Gradient Thin-Layer Chromatography and Densitometry Determination of Alternaria Mycotoxins

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Key Words

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Summary

Mycotoxins produced by the genus *Alternaria* in cereals, tomatoes and raspberries were analysed by stepwise gradient thin-layer chromatography (HPTLC plates). The chromatograms were suitable for direct quantitative densitometric determination of alternariotoxins. Extraction of samples was with ethyl acetate. Extracts of were purified on glass columns of silica gel Si 60 for column chromatography.

Introduction

Mycotoxins such as alternariol (AOH), alternariol methyl ether (AME), altenuen (ALT), altertoxin I, II, III (ATX-I, ATX-II, ATX-III) and tenuazonic acid (TA) which can occur in cereals, vegetables and fruits are produced by fungi of the genus *Alternaria* which are plant pathogens or saprophites [1]. Alternariol, alternariol methyl ether (Figure 1) and altenuen are dibenzopyrone derivatives, altertoxins I, II, III are perylene derivatives and tenuazonic acid is a tetraminic acid derivative.

These compounds, produced by some species of Alternaria in large amounts may have synergistic toxic activity. Recent investigations demonstrated toxicity of crude extracts or cultures of *Alternaria* spp. and toxicity of individual metabolites relative to mammals, cancerogenic and mutagenic activity and cytotoxicity. Therefore, interest in the fungi of the genus *Alternaria* and their metabolites is increasing. The level of mycotoxins occurring in some plant materials is high [2–4].

The most important properties of *Alternaria* mycotoxins, their natural occurrence and the analytical methods were reviewed by King, Shade [5, 6] and Palmisano, Visconti [7]. Recently investigations were carried out on the application and improvement of various analytical techniques for determination of mycotoxins in food products and on estimation of the contamination level of food by these toxins [8–12]. The most frequently applied techniques are thin-layer chromatography (TLC) [13–17], high-performance liquid chromatography (HPLC) and gas chromatography (GC).

HPLC and GC are expensive methods; therefore, in the present study an attempt to apply densitometry in combination with stepwise gradient TLC for the control of alternariotoxins in food products was made. Earlier investigations on the use of gradient TLC in the analysis of plant extracts [18–25] demonstrated that this technique gave good results in the separation of complex mixtures of natural origin (frequent double increase of the number of separated spots in comparison to isocratic elution, sharp zones) so that the chromatograms were well suited for quantitative densitometric determination.

Experimental

20 g sample was shaken with 90 mL ethyl acetate for 30 min, in a mechanical shaker. Mixtures were transferred to a separating funnel and the aqueous layer discarded. The organic phase extract was purified by shaking with an equal volume of aqueous 5 % $NaHCO_3$. The organic layer was filtered through a cellulose filter with a layer of anhydrous Na_2SO_4 to a spherical flask and evaporated under vacuum at 30 °C. The dry residue was dissolved in 5 mL chloroform and introduced into a 10 × 300 mm glass column containing 1 g Na_2SO_4, 2.5 g silica (Kiesegel 60, 0.063–0.20 mm for column chromatography, Merck, Darmstadt, Germany) and 1 g Na_2SO_4. To elute toxins from the column, 25 mL of a mixture of chloroform and methanol (9:1) was used. The first 5 mL of eluate was discarded and the next 15 mL.was collected in a spherical flask and evaporated under vacuum at 30 °C and the residue dissolved in methylene chloride. The sample solution in CH_2Cl_2 was transferred to a glass vessel and evaporated in a stream of nitrogen; the dry residue was dissolved in 200–400 µL methanol.

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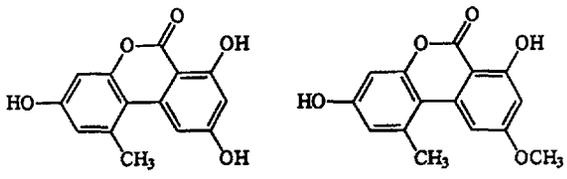
Glass, precoated HPTLC plates, 10 × 10 cm silica gel Si 60, 0.2 mm (E. Merck) were spotted with 5–40 μL samples of the purified extracts using a Desaga AS 30 automatic applicator under nitrogen at 2.5 atm for 5 s, as 5 mm streaks.

Chromatograms were developed with mixtures of chloroform and ethyl acetate, according to programs given in Table I, in horizontal Teflon DS chambers (Chromdes, Lublin, Poland) described in earlier publications [26–29]. The time of development was about 30 min. The spots were visualized under 243 nm UV.

The densitograms were obtained using a Desaga CD 60 densitometer (Desaga, Heidelberg, Germany) controlled with an Optimus PC 486 DX II 50 computer. Scans were obtained at 243 nm chosen from spectra in the 200–700 nm range determined with the same densitometer. For linear scanning the slit dimensions were 0.1 × 4 mm.

The standard solutions of AOH and AME (Alltech Unterhaching, Germany) contained 6 ng μL^{-1} of the solutes. 3–10 μL AOH and 10–40 μL AME solution were spotted (30–240 ng), whereas 30 ng was the smallest amount detectable. The detection limit in the samples was about 60 μg kg^{-1}.

The standard solution of AOH and AME in amounts given in Figures3, 4 and 5 were added to the samples of extracts from healthy tomatoes, wheat and oat before the spotting.



ALTERNARIOL ALTERNARIOL METHYL ETHER

Figure 1
Structures of two important toxins of *Alternaria.*

Results and Discussion

In reference [8] the authors separated alternariotoxins by two-dimensional TLC: the spots of mycotoxins as well as other components present in the extracts were rather diffuse, their identification was difficult and the chromatograms were not suitable for densitometric measurements. Better chromatograms with well defined zones can be obtained with stepwise gradient TLC which was applied in the present work.

The presence of mycotoxins was controlled in the weathered grains and in healthy grains from the 1993 crop from the northern part of Poland (Koszalin Voievodship) and in tomatoes and raspberries attacked by fungi and in healthy fruits; in the last case the samples were purchased in Warsaw market in 1994. For each sample type (grains, tomatoes, raspberries) the gradient programmes were chosen, taking into account R_F values of AOH and AME standards for isocratic elution. Thus, for the extract from naturally contaminated raspberries the gradient given in Table I was applied. The extract was separated into a large number of spots (15) whose characteristics are given in Table II. The densitogram, 'a', in Figure 2 shows the peak of alternariol, $hR_F = 12$. Its height is 219 relative absorbance units and its area is 48.4 mm (AU * mm) (for n = 7). Alternariol and alternariol methyl ether were identified against the standards for all extracts from the grains, fruits and vegetables on each chromatogram as shown in Figure 2 - the densitogram, 'b' corresponds to AOH (standard solution, 10 μL) and, 'c'. to AME (standard solution, 20 μL).

Figure 3 shows three densitograms from extracts of tomatoes purified on silica gel column [12]. Densitogram, 'a' corresponds to samples naturally contaminated by mycotoxins, densitogram, 'b' to a sample of helthy tomatoes spiked with AME (standard solution 10 μL), and, 'c' spiked with both standards; AOH (10 μL) and AME (10 μL). The gradient program is given in Table I was applied. The hR_F of AOH = 23 and AME = 58.

For samples of wheat spiked with mycotoxins AOH (3 μL) and AME(10 μL) (Figure 4), the gradient pro-

Table I. Four- and five-step gradient programs for analysis of extracts from raspberries, tomatoes, wheat and oats

Eluent	Extract from raspberries			Extract from tomatoes			Extract from wheat			Extract from oat		
No	Chlf % v/v	EtOAc %v/v	distance of development, cm	Chlf % v/v	EtOAc %v/v	distance of development, cm	Chlf % v/v	EtOAc %v/v	distance of development, cm	Chlf % v/v	EtOAc %v/v	distance of development, cm
1	100	–	4.5	100	–	4.5	100	–	4.5	100	–	4.5
2	100	–	9	100	–	9	100	–	9	100	–	9
3	95	5	9	95	5	9	95	5	9	95	5	9
4	95	5	9	90	10	9	95	5	9	90	10	9
5	–	–	–	–	–	–	90	10	9	85	15	9

Plates dried after each development for 15 min for every solvent mixture (Nos 1 to 5).

Table II. Displaying peaklist (S)

PeakNo.	hR_F	Y-Pos. [mm]	Area [AU*mm]	Area [%]	Height [AU]
1	69	33.2	129.8	1.5	114.84
2	63	37.4	3015.0	33.8	995.60
3	57	42.3	97.8	1.1	63.89
4	54	45.4	160.6	1.8	101.73
5	51	48.2	702.2	7.9	328.19
6	46	51.5	508.4	5.7	254.59
7	36	59.6	50.1	0.6	46.39
8	33	62.9	258.5	2.9	247.05
9	31	64.0	169.1	1.9	169.00
10	29	65.8	305.4	3.4	300.17
11	28	66.7	186.8	2.1	225.16
12	24	70.0	1488.7	16.7	897.00
13	19	73.5	1356.5	15.2	656.06
14	15	77.2	104.4	1.2	72.27
15	12	80.0	384.2	4.3	219.23

Area [%] – % content of component in whole sample.

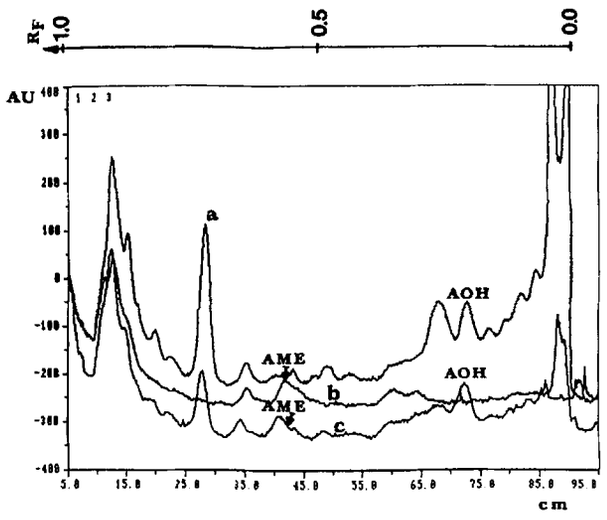


Figure 3
Densitograms: 'a' tomatoes naturally contaminated by AOH; 'b' healthy tomatoes which 10 µL AME added; 'c' healthy tomatoes to which 10 µL AME and 10 µL AOH added.

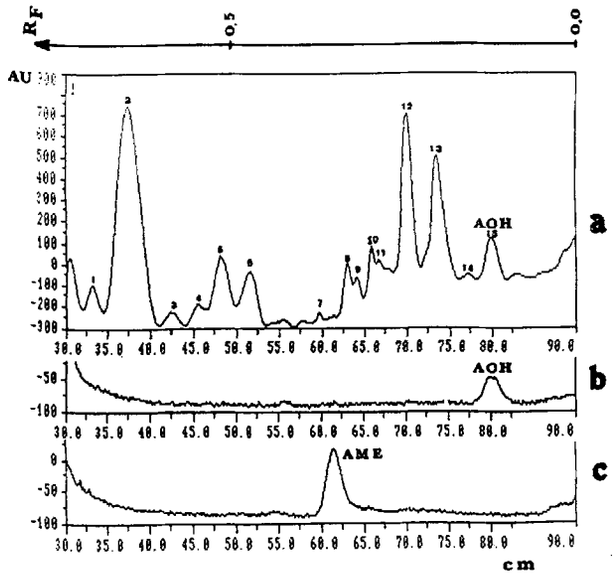


Figure 2
Densitogram, 'a' of raspberries-sample naturally contaminated by AOH.Densitogram, 'b' 10 µl standard solution AOH. Densitogram, 'c' 20 µL standard solution AME

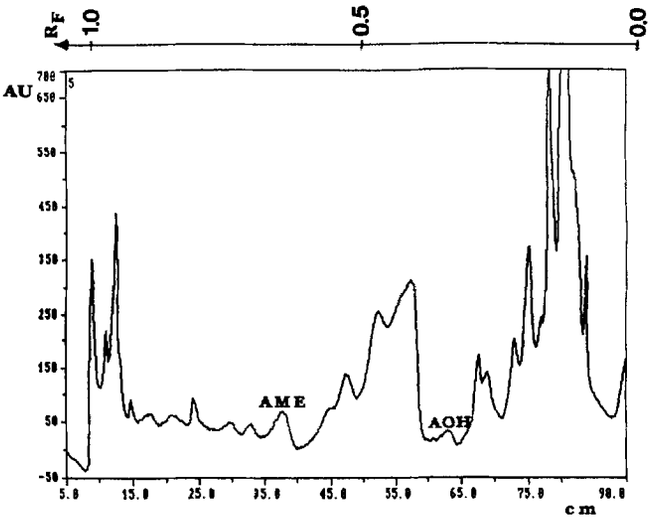


Figure 4
Densitogram of healthy wheat sample, to which 3 µL AOH and 10 µL AME added.

gram in Table I was applied. The spots of alternariotoxins were well separated from other components: hR_F AOH = 34 and hR_F AME = 64.

The samples of oat extracts could not be analysed with the preceding program because a component with an R_F close to that of AOH occurred in large quantities and its spot masked the narrow spot of AOH. However, the use of a gradient program of a higher elution strength (Table I) separated AOH and AME as separate peaks (Figure 5). To healthy samples of oat was added 10 µL standard AOH and 40 µL standard AME. The hR_F AOH = 43 and AME = 72.

The areas of peaks of AOH and AME determined from samples of raspberries, tomatoes, wheat and oats as well as the standard deviation for n = 7 are in Table III.

Conclusions

A direct method of determining alternariotoxins in food samples has been developed, using stepwise gradient elution in horizontal DS chambers combined with densitometry.

Table III. Areas of peaks AOH and AME in samples of raspberries, tomatoes, wheat and oats (7 measurements).

Compounds	Raspberries			Tomatoes			Wheat			Oats		
	Fig. No	Area [AU*mm]	Standard deviation	Fig. No	Area [AU*mm]	Standard deviation	Fig. No	Area [AU*mm]	Standard deviation	Fig. No	Area [AU*mm]	Standard deviation
AOH	2a	384.2	+/- 11.2	3a	168.0	+/- 12.3	4	56.2	+/- 14.2	5	150.0	+/- 16.2
	2b	160.4	+/- 9.8	3c	141.0	+/- 12.0						
AME	2c	318.0	+/- 6.5	3b	159.8	+/- 10.9	4	150.0	+/- 15.3	5	592.3	+/- 6.2
				3c	156.2	+/- 11.3						

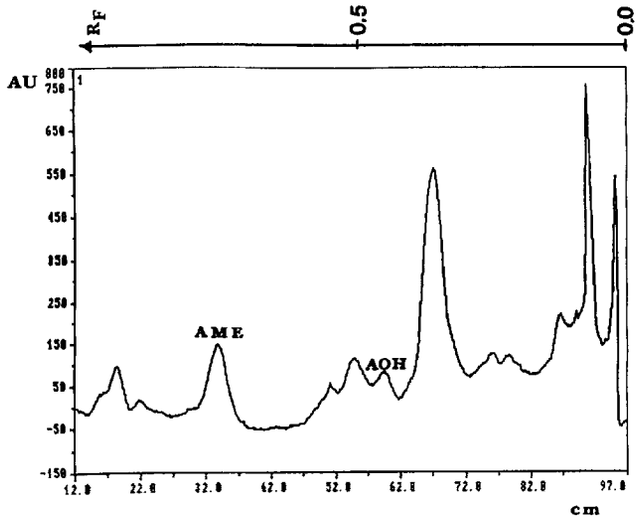


Figure 5
Densitogram of healthy oat sample, to which 10 µL AOH and 40 µL AME added.

The advantages of the method are: short development time (30 min) and limited consumption of solvents (ca. 3 mL for 10 × 10 cm plates).

The simplicity of the method enables its application in any laboratory concerned with detection of toxins in food products. The additional quantitative densitometric determinations can be made quite rapidly,directly from the chromatographic plate.

After development, narrow zones of the mycotoxins investigated as well as the accompanying compounds are obtained which is also seen from the densitograms in Figure 2; peaks are symmetrical and well-shaped.

References

[1] *M. E. Rhodes*, Food mycology, G. K. Hall and Co., Boston, 1979, 29.
[2] *J. Harwig, P. M. Scott, D. R. Stoltz, B. J. Blanchfield*, Appl. Environ. Microbiol. **38**, 267, (1979).
[3] *P. C. Mislivec, V. R. Bruce, M. E. Stack, R. Bandler*, J. Food Protection **50**, 38, (1987).
[4] *E. E. Stinson, S. F. Osman, E. G. Heisler, J. Siciliano, D. D. Bills*, J. Agric. Food. Chem. **29**, 790, (1981).
[5] *D. I. King, J. Schade*, J. Food Protection **47**, 886 (1984).
[6] *J. Schade, D.I. King*, J. Food Protection **47**, 978 (1984).
[7] *F. Palmisano, A. Visconti*, II Prodotto Chimico **3**, 29 (1998).
[8] *A. Visconti, A. Logrieco, A. Bottalico*, Food Additives Contam. **3**, 323 (1986).
[9] *M. Witkowski, W. Baltes, W. Kronert, R. Weber*, Z. Lebensm. Unters. Forsch. **177**, 447 (1983).
[10] *A. Torres, S. Chulze, E. Varsavasky, M. Rodriguez*, Mycopathologia **121**, 17 (1993).
[11] *H. Giryn, B. Szteke*, Annals of State Institute of Hygiene (Roczniki PZH), Warsaw, 46, 129 (1995).
[12] *H. Giryn*, Annals of State Institute of Hygiene (Roczniki PZH), Warsaw, in press.
[13] *M. E. Stuck*, Toxins, Ch. 29 in "Handbook of Thin-Layer Chromatography", J. Sherma, B. Fried, Eds., Marcel Dekker, New York 1991, p. 971.
[14] *R. W. Pero, D. Harvan, M. C. Blois*, Tetrahedron Lett. **12**, 945 (1973)
[15] *A. Sroessl*, Can. J. Chem. **47**, 767 (1969)
[16] *D. Robeson, G. Strobel, G. K. Matsumoto, E. L. Fisher, M.H. Chen, J. Clardy*, Experientia **40**, 1248 (1984).
[17] *M. E. Stack, M. J. Prival*, Appl. Environ. Microbiol. **52**, 718, (1986).
[18] *W. Golkiewicz*, in "Handbook of Thin-Layer Chromatography", J. Sherma, B. Fried, Eds., Marcel Dekker, New York 1991, p. 135.
[19] *G. Matysik, L. Jusiak*, J. Chromatogr. **518**, 273 (1990).
[20] *G. Matysik, M. Benesz*, Chromatographia **32**,19 (1991).
[21] *G. Matysik, W. Markowski, E. Soczewinski, B. Polak*, Chromatographia **34**, 303 (1992).
[22] *E. Soczewinski, G. Matysik*, J. Planar Chromatogr. **5**, 388 (1992).
[23] *G. Matysik, E. Wojtasik*, J. Planar Chromatogr. **7**, 34 (1994).
[24] *G. Matysik*, Chromatographia **38**, 109 (1994).
[25] *G. Matysik*, J. Planar Chromatogr. **5**, 146 (1992).
[26] *E. Soczewinski* Anal. Chem. **41**, 179 (1989).
[27] *E. Soczewinski*, J. Chromatogr. **388**, 91 (1987).
[28] *T. H. Dzido*, J. Planar Chromatogr. **3**,199 (1990)
[29] *T. H. Dzido, E. Soczewinski*, J. Chromatogr. **516**, 461 (1990).

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