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New method of stationary phase preparation for silver ion column chromatography: Application to the isolation of steroidal hydrocarbons in vegetable oils

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Abstract

A simple method to prepare silver nitrate loaded silica gel for low pressure silver ion column chromatography is presented. To prepare the packing material, activated silica gel is homogenised with a small volume of aqueous solution of silver nitrate. The effects of water and silver content on the column efficiency, and of elution solvent and light on the recovery, were studied. The procedure was applied to the isolation of steroidal hydrocarbons in olive oils. Separation between 3,5-, 2,4- and 2,5-sterene isomers was achieved.

Keywords: Silver ion liquid chromatography; Olive oil; Silica gel, silver nitrate loaded; Vegetable oils; Stationary phases, LC; Oils; Steroids

1. Introduction

Since its introduction in 1962, silver ion chromatography has been largely used to resolve unsaturated compounds containing double bonds. The property of silver compounds to form polar complexes reversibly with the double bonds of aliphatic moieties is used as a means of separating them according to the number, configuration (*cis* or *trans*), and to some extent the position of those double bonds. This technique is a well established and important method for the separation of unsaturated lipid species, such as triglycerides, wax esters, fatty acid methyl esters, and steroids [1].

The technique has been adapted to thin-layer chromatography, high-performance liquid chromatography (HPLC), solid-phase extraction, and low-pressure column chromatography. However, the latter has found limited application due to the difficulty in obtaining a homogeneous and unaltered packing material.

When considering the preparation of the packing material for silver ion column chromatography, the effect of the supporting material must be taken into account. Silica gel, the most widely used supporting material, possesses appreciable polarity and adsorption activity. Therefore, the elution order cannot be ascribed solely to the complexion reaction of silver ion and double bond. The retention must be a result of a mixed mechanism, via complexion of silver ions and interaction with silanol moieties. In general, the column is prepared by impregnation of the supporting material with an aqueous silver nitrate solution, followed by drying at 120°C. The activated material is suspended in the mobile phase and used to pack the column [2]. The main problems are the partial

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decomposition of silver nitrate during the activation process, and the excessive adsorption activity.

As an alternative, macroreticular sulphonic acid ion-exchange resins are used as sorbents. These are prepared by passing an aqueous silver nitrate solution through a column of resin until excess silver ions start to elute. Separations in silver loaded resin have generally been found to be poor, because there is no adsorption activity of the sorbent [1].

In the present work, a new simple, rapid method to prepare silver loaded silica gel for column chromatography is discussed. Application of this method to the isolation of the steroidal hydrocarbons of vegetable oils is presented. The unsaturated hydrocarbons appear in refined oils as the result of dehydration of sterols during the refining process. 3,5-Stigmastadiene and 3,5-campestadiene are the main components deriving from β-sitosterol and campesterol, respectively [3,4]. Minor amounts of the 2,4- and 2,5-isomers have been also detected [5]. The determination of stigmastadienes is used to reveal the presence of refined vegetable oils in crude oil [6]. Furthermore, the ratio between stigmastadienes and campestadienes (*R*1) is used to detect desterolised seed oils in refined olive oil [4].

2. Experimental

2.1. Material and reagents

All the reagents should be of analytical grade unless otherwise specified. The water used should be distilled water, or water of at least equivalent purity. Hexane or mixture of alkanes of b.p. range 65–70°C, distilled with rectifying column. Silica gel 60 for column chromatography, 70–230 mesh, (Merck ref. 7734 or similar), heated in the oven at 110°C for 2 or more h and allowed to cool in a desiccator.

2.2. Preparation of the packing material

The preparation of argentated silica gel for two columns is as follows. In a 500 ml round-bottomed flask wrapped in aluminium foil, weigh 30 g of activated silica gel. In a 25 ml Erlenmeyer flask, dissolve 3.0 g of silver nitrate in 7 ml of distilled water, and with a pipette add the solution drop-wise onto the silica gel, shaking from time to time. Place the stopper in the flask and shake vigorously for 20 s, then place the flask in the rotary evaporator and rotate for 30 min under atmospheric pressure and room temperature. The silica gel can be kept in the stoppered flask in darkness.

2.3. Preparation of the column

To prepare the silica gel column, pour elution liquid (hexane with 0.2% of absolute ethanol) into the column to a depth of approximately 5 cm, and then fill with a slurry of argentated silica gel in the elution liquid (20 g in 40 ml) with the help of elution liquid portions. Allow to settle, and finish settling by applying slight vibration. Add anhydrous sodium sulphate to a height of approximately 0.5 cm, finally, elute the excess eluent. Protect the argentated silica gel from light by wrapping the column in black paper.

2.4. Separation of steroidal hydrocarbon fraction

Weigh 1 ± 0.01 g of oil in a 10 ml beaker, add 1 ml of standard solution of 3,5-cholestadiene (20 μg), take the mixture to the fractionating column with the aid of two 1 ml portions of hexane, and run the sample onto the column by allowing the solution level to drop to the top of the sodium sulphate. Start the chromatographic elution with the elution liquid at a flow-rate of 1 ml/min approximately. Discard the first 35 ml of elution, and then collect fractions of 20 ml. Transfer the fractions to a 50 ml round-bottomed flask, and evaporate in a rotary evaporator at 30°C, under reduced pressure until dryness, and immediately dissolve the residue in 0.2 ml of elution liquid. Keep the solution in the refrigerator until gas chromatographic (GC) analysis. To quantify the sterenes, the second and third fractions must be evaporated together.

2.5. GC

GC analysis of the steroidal hydrocarbons was performed using a Carlo Erba (Milan, Italy) HRGC MEGA 2 gas chromatograph fitted with a flame ionisation detection (FID) system and a split injection system (split ratio 1:15). Separations were

carried out on a high-temperature fused-silica capillary column (25 m×0.25 mm I.D.) coated with 5% phenylmethylsilicone, with a thickness of 0.25 μm (Sugerlabor, Spain). The operating conditions were the following: injector temperature, 300°C; detector temperature, 320°C; oven programming temperatures, initial 235°C for 6 min and then rising at 2°C/min to 285°C. The amount of solution injected was 2 μl. Sensitivity was about 16-times the minimum attenuation. The gas carrier was hydrogen at about 80 kPa of pressure. Data acquisition and processing were carried out using a Chrom-Card Data System (Fisons, Altricham, UK).

3. Results and discussion

The official method for determination of steroidal hydrocarbons in vegetable oils [7] uses a silica gel column for fractionating. When the method is applied to strongly refined olive oils, the gas chromatograms show peaks interfering in the determination and quantification of the steroidal hydrocarbons (Fig. 1). These peaks arise from the degradation of the squalene during refining [8].

In order to improve the separation, silver nitrate loaded silica gel was used. The packing material was prepared by simple admixture of silica gel with a small volume of silver nitrate solution, followed by homogenisation of the solid. With this method, a clean chromatogram was obtained (Fig. 2), allowing quantification of even the minor peaks.

The isolation of steroidal hydrocarbons from a refined olive oil spiked with 17 mg/kg of 3,5-cholestadiene was used to study the factors affecting column efficiency. For each run, just prepared packing material was used. High percentages of water present in the silica gel decrease the efficiency in separating unsaturated hydrocarbons, while low percentages require a great volume of eluent. However, the elution volume of saturated hydrocarbons is only slightly affected by the water concentration. Therefore, control of the water content allows the column efficiency to be set according to the characteristic of the separation. For the hydrocarbon fractionation, 23% (w/w) of water in the silica gel gives a good separation with small eluent volume. This percentage of water is high compared with that used in unloaded silica gel columns (≈2%).

Another factor affecting column efficiency is the

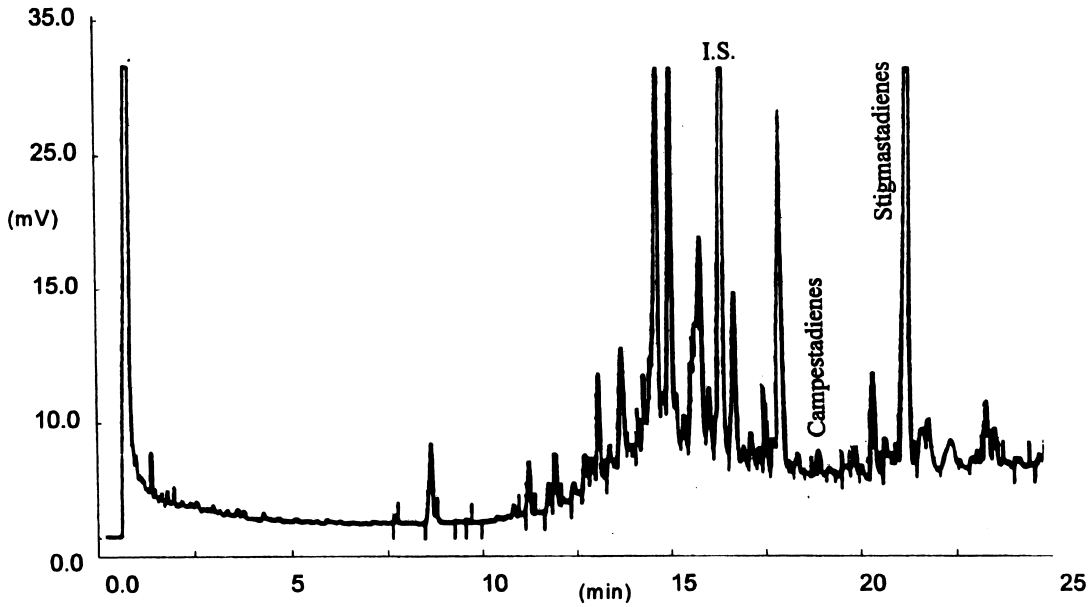

Fig. 1. GC–FID profile of the steroidal hydrocarbon fraction isolated by silica gel column chromatography from a refined olive oil, using a fused-silica capillary column (25 m×0.25 mm I.D.) coated with 5% phenylmethylsilicone.

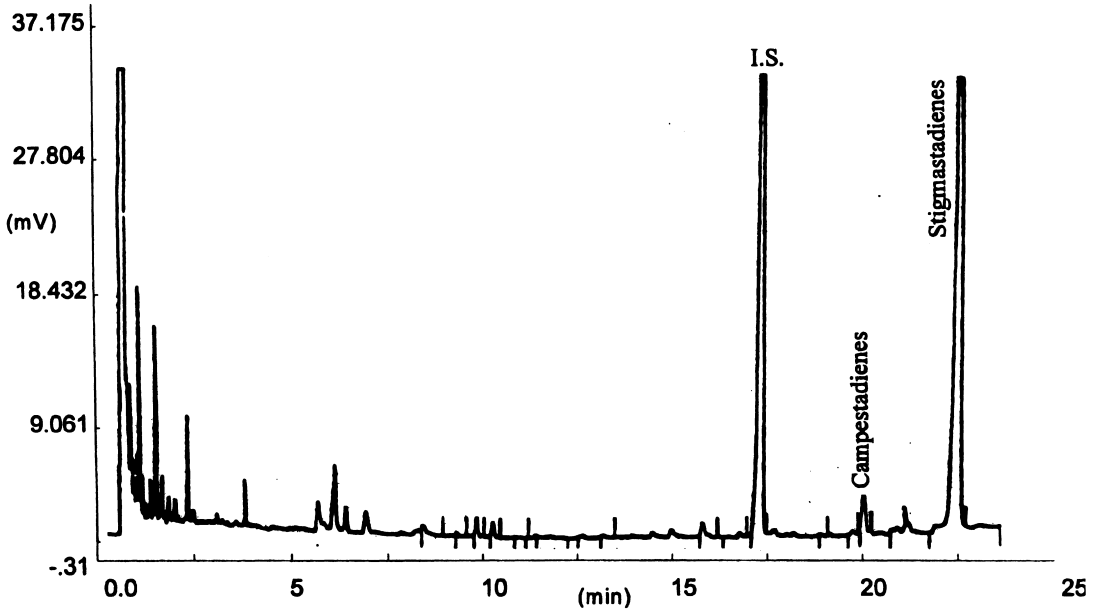

Fig. 2. GC–FID profile of steroidal hydrocarbon fraction isolated by silver nitrate loaded silica gel column chromatography from a refined olive oil, using a fused-silica capillary column (25 m×0.25 mm I.D.) coated with 5% phenylmethylsilicone.

amount of silver nitrate. Percentages higher than 10% (w/w) of silver nitrate on the silica gel did not improve the separation, but lower concentrations worsened it. A silica gel loaded with 23% of water and 10% of silver nitrate was finally chosen.

Using unsilvered silica gel, a total recovery of 79% of 3,5-cholestadiene was achieved. A loss of approximately 15% occurs during evaporation of the liquid fraction under reduced pressure, therefore, minor losses can be ascribed to the column chromatography. Nevertheless, the recovery diminished significantly with increasing silver nitrate percentage (Table 1). This fall could be due to the oxidant activity of the silver ions on the steroidal hydrocarbons, since these compounds are very labile in solution. Eluents containing small proportions of ethanol as stabilising agent were used. The recovery improved with increasing ethanol percentages, but with a loss of efficiency for the separation between steroidal hydrocarbon and squalene degradation products (Table 1). However, fractionations carried out in absence of light yielded similar recoveries to

Table 1
Factors affecting the recovery and isolation of the 3,5-cholestadiene in olive oil using silvered and unsilvered silica gel column chromatography, containing 23% and 2% (w/w) of water, respectively

Packing material	Eluent	Recovery (%)	Separation[a]
Si	Hexane	79	None
Si-Ag (5%)	Hexane	51	Incomplete
Si-Ag (10%)	Hexane	42	Complete
Si-Ag (10%)	Hexane–ethanol (0.2%)	54	Complete
Si-Ag (10%)	Hexane–ethanol (0.5%)	59	Incomplete
Si-Ag (10%)	Hexane–ethanol (1%)	75	None
Si-Ag (10%) absence of light	Hexane	80	Complete
Si-Ag (10%) absence of light	Hexane–ethanol (0.2%)	84	Complete

[a] Separation between 3,5-cholestadiene and squalene degradation products.

Table 2
Recovery of 3,5-cholestadiene in olive oil using silvered silica gel column chromatography prepared with 23% (w/w) of water and 10% (w/w) of silver nitrate

Concentration (mg/kg)	Recovery (%)
4.3	87
7.4	84
17	82
25	86

those obtained using unsilvered silica gel. These facts indicate that the loss of steroidal hydrocarbons is due to a combined effect of silver ions and light. The best separation and recovery were obtained in elutions using hexane with 0.2% of absolute ethanol in darkness. With this operating mode, the total recoveries of various concentrations of 3,5-cholestadiene in olive oil were constant, showing negligible losses during column chromatography (Table 2).

The method was standardised by means of an interlaboratory ring test carried out on two samples of each oil. The statistical evaluation of the results, in accordance with ISO 5725-1986E, is summarised in Tables 3 and 4. For the stigmastadiene determination, the Horwitz ratios (Ho_R)≤1 indicate good reproducibility of the entire analytical method [9]. The reproducibility of the ratio *R*1 is acceptable, taking into account that for high *R*1 values, the campestadiene concentration is very low. The pro-

Table 3
Statistics of stigmastadienes determination derived from the results of the interlaboratory test

	Samples			
	Olive oil	Mixture of refined olive and refined olive residue oils	Refined high oleic sunflower oil	Mixture of olive and desterolised sunflower oils
Level (mg/kg)	15.80	82.78	10.39	8.61
n (Outliers)	10 (1)	8 (2)	10 (1)	9 (1)
r (Repeatability)	1.22	7.95	1.28	0.81
S_r [a]	0.44	2.84	0.46	0.29
R.S.D.$_r$ (%)	2.77	3.43	4.41	3.35
R (Reproducibility)	4.72	8.67	3.60	2.54
S_R [b]	1.69	3.10	1.29	0.91
R.S.D.$_R$ (%)	10.66	3.74	12.38	10.55
Ho_R [c]	1.01	0.45	1.10	0.91

[a]Standard deviation of repeatability.
[b]Standard deviation of reproducibility.
[c]Horowitz ratio.

Table 4
Statistics of stigmastadienes/campestadienes ratio (*R*1) derived from the results of the interlaboratory test

	Samples			
	Olive oil	Mixture of refined olive and refined olive residue oils	Refined high oleic sunflower oil	Mixture of olive and desterolised sunflower oils
Level (mg/kg)	29.30	25.50	7.17	3.62
n (Outliers)	11	10	9 (2)	9 (1)
r (Repeatability)	10.68	9.13	0.92	0.52
S_r [a]	3.81	3.26	0.33	0.19
R.S.D.$_r$ (%)	13.01	12.79	4.61	5.12
R (Reproducibility)	17.15	20.18	1.62	0.62
S_R [b]	6.13	7.21	0.58	0.22
R.S.D.$_R$ (%)	20.91	28.96	8.09	6.08

[a]Standard deviation of repeatability.
[b]Standard deviation of reproducibility.

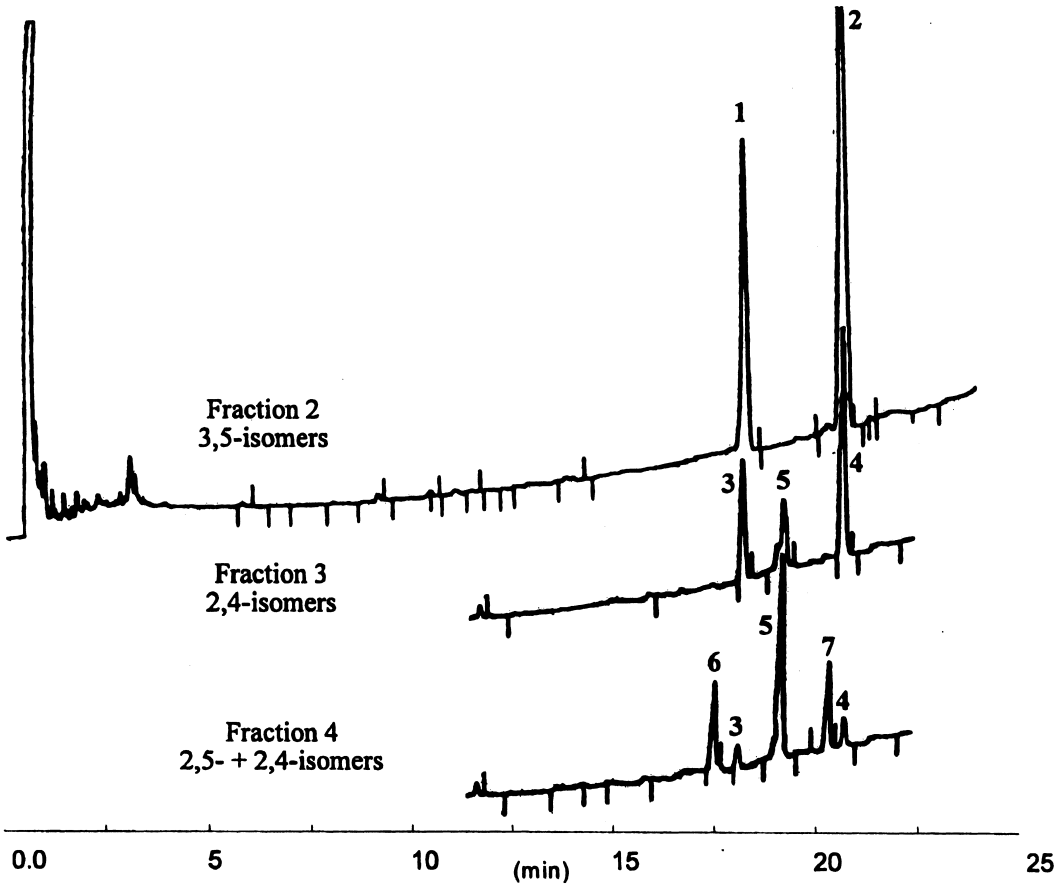

Fig. 3. GC–FID profiles of steroidal hydrocarbon isomers present in the second, third and fourth fractions (20 ml) isolated by silver ion column chromatography from a mixture of refined olive oil and desterolised sunflower oil, using a fused-silica capillary column (25 m×0.25 mm I.D.) coated with 5% phenylmethylsilicone. (1) 3,5-Campestadiene; (2) 3,5-stigmastadiene; (3) 2,4-campestadiene; (4) 2,4-stigmastadiene; (5) stigmastatriene; (6) 2,5-campestadiene; (7) 2,5-stigmastadiene.

cedure has been adopted by the International Olive Oil Council for the determination of desterolised seed oils in refined olive oil [10].

The method allows the isolation of the isomers of the steroidal hydrocarbons for their subsequent characterisation by GC–mass spectrometry. The isomers elute in the order 3,5-, 2,4- and 2,5-isomers (Fig. 3). This order is different to that obtained using HPLC on silica gel (2,4-, 3,5- and 2,5-isomers) [5], indicating that the silver ion complexion effect predominates over the silica gel retention.

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