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Determination of Petroselinic, *cis*-Vaccenic and Oleic Acids in Some Seed Oils of the Umbelliferae by Silver Ion Thin Layer Chromatography of their Phenacyl Esters

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A method is proposed for the determination of petroselinic, oleic and *cis*-vaccenic acids in plants of the Umbelliferae by using silver ion thin layer chromatography and densitometry. The fatty acids are first converted into phenacyl esters and this enables base line separation at ambient temperature on a plate impregnated with 1% methanolic silver nitrate. Conditions were found for the simultaneous determination of the saturated and dienoic fatty acids while maintaining the resolution of petroselinic and oleic acids. The procedure is suitable for screening plants of the Umbelliferae in phytochemical and selection studies, and may be of value as a small-scale preparative technique.

Keywords: Umbelliferae seed oils; *Petroselinum sativum; Pimpinella anisum*; petroselinic acid; oleic acid; silver ion thin layer chromatography; densitometry.

INTRODUCTION

The seed oils of Umbelliferae species are characterized by a high content (over 50%) of petroselinic acid (*cis*-6-octadecenoic acid). This acid is the only natural positional isomer of significance of the most abundant octadecenoic fatty acid, oleic acid (*cis*-9-octadecenoic acid), in plants (Gunstone *et al.*, 1986; Placek, 1963). The position of the double bond in petroselinic acid provides an opportunity to produce a variety of valuable raw materials for industry (Placek, 1963; Kleiman and Spencer, 1982; Princen and Rothfuss, 1984), and plants of the Umbelliferae family have been identified as promising sources (Thies, 1993).

In seed oils of the Umbelliferae, petroselinic acid is always accompanied by small amounts of oleic acid. The resolution and correct quantification of these two acids has been a difficult analytical task because of the great similarity in their properties, including their chromatographic behaviour. Despite the rapid development of chromatographic techniques and continuously improved instrumentation, a routine, simple and rapid method for analysis of the fatty acids of these oils has not been available until recently. Three gas liquid chromatographic (GC) procedures have been reported recently in which petroselinic and oleic acids were reasonably well resolved (Griffiths *et al.*, 1992; Wolff and Vandamme, 1992; Thies, 1993) and two of these were applied for the determination of the petroselinic acid/oleic acid ratio in the seed oil of *Coriandrum sativum* (Umbelliferae).

Among the different approaches for the separation of positionally isomeric monoenoic fatty acids, silver ion chromatography has a significant place and its capabilities in this field have been recently reviewed (Nikolova-Damyanova, 1992). The method is based on the ability of silver ion to complex with double bonds, and species are resolved according to the number, position and geometry of their double bonds. Silver ion thin layer chromatography (Ag-TLC) is the technique that has been most often used in attempts to separate positionally isomeric fatty acids. Petroselinic and oleic acids were indeed successfully resolved by this means, although the procedures were not easy to perform since a satisfactory result was possible only at temperatures of about −20°C (Morris *et al.*, 1967; Breuer *et al.*, 1987).

A common feature of all procedures for fatty acid analysis, including Ag-TLC, is that fatty acids are first converted into methyl esters. A new perspective on the resolution of positionally isomeric fatty acids by means of silver ion chromatography was found while studying the retention of other fatty acid derivatives under the conditions of silver ion high performance liquid chromatography (Ag-HPLC) (Nikolova-Damyanova *et al.*, 1992). An improved separation of petroselinic, oleic and vaccenic acid as phenacyl esters with base-line resolution was achieved at ambient temperatures. Later the same result was obtained using Ag-TLC (Nikolova-Damyanova *et al.*, 1994).

Here we report a procedure for the separation and quantification of petroselinic and oleic acid as phenacyl esters by Ag-TLC and densitometry. It has been applied to determine the fatty acid compositions of the seed oils of two species of the Umbelliferae namely parsley (*Petroselinum sativum*) and aniseed (*Pimpinella anisum*). All fatty acids were separated and determined on a single plate after a two-stage development. The procedure is suitable for rapid screening of the seed oils in phytochemical or plant selection studies, or as a micropreparative procedure.

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EXPERIMENTAL

Materials. All reagents and solvents were analytical grade. Acetone and methanol were redistilled, and chloroform was washed to remove the stabilizing alcohol, prior to use as components of a mobile phase. Stearic, palmitic, linoleic, petroselinic, *cis*-vaccenic and oleic acids were purchased from Sigma Chemical Co. (Poole, UK), and silica gel 60G was from Merck (Darmstadt, Germany). The seeds of *Petroselinum sativum* and *Pimpinella anisum* were purchased from a local market and were from the crop of 1993.

Extraction of the oils. The seeds (2.0 g) were ground and extracted with light petroleum (25 mL; b.p. 40–70°C) in a Soxhlet apparatus for 12 h. The extracts were filtered and the oils were dried under vacuum at 40°C.

Isolation of the triglycerides by preparative TLC. Each oil was dissolved in hexane to give a solution containing 100 mg/mL. In order to isolate the respective triglycerides, three aliquots (0.3 mL each) of the crude oil solution (about 90 mg) were applied to a 20×20 cm glass plate covered with a 1 mm thick layer of silica gel G as an 18 cm band. The plate was developed in a closed standard Desaga tank with 60 mL of a mobile phase consisting of light petroleum (b.p. 40–70°C):acetone (100:12, v/v). Triglycerides were recovered from the adsorbent with diethyl ether; the solvent was removed under vacuum and the triglycerides were redissolved in hexane to give a 5 mg/mL solution.

Preparation of phenacyl esters. Triglycerides (2 mg) were hydrolysed with 0.1 M potassium hydroxide (0.5 mL), in 90% ethanol at room temperature overnight. The sample was then acidified with two drops of acetic acid and the free fatty acids were extracted with diethyl ether (3 mL) and hexane (3 mL), before being washed with water (2 mL). The organic solvent was removed in a gentle stream of nitrogen and the fatty acids were converted into phenacyl derivatives as described by Wood and Lee (1983). Briefly, the free acids were reacted with 0.5 mL each of phenacyl bromide (10 mg/mL in acetone) and triethylamine (10 mg/mL in acetone) for 15 min in a boiling water bath. Acetic acid (50 μL) was added and the sample was heated for an additional 5 min. The sample was then purified on a Florisil Sep-Pak cartridge (Waters-Millipore Corporation, Milford, MA, USA). The cartridge was first washed with 8 mL hexane:diethyl ether (95:5, v/v) and the pure phenacyl esters were then eluted with hexane:diethyl ether (80:20, v/v). The solvent was removed in a stream of nitrogen, and the phenacyl derivatives were redissolved in dichloroethane to give a final concentration of 2.5–5 mg/mL.

Silver ion thin layer chromatography (Ag-TLC). An aliquot (10 μL) of the derivatized fatty acids was applied by micropipette to a 4×19 cm home-made glass TLC plate coated with an 0.2 mm thick silica gel G layer and impregnated (by dipping) in 1.0% methanolic silver nitrate (Chobanov *et al.* 1976, 1992). The plate was developed twice in a non-saturated closed cylindrical tank (22×4.5 cm i.d.) with either 3.0 mL chloroform:acetone (100:0.25, v/v) or in an open tank (of the same dimensions) with 3.5 mL dichloroethane. The plate was then treated for 30 min successively with vapours of bromine and sulphuryl chloride in a closed chamber (fume cupboard) and heated at 180–200°C on a metal plate with temperature control. The phenacyl esters of the fatty acid appeared as black spots on a nearly white background.

Densitometric quantification. The densities of the charred spots were measured with a Shimadzu CS-930 densitometer (Shimadzu Corporation, Kyoto, Japan) by zigzag scanning in the reflection mode at 450 nm with a slit of 0.4×0.4 mm. The quantities of the fatty acids were expressed as relative area percentages as derived from the integrator (Shimadzu DR-2).

Gas liquid chromatography (GC). Part of the purified triglycerides (2 mg) was trans-butylated with 1% sulphuric acid in *n*-butanol (Christie, 1989). Fatty acid analysis was performed on a Pye Unicam Model 304 (Pye Unicam Ltd., Cambridge, UK) gas chromatograph with a flame ionization detector and a fused silica capillary column (15 ×0.2 mm i.d.) coated with SP-2340 (Supelco Inc., Gland, Switzerland) at 160°C (isothermally):detector temperature — 260°C; injector temperature — 260°C; carrier gas (nitrogen) flow-rate — 10 cm/s.

RESULTS AND DISCUSSION

The petroselinic/oleic acid ratio in plants of the Umbelliferae presents one of the most difficult cases for chromatographic determination, i.e. when the major component (petroselinic acid) migrates behind the minor component (oleic acid). The resolution becomes even more complicated when *cis*-vaccenic acid accompanies the other isomers, as is the case with aniseed oil. Conditions to resolve such pairs sufficiently well in a way to be sure of their correct quantification can rarely be found, and the minor component appears as a more or less well defined shoulder on the chromatographic peak of the major component. This was the case when the above mentioned acids were separated as their methyl esters by Ag-TLC. When converted into the phenacyl derivatives, however, petroselinic and oleic acids were completely resolved at ambient temperature on a plate impregnated with 1% methanolic silver nitrate and developed in an open cylindrical tank with dichloroethane as solvent. A typical densitogram of *Petroselinum sativum* seed oil is presented in Fig. 1. The separation achieved is by far the best reported so far when applying Ag-TLC and is comparable to that obtained by Ag-HPLC (Nikolova-Damyanova *et al.*, 1992).

However, a remaining problem was that, under these conditions, it was not possible to perform a complete fatty acid analysis of the samples in a single run, as the saturated fatty acids were washed out from the plate while the dienoic derivative had an R_f value of 0.6 (in comparison to petroselinic (0.41) and oleic (0.55) acid derivatives). We tested other solvents as components of a mobile phase, therefore, and mixtures based on chloroform appeared very promising. Methanol, ethanol, acetone and acetonitrile were examined as modifiers, and acetone and acetonitrile provided the most satisfactory results. Acetone was chosen for further work as it is less toxic. It also appeared that the separation was more reproducible if development was carried out in closed tanks, not saturated with solvent vapour, but more than one development was necessary. Thus, the optimal separation of all fatty acid components of the two species of Umbelliferae tested was achieved on a single plate by developing it twice with a mobile phase of

chloroform:acetone (100:0.25, v/v) to a solvent front of 17.5 cm. The separation is illustrated in Fig. 2 with the derivatives of the fatty acids of *Pimpinella anisum* as an example. Under these conditions the R_f values of the derivatives were: 0.17 (diene), 0.57 (petroselinic), 0.66 (oleic), 0.71 (*cis*-vaccenic), 0.91 (saturated), i.e. petroselinic, oleic and *cis*-vaccenic acid derivatives were clearly resolved.

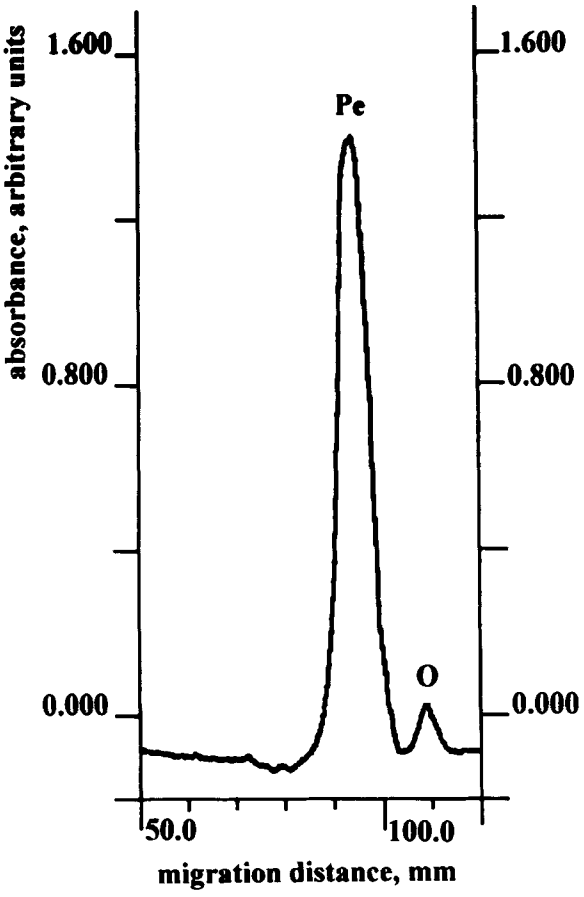

Figure 1. Separation of phenacyl esters of petroselinic and oleic acids from *Petroselinum sativum* seed oil. Conditions: home-made silica gel G plate; layer impregnated with 1.0% methanolic silver nitrate; development with 3.5 mL dichloroethane in open cylindrical tank; detection by charring with sulphuryl chloride vapours and heating at 180–200°C. (Key to peak identity: Pe — petroselinic; O – oleic fatty acids.)

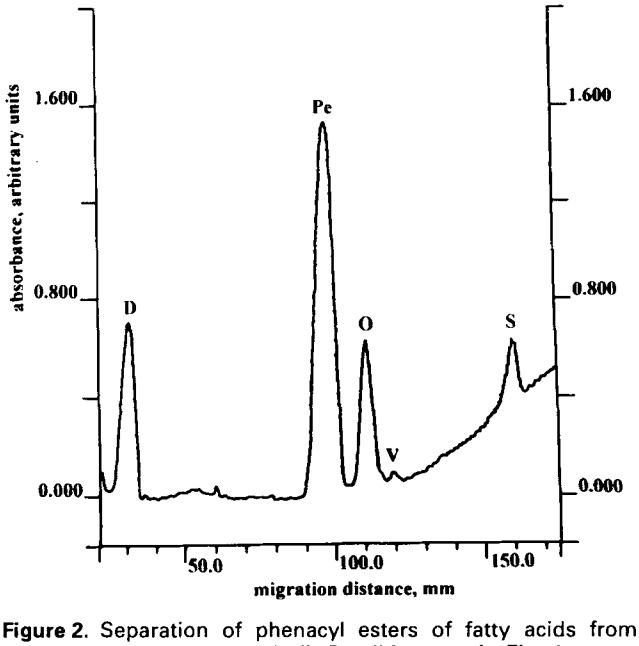

Figure 2. Separation of phenacyl esters of fatty acids from *Petroselinum sativum* seed oil. Conditions: as in Fig. 1 except that a two-stage development was performed in closed cylindrical tanks with a mobile phase of chloroform:acetone (100:0.25, v/v). (Key to peak identity: Pe — petroselinic; O — oleic; D — dienoic, V - *cis*-vaccenic, S — saturated fatty acids.)

As TLC is influenced by the laboratory environment, mainly by temperature and humidity, small variations in the composition of the mobile phase are possible. Thus, at higher ambient temperatures (about 22°C to 30°C) and higher humidity (75% and over) the proportion of acetone in the mobile phase can be lowered to 0.1%.

The concentration of the silver nitrate in the methanolic impregnating solution was 1%. For qualitative purposes, it could be lowered to 0.5%, but in this case the spots were diffuse and densitometric peaks were broad and badly shaped. It is interesting to note that impregnation of the layer with highly concentrated silver nitrate solutions in methanol or incorporation of about 30% by weight of silver nitrate into the layer is a common practice in Ag-TLC of lipids (see for example, Breuer *et al.*, 1987). It has been shown in a series of papers, however, that silver nitrate can successfully govern the processes at a much lower level than is usually accepted (Chobanov *et al.*, 1976, 1992; Tarandjiiska and Nguen Hien, 1988; Nikolova-Damyanova *et al.*, 1993). Moreover, very fine and difficult resolutions of fatty acid and triglyceride isomers can be achieved at low concentrations of silver nitrate (Chobanov *et al.*, 1992, Nikolova-Damyanova *et al.*, 1993). The great advantage of this is not only the lower cost of the analysis but also that plates are handled more easily. There is no need to keep them or use them in the dark. Most important for analysis is that plates can be treated with charring reagents and then heated to produce the charred fatty acid spots with the background of the plate remaining white. This enables the final densitometric scanning. That there is no influence of the layer is evident from Fig. 1, which presents the smooth base-line usually obtained. A sloping base-line (c.f. Fig. 2) observed on some occasions was due to the home-made layer and not to the impregnation with silver nitrate.

Table 1 lists the quantitative densitometric results for the fatty acid composition of the seed oils of *Petroselinum sativum* and *Pimpinella anisum*. The data were compared to those obtained for the same sample by GC of the respective butyl esters.

The reproducibility and the accuracy of Ag-TLC/densitometry in determining fatty acids (as methyl esters) after charring was determined earlier (Chobanov *et al.*, 1992). It was shown that the standard deviation between actual and experimental values for a fatty acid (as its methyl ester derivative) with zero to three double bonds were less than 2%, and the relative error was less than 10%. Similar

Table 1. Fatty acid composition of *Petroselinum sativum* and *Pimpinella anisum* seed oils as determined by silver ion thin-layer chromatography and densitometry and by gas chromatography

	Petroselinum sativum		Pimpinella anisum	
Fatty acids	TLC[a]	GLC	TLC	GLC
Dienoic	12.3±0.2	11.6	19.0±2.7	18.6
Petroselinic	74.4±1.8	77.7	56.0±1.9	60.0
Oleic	5.0±0.9	4.1	18.5±0.9	15.4
Vaccenic	–	–	1.4±0.1	1.4
Saturated[b]	8.2±1.8	6.6	5.1±1.3	5.1

[a] Mean ±SD for 3 separate determinations.
[b] Sum of palmitic acid and stearic acids.

reproducibility was obtained in this study for phenacyl esters. There was a good agreement between the GC and densitometric data, confirming the accuracy of the former technique.

We believe, therefore, that Ag-TLC of fatty acid phenacyl esters provides a simple to perform, low-cost approach for qualitative and quantitative screening of seed oils which contain petroselinic, oleic and vaccenic acids for use in laboratories which do not have access to more sophisticated techniques. However, an important advantage of Ag-TLC is that it is not merely an analytical technique: it is also applicable on a micro-preparative scale, and it has an advantage over alternative GC methods in this respect. For example, our Ag-TLC method could be used in biochemical studies in which isotopically labelled substrates are used to study the biosynthesis of these acids.

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