16], or another HPLC column, such as polystyrene–divinylbenzene employed by Smith and Sanagi [17], will have to be used.

Capillary column separation of Fusarium mycotoxins

Separations of selected *Fusarium* mycotoxins on capillary columns under SFC conditions were investigated. The variables included eluent density and column polarity.

The mycotoxins investigated eluted faster at higher eluent density, due to the increased solvating power of the supercritical CO_2. Because of the substantial differences in compound polarities, density programming was employed to achieve elutions in the least possible times while retaining sufficient resolution. The retention times for capillary column SFC were much longer than those observed for packed-column SFC (see below).

There were no apparent trends upon going from low- (methyl) through mid- (biphenyl) to high-polarity (Carbowax) columns. The best chromatography (resolution and peak shape) was achieved on Carbowax (Fig. 3). Relative and absolute k' values were highly variable and column dependent (Fig. 4). The order of elution on all the columns investigated tended to follow increased solute polarity (*e.g.* triacetyldeoxynivalenol < 15-acetyldeoxynivalenol < 3-acetyldeoxynivalenol < deoxynivalenol and T-2 toxin < HT-2 toxin < T-2 triol) although a few compounds (*e.g.* butenolide and T-2 toxin) showed highly variable relative elution orders from column to column (Fig. 4).

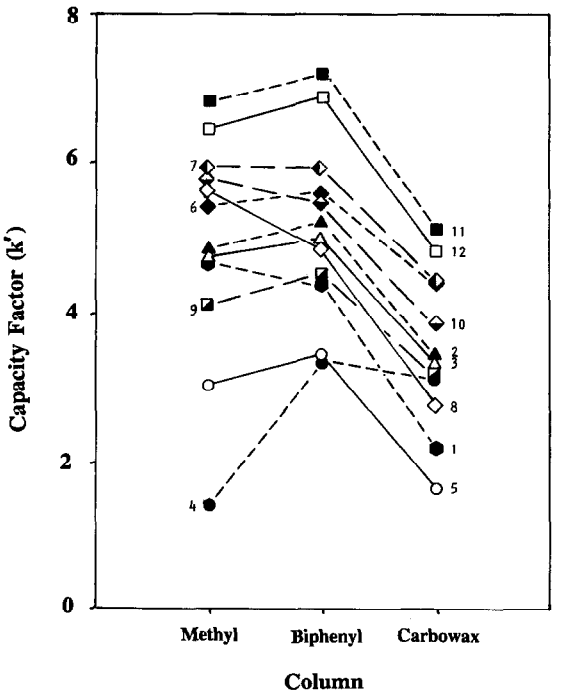


Fig. 4. Capacity factors for mixture of *Fusarium* mycotoxins (1) triacetyl-deoxynivalenol, (2) 3-acetyldeoxynivalenol, (3) 15-acetyldeoxynivalenol, (4) butenolide, (5) culmorin, (6) deoxynivalenol, (7) T-2 triol, (8) T-2 toxin, (9) sambucinol, (10) HT-2 toxin, (11) zearalenone and (12) zearalenone isomer separated by SFC with eluent supercritical CO_2 at 100°C on 10 m × 50 μm I.D. capillary columns with 0.25 μm film of SB-Methyl-100 and SB-Biphenyl-30 density programmed from 0.2 to 0.7 g/ml at 0.01 g/ml/min and Carbowax density programmed from 0.2 to 0.7 g/ml at 0.015 g/ml/min. Detection by flame ionization.

Packed-column separation of Fusarium mycotoxins

In addition to the conventional HPLC variables (adsorbent, eluent and temperature), packed-column SFC also has eluent density available. Each of these parameters was investigated for those UV absorbing mycotoxins that were available.

Differences in resolving power under identical operating conditions for the six packings are illustrated in Fig. 5. The phenyl, amino and diol columns, which gave higher k' values, showed the best resolution. The best chromatography was achieved on the amino column (Fig. 6), the only column to give baseline resolution of the zearalenone isomers, whereas silica appeared to be the least efficient. The diol and phenyl columns also gave relatively sharp peaks with only slight tailing. Under SFC conditions, all packings behave as normal phases and k' values increase with increased solute polarity. As noted for the capillary columns, butenolide showed variable relative elution order on the packed columns.

Capacity factors became smaller as pressure increased (Fig. 7) due to increased solvating power of supercritical CO_2. The rate of change of k' values for butenolide was different from the other compounds. Resolution, with respect to deoxynivalenol, decreased with increased pressure on the phenyl, cyano and ODS columns.

Addition of increasing amounts of methanol as a modifier to the CO_2 also resulted in reduced capacity factors (Fig. 8) due to increased solvating power.

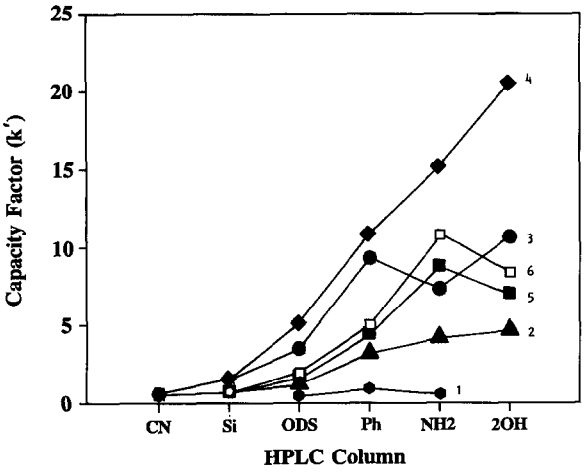


Fig. 5. Capacity factors for mixture of *Fusarium* mycotoxins (1) triacetyldeoxy-nivalenol, (2) 3-acetyldeoxynivalenol, (3) butenolide, (4) deoxynivalenol, (5) zearalenone and (6) zearalenone isomer separated by SFC with eluent supercritical CO_2 containing 5% methanol at 60°C and a pressure of 265 bar on 250 × 4.6 mm I.D. stainless steel columns containing (CN) Spherisorb Cyano 3 μm, (Si) Spherisorb Silica 5 μm, (ODS) Partisil 10 ODS3, (Ph) Apex Phenyl S5B, (NH_2) Spherisorb Amino 5 μm and (2OH) Apex II Diol. Detection by UV at 224 nm.

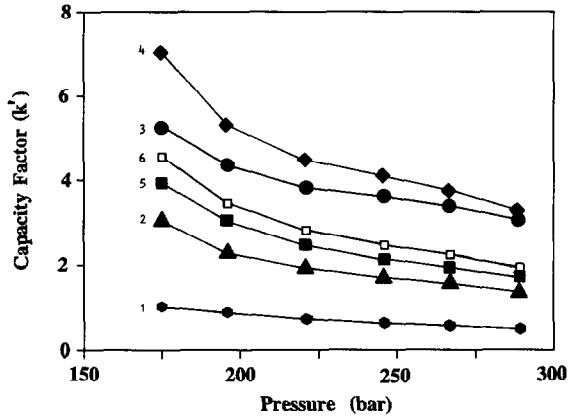


Fig. 7. Effect of pressure on capacity factors for mixture of *Fusarium* mycotoxins (1) triacetyldeoxynivalenol, (2) 3-acetyldeoxynivalenol, (3) butenolide, (4) deoxynivalenol, (5) zearalenone and (6) zearalenone isomer separated by SFC with eluent supercritical CO_2 containing 10% methanol and at a temperature of 60°C on 250 × 4.6 mm I.D. stainless-steel column of Apex Phenyl S5B. Detection by UV at 224 nm.

The influence on resolution appears to be solute dependent; higher concentrations of methanol resulted in reduced resolution between butenolide and deoxynivalenol but increased resolution between the zearalenone isomers.

The effect of temperature is opposite to that typically observed in conventional GC and HPLC; both k' and resolution increased with temperature (Fig. 9). The reason is that at constant pressure, as the temperature increases, the density of CO_2 de-

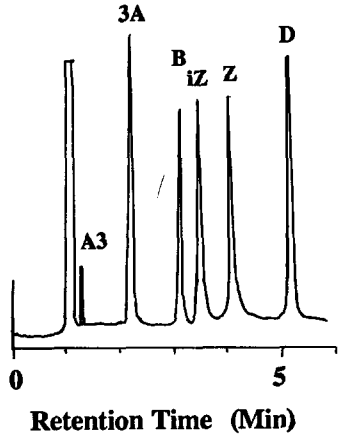


Fig. 6. Chromatogram of mixture of *Fusarium* mycotoxins (A3) triacetyldeoxynivalenol, (3A) 3-acetyldeoxynivalenol, (B) butenolide, (D) deoxynivalenol, (Z) zearalenone and (iZ) zearalenone isomer separated by SFC with eluent supercritical CO_2 containing 10% methanol at 70°C and a pressure of 293 bar on 250 × 4.6 mm I.D. stainless-steel column of Spherisorb Amino 5 μm. Detection by UV at 224 nm.

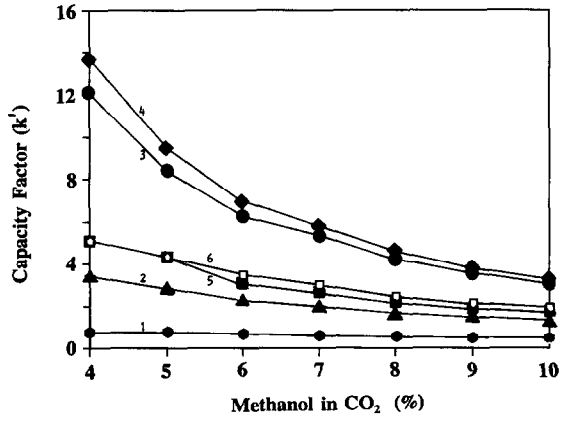


Fig. 8. Effect of modifier concentration on capacity factors for mixture of *Fusarium* mycotoxins (1) triacetyldeoxynivalenol, (2) 3-acetyldeoxynivalenol, (3) butenolide, (4) deoxynivalenol, (5) zearalenone and (6) zearalenone isomer separated by SFC with eluent supercritical CO_2 containing methanol at a temperature of 60°C and pressure of 289 bar on 250 × 4.6 mm I.D. stainless-steel column of Apex Phenyl S5B. Detection by UV at 224 nm.

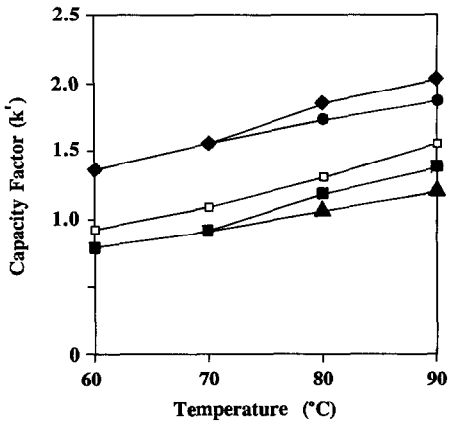


Fig. 9. Effect of temperature on capacity factors for mixture of *Fusarium* mycotoxins (▲) 3-acetyldeoxynivalenol, (●) butenolide, (◆) deoxynivalenol, (■) zearalenone and (□) zearalenone isomer separated by SFC with eluent supercritical CO_2 containing 3% methanol and a pressure of 276 bar on 250 × 4.6 mm I.D. stainless-steel column of Spherisorb Cyano 3 μm. Detection by UV at 224 nm.

creases with a concomittent loss of solvation power.

Compared with conventional HPLC, packed-column SFC results in an order of magnitude greater resolution per unit time (*i.e.* shorter retention times) and narrower peaks (only several seconds as compared with 0.5 to 1 min reported by Kostiainen *et al.* [22]).

CONCLUSIONS

A wide variety of *Fusarium* mycotoxin structure types can be readily separated by SFC on both capillary and packed columns. The best resolution and sharpest peaks were obtained on the Carbowax capillary and Spherisorb Amino packed columns. Resolution was influenced by column temperature, pressure/density of the liquid carbon dioxide eluent and by concentration of modifier (in this case, methanol) added to the eluent; the specific effect was dependent upon the solute and column packing material.

SFC retention times on packed columns are up to 8 times shorter than those on capillary columns. Compared with conventional HPLC, packed-column SFC offers faster analysis times and gives narrow peak widths usually associated with capillary column GC.

Homologous *n*-alkylphenones could be used to generate retention index values on two of the capillary columns investigated. However they were not sufficiently retained on any of the packed columns. A more polar homologous series is required for SFC packed column indices.

ACKNOWLEDGEMENTS

J.C.Y. wishes to thank D.E.G. for being able to spend a year on sabbatical leave in the latter's laboratory.

This paper is PRC Contribution No. 1426.

REFERENCES

1 M. E. Savard, R. Greenhalgh and J. W. ApSimon, in Atta-ur-Rahman (Editor), *Studies in Natural Products Chemistry*, Vol. 6, Part D, Elsevier, Amsterdam, New York, 1990, p. 213.
2 J. F. Grove, *Nat. Prod. Rep.*, 5, (1988) 187.
3 R. J. Cole (Editor), *Modern Methods in the Analysis and Structural Elucidation of Mycotoxins*, Academic Press, Orlando, FL, 1986.
4 E. Kováts, *Helv. Chim. Acta*, 41 (1958) 1915.
5 L. S. Ettre, *Anal. Chem.*, 36 (1964) 31A.
6 R. M. Smith, *Adv. Chromatogr.*, 26 (1987) 277.
7 J. K. Baker and C.-Y. Ma, *J. Chromatogr.*, 169 (1979) 107.
8 R. M. Smith and N. Finn, *J. Chromatogr.*, 537 (1991) 51.
9 C. F. Culberson, W. L. Culberson and A. Johnson, *Mycologia*, 78 (1986) 888.
10 M. Bogusz and R. Anderjan, *J. Chromatogr.*, 435 (1988) 43.
11 M. Bogusz, *LC · GC*, 9 (1991) 290.
12 R. M. Smith, *J. Chromatogr.*, 236 (1982) 313.
13 D. W. Hill, T. R. Kelley, K. J. Langner and K. W. Miller, *Anal. Chem.*, 56 (1984) 2576.
14 J. C. Frisvad and U. Thrane, *J. Chromatogr.*, 404 (1987) 195.
15 C. R. Yonker and R. D. Smith, *Anal. Chem.*, 59 (1987) 727.
16 R. Kostiainen and P. Kuronen, *J. Chromatogr.*, 543 (1991) 39.
17 R. M. Smith and M. M. Sanagi, *Chromatographia*, 26 (1988) 77.
18 R. D. Smith, H. R. Udseth and B. W. Wright, *J. Chromatogr. Sci.*, 23 (1985) 192.
19 H. T. Kalinowski, H. R. Udseth, B. W. Wright and R. D. Smith, *Anal. Chem.*, 58 (1986) 2421.
20 J. A. Roach, J. A. Sphon, J. A. Easterling and E. M. Calvey, *Biomed. Environ. Mass Spectrom.*, 18 (1989) 64.
21 D. R. Gere, R. D. Board and D. McManigill, *Anal. Chem.*, 54 (1982) 736.
22 R. Kostiainen, K. Matsuura and K. Nojima, *J. Chromatogr.*, 538 (1991) 323.