Journal of Chromatography, 268 (1983) 461–468
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,042

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF XANTHOMEGNIN AND VIOMELLEIN

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(Received June 1st, 1983)

SUMMARY

Preliminary separations of xanthomegnin and viomellein demonstrated some irreversible binding to silica. However, reversed-phase high-performance liquid chromatographic procedures on C_{18} columns showed that binding could be minimized by the inclusion of sodium dodecyl sulfate in the mobile phase. Capacity factors for xanthomegnin and viomellein in several solvent systems are reported. Baseline resolution of the two mycotoxins was achieved with water–acetonitrile–sodium dodecyl sulfate–sulfuric acid (55:45:0.02:0.04, v/v/w/w). Utilization of radial compression technology provided for separation times of 14–16 min. Quantitative techniques were established for the substances based on solvent extraction from the natural substrate followed by reversed-phase high-performance liquid chromatographic separation. In addition to effective separation, the procedure appeared to partially resolve two isomeric forms of viomellein. The toxin occurred in a single form in the initial, crude fungal extract but apparently the compound was racemized during exposure to silica.

INTRODUCTION

Xanthomegnin and viomellein are toxic metabolites of a number of fungi that include *Aspergillus* and *Penicillium* species; these microbes are of particular interest because they are routinely implicated in toxin contamination of foods and feeds[1–3]. Extensive investigations by scientists at Purdue University have identified xanthomegnin and viomellein as hepatic and renal toxins in animals[3–5]. Renal toxicity of the two compounds has attracted considerable research interest since a nephropathy of swine in Scandinavia has been linked to ingestion of feeds containing species that produce the toxins, *Penicillium viridicatum* Westling and *Aspergillus ochraceus* Wilhelm. Krogh and Hald[6] have recently documented the natural occurrence of viomellein in a feed associated with the kidney disorder.

In addition to nephropathy in domestic and laboratory animals, mycotoxins have also been associated with a fatal kidney disease of humans (Balkan endemic nephropathy) which occurs in certain areas of Yugoslavia, Bulgaria and Rumania[1,7]. A similarity between the kidney disease observed in swine and humans has been

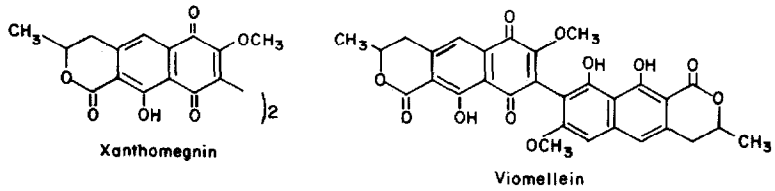

Fig. 1. Structures of xanthomegnin and viomellein.

observed. Since presence of xanthomegnin- and viomellein-producing species of *Penicillium* in commodities has been associated with high levels of the kidney disease, the toxic metabolites have been implicated in the disorder[7].

Broad screening for xanthomegnin and viomellein in foods and feeds has been restricted by limited quantitative techniques. Prior investigations utilized thin-layer chromatographic (TLC) methods for separation along with treatment of developed plates in ammonia vapors to enhance the color identification of the two toxins[8]. Preliminary studies of high-performance liquid chromatographic (HPLC) methods were characterized by normal-phase separation techniques using silica gel columns and UV detection at 405 nm[8,9]. However, little has been done to separate completely all the fungal compounds in crude extracts.

Structural similarity of xanthomegnin and viomellein (Fig. 1)[10] has restricted development of systems to determine simultaneously both substances. Ciegler *et al.*[9] wrote the only previous report of quantitative values for both compounds using HPLC methods. The objectives of the current study were to compare the efficiency of normal- *vs.* reversed-phase techniques and to develop additional HPLC methods for simultaneous quantitation of xanthomegnin and viomellein since they represent potential cocontaminants in foods and feeds.

EXPERIMENTAL*

Production of fungal metabolites

A. ochraceus (M-298; from J. Tuite, Purdue University) was utilized for toxin production because the microbe synthesizes large quantities of xanthomegnin and viomellein. A spore inoculum was prepared on potato dextrose agar. Spores were removed from the agar surface in sterile water (0.01% Triton X), filtered through sterile glass wool, collected and adjusted to $1.0 \cdot 10^8$/ml. Autoclaved, converted rice (40% moisture) was inoculated with 1.0 ml of spores per 100 g of substrate and incubated statically for 10 days at 28°C. The moldy rice was extracted by suspension in methylene chloride and grinding in a Waring Blendor. The extract was filtered and the solvent removed by vacuum evaporation. These crude oils were sequentially extracted three times with acetonitrile using a 1:5 oil–solvent ratio. Acetonitrile solutions were used for chromatographic determinations.

TLC

For quantitative TLC separations, samples were spotted on activated TLC

* Mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

plates coated with 0.5 mm of Absorbosil-1. Plates were developed with benzene–methanol–acetic acid (18:1:1, v/v/v) and dried plates were exposed to ammonia vapors. Quantities of xanthomegnin and viomellein were determined by visual comparison with reference standards (M. E. Stack, Food and Drugs Administration, Washington, DC, U.S.A.; L. S. Lee, U.S. Department of Agriculture, New Orleans, LA, U.S.A.). Standards of the two metabolites were obtained from preparative plates (Brinkmann G-200, 2 mm silica gel) that were developed in the B–M–A solvent system. Appropriate bands were scraped from the plates and compounds were eluted with methylene chloride. The solvent was removed under a stream of nitrogen and standards were stored as dry films in a freezer. Purity of standards was determined by TLC and HPLC comparisons with reference compounds.

HPLC

A Waters Assoc. 6000A solvent delivery system was used with a WISP 710 auto injector and a Waters RCM 100 with a C_{18} (10-μm silica) Radial Pak column. Detection was achieved with a Waters Model 440 UV detector. Samples were monitored at 405 nm with an amplitude of 0.2 a.u.f.s. Peak height and area determinations were made with a Hewlett-Packard LAS 3356.

The column was equilibrated for approximately 1 h with a mobile phase of water–acetronitrile–sodium dodecyl sulfate (SDS)–sulfuric acid (55:45:0.02:0.04, v/v/w/w) at a flow-rate of 3 ml/min. All separations were done isocratically.

RESULTS AND DISCUSSION

Substrates for separation

The use of silica for open-bed columns and TLC for the preliminary clean-up of fungal metabolites has been employed[5,9]. Xanthomegnin and viomellein are quinone color indicators that cannot be completely eluted from open-bed columns. Irreversible binding of these pigments to silica columns prompted concern for similar binding in HPLC separations which might compromise quantitative measurements. Visual examination of silica HPLC columns with semitransparent PTFE walls demonstrated a gradual color build-up.

The limitations of silica suggested that C_{18}, or some other bonded phase, might promote better elution and reproducible separations. Initial tests demonstrated a general color build-up on a C_{18} column but was less than that observed on unbonded silica. It appeared that residual silanols on C_{18} columns were affecting the chromatographic separation of the fungal extracts. C_{18} silica used in Radial-Pak columns has undergone no endcapping reaction and might display greater residual silanol interactions than that used in steel columns. However, a steel column was dismantled after several fungal extract separations and the C_{18} silica, endcapped with trimethylsilane, also had a significant color build-up. The inclusion of a small amount of SDS minimized the binding of pigments to both types of C_{18}-bonded silica. Subsequently, complete recoveries of xanthomegnin and viomellein standards were achieved on HPLC C_{18} columns; recoveries of the compounds were monitored by TLC determinations.

HPLC

The isocratic mobile phase used to separate *A. ochraceus* metabolites on C_{18}

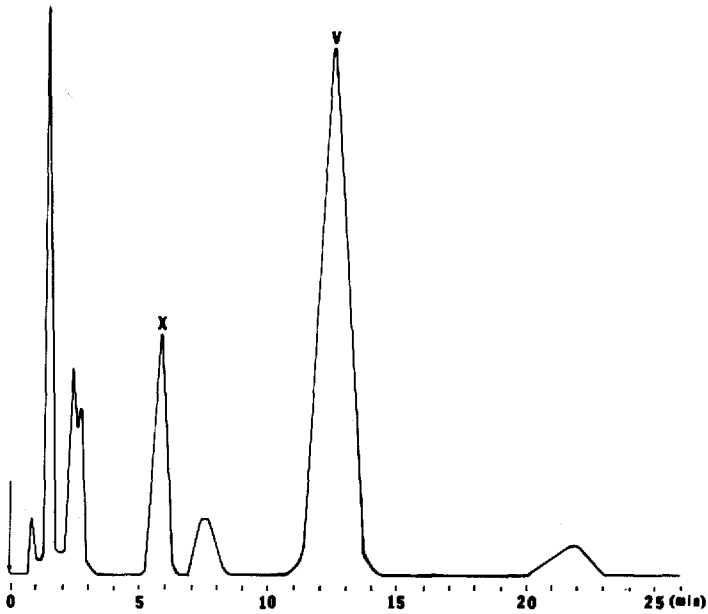

Fig. 2. Separation of an *A. ochraceus* extract on a reversed-phase C_{18} column with a mobile phase of water–acetonitrile–SDS–sulphuric acid (55:45:0.02:0.04, v/v/w/w) and a flow-rate of 3 ml/min.

silica included 0.04% sulfuric acid to maintain the dihydroxy form of both xanthomegnin and viomellein. The increase in acid strength in the mobile phase had no effect on the separation. Apparently, SDS eliminated residual silanol interactions and concentrations greater than 0.02% (w/w) had no effect on the separation. Acetonitrile was selected as the organic modifier and samples were introduced into the system

TABLE I

k' VALUES OF XANTHOMEGNIN AND VIOMELLEIN

C_{18} Radial Pak column with a mobile phase flow-rate of 3 ml/min. THF = tetrahydrofuran; SDS = sodium dodecyl sulfate.

Mobile phase		*k'*	
Components	*Composition (v/v/w/w)*	*Xanthomegnin*	*Viomellein*
Water–THF–SDS–sulphuric acid	(50:50:0.02:0.04)	1.03	1.03
Water–acetone–SDS–sulphuric acid	(50:50:0.02:0.04)	3.40	8.00
Water–acetonitrile–SDS–sulphuric acid	(50:50:0.02:0.04)	5.10	11.20
Water–acetonitrile–SDS–sulphuric acid	(65:35:0.02:0.04)	21.05	–
Water–acetonitrile–SDS–sulphuric acid	(60:40:0.02:0.04)	10.40	28.90
Water–acetonitrile–SDS–sulphuric acid	(55:45:0.02:0.04)	5.35	12.78
Water–acetonitrile–SDS–sulphuric acid	(45:55:0.02:0.04)	1.55	3.34
Water–acetonitrile–SDS–sulphuric acid	(40:60:0.02:0.04)	1.33	2.65
Water–acetonitrile–SDS–sulphuric acid	(34:65:0.02:0.04)	0.95	1.78

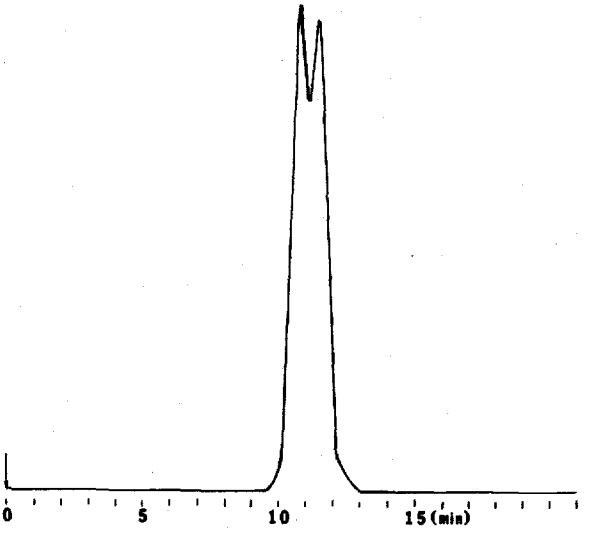

Fig. 3. Separation of a viomellein standard on a reversed-phase C_{18} column with a mobile phase of water–acetonitrile–SDS–sulphuric acid (55:45:0.02:0.04, v/v/w/w) and a flow-rate of 3 ml/min.

dissolved in acetonitrile. Fig. 2 is a typical chromatogram of a crude fungal extract. The procedure effected separation of the fungal metabolites in 14–16 min.

The efficiency of other solvent systems was also examined. The capacity factors (k') for xanthomegnin and viomellein varied in a typical reversed-phase manner, depending on the concentration of the organic phase (Table I). Although a water–acetone mobile phase provided for effective separation of the two compounds, use of water and acetonitrile provided the best resolution.

A series of standards of xanthomegnin and viomellein were chromatographed to establish a basis for quantitation. Standards were prepared by TLC methods and the purity was verified by TLC and HPLC comparisons with reference compounds (M. E. Stack; L. S. Lee). Viomellein in all three standards yielded a double peak in the SDS–reversed-phase HPLC system (Fig. 3). An earlier nuclear magnetic resonance investigation characterized the existence of diastereoisomers of viomellein due to restricted rotation about the dimeric linkage[12]. Separation of these isomers could account for the two peaks in the chromatograms of the viomellein standards (Fig. 3). The basic difference between the crude extract (Fig. 2) and the standard solution (Fig. 3) was the absence of exposure of the extract to silica whereas reference compounds were prepared on TLC plates coated with silica. Preliminary clean-up of the crude fungal extract on open-bed silica columns also yielded viomellein that exhibited a double peak in subsequent HPLC determinations. Although viomellein exposure to silica yielded a double peak in the HPLC separation, identical samples provided a single spot in TLC determinations and only one peak in normal-phase HPLC separations[11].

Response curves for varied quantities of xanthomegnin and viomellein were prepared using peak area as the dependent variable (Figs. 4 and 5). The correlation coefficients for area *vs.* quantity of toxin exceeded 0.99 for both metabolites. Cursory

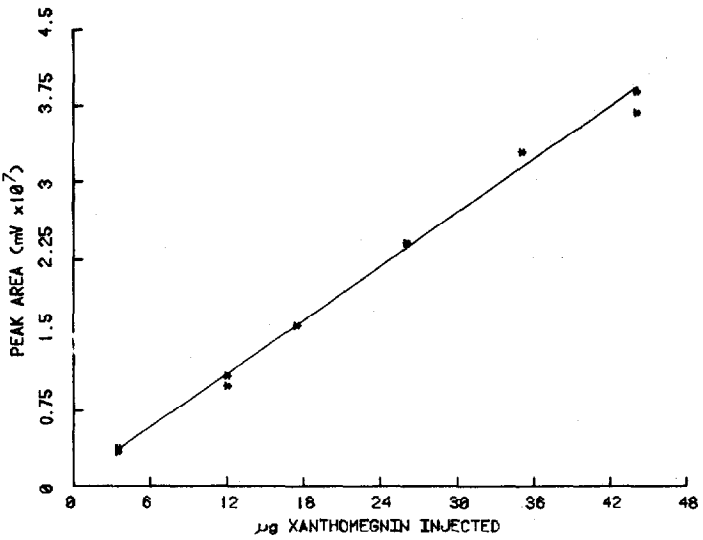

Fig. 4. Reference plot of xanthomegnin *vs.* area (mV) under the peak at 405 nm from known amounts of the compound.

examination showed that peak height could be effectively substituted for areas. The total peak area for the double peak representing standard viomellein was used with little loss of accuracy since both isomers probably have the same extinction coefficient. The lower limit of detection for both xanthomegnin and viomellein was approximately 0.3 µg with 405 nm UV detection and maximum detector amplitude.

Based on quantitative estimation of xanthomegnin and viomellein (Figs. 3 and

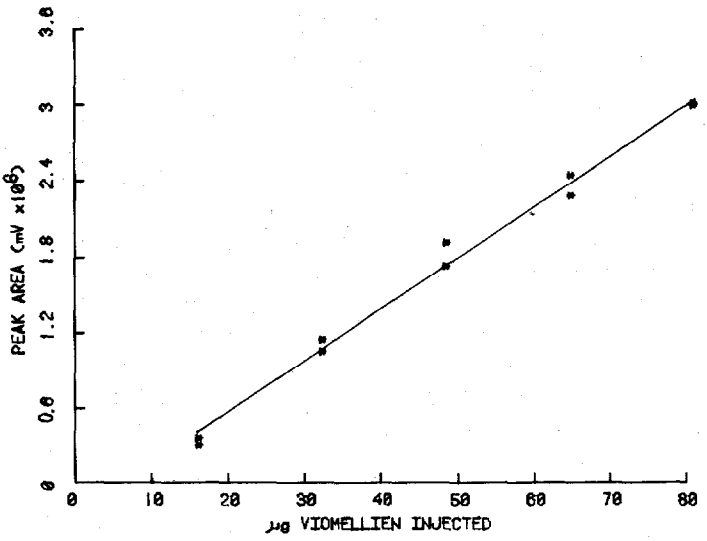

Fig. 5. Reference plot of viomellein *vs.* area (mV) under the peak at 405 nm from known amounts of the compound.

4) the quantity of toxin in a contaminated material can be calculated from the response figures with the equation: μg toxin per g sample = $A/B \times C/D$ where A = toxin level (μg) from response plot, B = volume (ml) of sample injected into HPLC system, C = dilution of extracted material (ml) and D = quantity (g) of original, toxin-contaminated material.

Extraction and recoveries

Initial extractions (Fig. 2) represented sample material obtained from 1 g of the residual oil obtained from 75 g of fungal-contaminated rice; the oil was extracted by gentle agitation on a reciprocal shaker for 15 min at 50°C with 15 ml of acetonitrile. To determine the efficiency of the acetonitrile extraction procedure, a 1-g sample of oil was sequentially extracted with the solvent. Since only trace quantities of either toxin were detected in the fourth extraction it appeared that most of the extractable toxins were solubilized in the initial three stages. The percentage of xanthomegnin (x) and viomellein (v) recovered in the three steps was: (1) x = 78.9, v = 64.1; (2) x = 18.9, v = 24.5; and (3) x = 1.4, v = 10.4. The residual, acetonitrile-extracted oil was allowed to stand for several days, heated and re-extracted with acetonitrile; the absence of toxin in the solvent phase indicated that a slow release mechanism of the compounds was not present in the extracted oil.

Sep-Pak recoveries

The advent of the Sep-Pak (Waters Assoc.), and other microcolumns, has assisted in solving two of the most difficult problems the chromatographer encounters, *i.e.* trace enrichment and sample clean-up. Use of Sep-Paks with crude fungal extracts was investigated. Silica Sep-Paks retained 100% of both metabolites from 2 ml of acetonitrile solutions used for HPLC analysis. No solvents were found that could elute the compounds in a volume required to effect trace enrichment. C_{18} Sep-Paks activated in a routine manner and with 10 ml of the mobile phase partially retained both compounds and no solvents tested completely eluted the toxins. Since analytical HPLC results demonstrated complete recovery of the two metabolites from C_{18} columns, the Sep-Pak investigation was extended. Subsequent exhaustive equilibration of the C_{18} Sep-Pak with the mobile phase (0.2 ml/min for 3 h) provided conditions for complete recovery of the compounds from microcolumns.

Chromatograms of extracts passed through C_{18} Sep-Paks were less complex than those of total extracts and Sep-Pak use provided a clean-up for qualitative analysis. Silica Sep-Paks for qualitative work were not considered since the usefulness of silica is well established with fungal metabolites clean-up in open-bed liquid chromatography and TLC. The use of Sep-Paks, silica or C_{18} was found not to be practical for quantitative analytical schemes.

CONCLUSIONS

An effective reversed-phase system for separation of xanthomegnin and viomellein was developed. The pH of the mobile phase was adjusted to provide conditions that favored the dihydroxy form of the quinone structures of the compounds. Complete elution of the toxins from a C_{18} column required the presence of SDS in the eluent. *A. ochraceus* appeared to produce a single form of viomellein that is

racemized by exposure to silica; the reversed-phase HPLC system provided partial separation of the two isomeric forms.

ACKNOWLEDGEMENT

We thank E. J. Bowers for assistance in carrying out HPLC determinations.

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