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SEPARATION OF LIPOPHILIC FRACTIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An approach to the preparative isolation of different lipid classes is described. It was applied to the separation of a lipophilic fraction from Antarctic krill on a silica column using a multistep gradient (both the mobile phase composition and flow-rate were changed). Hydrocarbons (including those of the carotinoid type), sterol esters, waxes, triglycerides, free fatty acids, sterols, mono- and diglycerides, phosphatidylinositols, phosphatidic acids, phosphatidylethanolamines, lecithin and corresponding lyso-derivatives were isolated on a single column and some of them were identified by high-performance thin-layer chromatography, gas–liquid chromatography and mass spectrometry. These results are considerably in advance of those previously published.

INTRODUCTION

The application of high-performance liquid chromatography (HPLC) to lipids separation has much potential for the detailed study of this class of compounds, having different polarities. In spite of various problems, the separation of neutral lipids has already been described[1], using columns with reversed phases[2]; alkylated Sephadexes[3] and silica gel[4,5]. Phospholipids have been separated on silica gel[5,6], reversed phases[7] and ion-exchange resins[8,9]. In some cases the separation of both polar and neutral lipids is reported[10].

For further identification of lipids various types of detectors have been applied: UV-absorption detectors (operated at wavelengths of 206, 210 and 200 nm)[5,11,12]; refractometric detectors[13,14]. In the majority of cases reported, HPLC was performed with a constant mobile phase, *i.e.*, isocratic elution. However, the application of gradient elution has also been described[15,16].

The present work concerns the separation of lipophilic fractions from Antarctic krill by preparative HPLC on a silica gel column with stepwise gradient switching of a four-component mobile phase. Hydrocarbons (including those of the carotenoid type), sterol esters, waxes, triglycerides, free fatty acids, sterols, mono- and diglycerides, phosphatidylinositols, phosphatidic acids, phosphatidylethanolamines, lecithin and corresponding lyso derivatives were isolated and some of them were identified

by high-performance thin-layer chromatography (HPTLC) and gas–liquid chromatography (GLC) [in some cases, GLC data were confirmed by mass spectrometric (MS) analysis]. The HPLC preparative separation took approximately 2 h.

EXPERIMENTAL

Materials

Lipids were extracted according to Bligh and Dyer[17] from Antarctic krill *Ephausida superba*, caught in 1984 during an expedition in the Indian Antarctic sector. The extract was evaporated to dryness under a flow of nitrogen and redissolved in isopropanol–hexane–0.01 *M* acetate buffer (500:500:5) to give a final concentration of 200 mg/ml.

All solvents used were purified according to appropriate procedures[18].

Plates used for HPTLC were 10 × 10 and 5 × 5 cm (Merck, Darmstadt, F.R.G.). All solvents used were saturated with helium and stored under an helium atmosphere. Standard kits of lipids for TLC were obtained from Sigma, (St. Louis, MO, U.S.A.), Gasukuro Kogio (Japan), Helena Labs. (U.S.A.) and Latron Laboratory (Japan).

Methods

The separation was performed on an Analyst-7900 HPLC chromatograph (LDC, Riviera Beach, FL, U.S.A.), consisting of a ConstaMetric III pump, an UV-SpectroMonitor III and a chromatographic control module CCM II. A 500 mm × 21.5 mm (2 × 250 mm in series) Zorbax Sil column (DuPont, Wilmington, DE, U.S.A.) was used. The mobile phases were as follows: (a) isopropanol–hexane–0.01 *M* acetate buffer (500:500:5); (b) isopropanol–hexane–0.01 *M* acetate buffer (500:500:15); (c) isopropanol–hexane–0.01 *M* acetate buffer (500:500:37.5); (d) isopropanol–0.01 *M* acetate buffer (1000:37.5). Separation was performed at ambient temperature using a stepwise gradient of mobile phase according to the following programme: 0–33 min, solvent a, gradual linear increase of flow-rate from 3 to 10 ml/min; 33–40 min, solvent a switched off, solvent b at 10 ml/min, gradual linear increase in the flow-rate of solvent a from 0 to 10 ml/min (total flow-rate increased to 20 ml/min and the buffer content in the feed linearly increased to 10 ml/l); 40–100 min, solvent a switched off, solvent c, flow-rate 10.0 ml/min (total flow-rate 20 ml/min; buffer content 26.25 ml/l); 100–130 min, solvent b switched off, solvent d, flow-rate 10.0 ml/min [the mobile phase composition isopropanol–hexane–acetate buffer (750:250:37.5), total flow-rate 20 ml/min]; finally, solvent d only, flow-rate 10.0 ml/min.

Detection was performed with either a spectrophotometric detector operated at a wavelength of 220 nm (sensitivity 0.1 a.u.f.s.) and equipped with a semipreparative cell, or a diode-array detector (Model SPD-M1A; Shimadzu, Japan), operated at wavelengths ranging from 200 to 600 nm (sensitivities 1.0 and 0.5 a.u.f.s., switched during the course of recording). The flow-rate to the diode-array detector was half that to the spectrophotometric detector and did not exceed 10.0 ml/min.

The volume of the lipid sample injected was 1 ml. Fractions were collected with a Gilson Model 202 fraction collector, each during a period of 0.5 min. This collector was connected after the diode-array detector. The fractions obtained were

analyzed by HPTLC employing the lipid standards. The mobile phase for neutral lipids was hexane–diethyl ether–acetic acid (85:14:1)[19], and for polar lipids was chloroform–methanol–water (65:25:4)[20]. For the identification of the lipids, circular, anticircular and linear HPTLC was applied using the above-solvent systems.

RESULTS AND DISCUSSION

Preliminary experiments were carried out on one column (250 mm × 21.5 mm) filled with Zorbax SIL. A gradient of changing flow-rate from 3 to 10.0 ml/min was used, but without changing the mobile phase composition. The elution of the most polar components (phospholipids) was accomplished by passing pure isopropanol through the sample loop (three successive injections through the 1-ml loop). Then, for a better identification (by improving the selectivity and efficiency of the system) and for a more complete separation of the components into classes, the described procedure was applied. The increase in the column length led to a corresponding increase in the number of theoretical plates. The elution was performed by beginning with a less polar mobile phase and finishing with the most polar solvent, isopropanol. Furthermore, in order to obtain a separation efficiency close to the theoretical one, the linear velocity of the mobile phase was adjusted to be similar to that required by the Knox curve[21]. Such an increase in the flow-rate required flow splitting when applying the diode-array detector to prevent damage from high backpressure. It was possible to collect all fractions only after halving the flow-rate.

The necessity for the increase in flow-rate to 20.0 ml/min dictated a modification in the chromatograph used, which allowed flow-rates only up to 10.0 ml/min. Thus, a third pump was inserted and a special programme (non-typical for such an instrument) for control of the automatic switching of the pumps was elaborated.

It was shown during preliminary experiments that due to the presence of unsaturated compounds in the pooled lipophilic fraction it was also possible to use common fixed-wavelength detectors (at a wavelength of 254 nm). With the chromatogram obtained using such a detector it was possible to assign the fractions collected to corresponding classes of lipids. Such a chromatogram is not shown, however

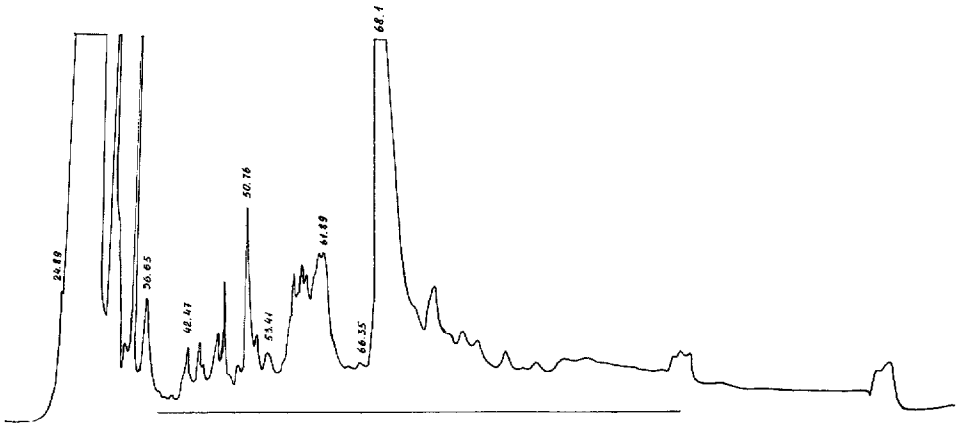

Fig. 1. Chromatogram of total krill lipids obtained by HPLC with a photometric detector operated at λ = 254 nm. Conditions as described in the text.

the possibility of recording it is clearly illustrated by the record obtained with the diode-array detector (Fig. 2). Generally speaking, a diode-array detector is advantageous for obtaining preliminary data concerning the sample analyzed for the purpose of further elaboration of optimum conditions for detection of desired compounds. Thus, the detection of carotinoids (see Fig. 2) can be performed by operating the detector at wavelengths in the range 400–450 nm, without significant interference from other substances present in the complex mixture. Furthermore, Fig. 2 clearly illustrates the complexity of the major components of the lipophilic fraction of interest; detection is possible only in the far-ultraviolet region (wavelengths 210–220 nm).

It is of particular interest that when detectors of the type SpectroMonitor III (LDC) and SPD-M1A (Shimadzu) were applied, compensation for the mobile phase background to 2.7 absorbance units was possible, thus enabling detection in a rather sensitive region (0.05 a.u.f.s. for SpectroMonitor III and 0.05 a.u.f.s. for SPD-M1A). This then revealed another interesting fact: generally used textbook data attesting to the applicability of a great number of solvents when applying detectors operated in the ultraviolet and far-ultraviolet regions need revision. The generally accepted opinion about the "intransparency" of solvents disregards the modern development of

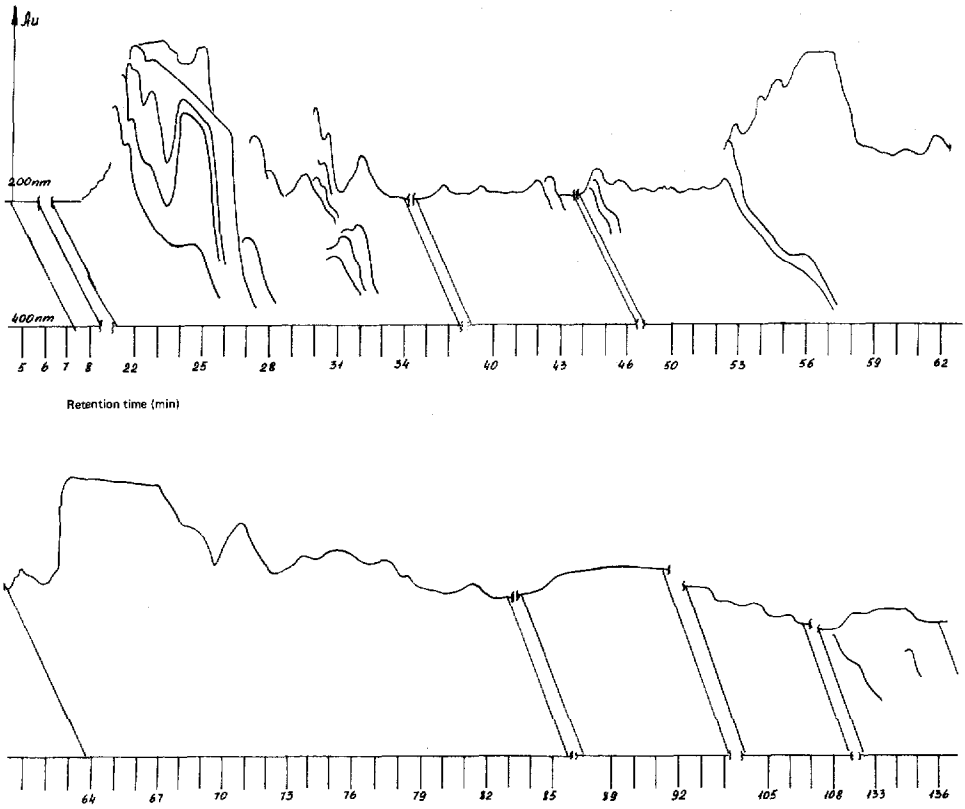

Fig. 2. Chromatogram of total krill lipids obtained with a diode-array detector. Conditions as described in the text.

data-handling systems and the availability of special compensators (such as the supplied by, for example, Laboratory Data Control). For example, we have shown the possibility of application of acetone as a mobile phase with the detector operated at a wavelength of 212 nm where there is a characteristic minimum in the absorbance curve. Thus, the possibilities for analysis of, for example, triglycerides are substantially increased: for triglycerides detection rather insensitive refractometric detectors are usually employed.

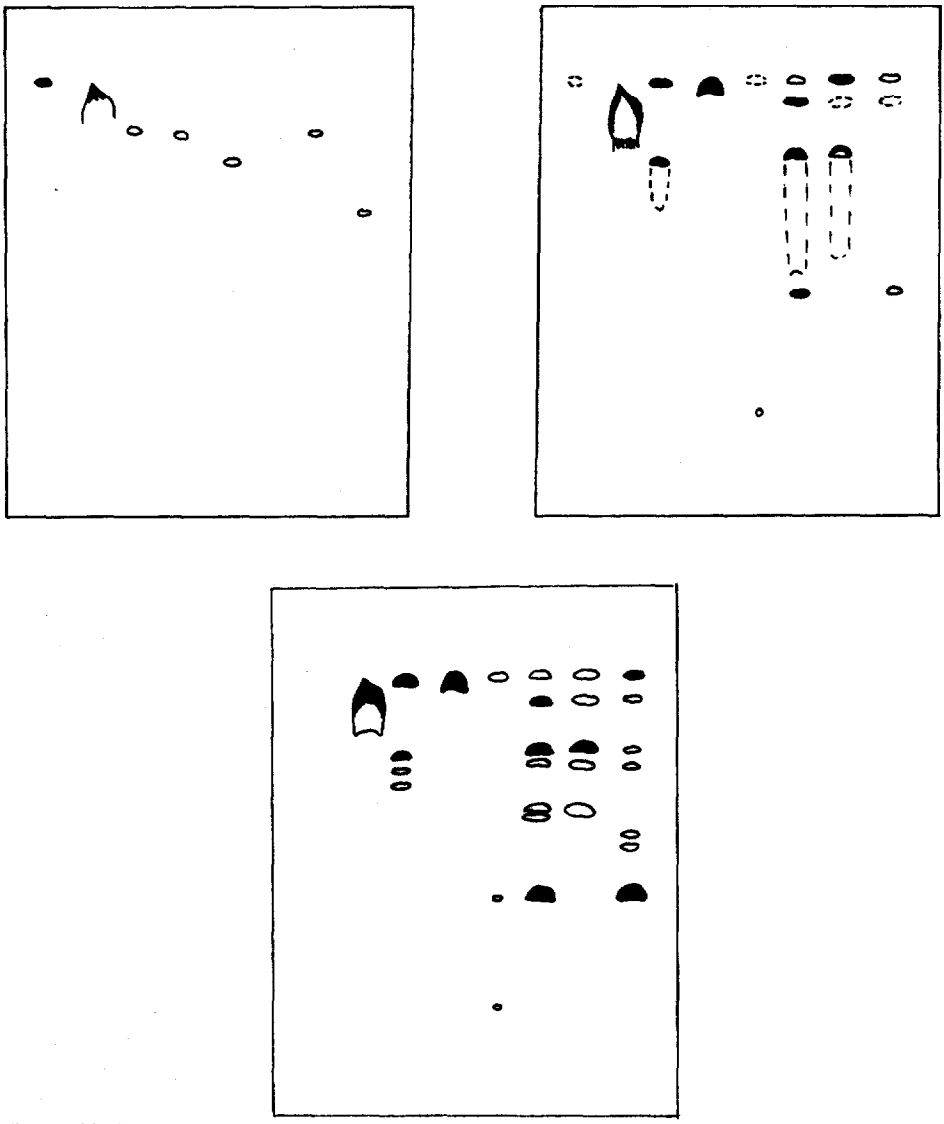

Fig. 3. Thin-layer chromatograms of the neutral lipid fractions 6–13 with retention times from 30 to 34.03 min: (A) without visualization; (B) with visualization using iodine vapour; (C) with visualization using phosphomolybdic acid. Fractions: 6 = α-carotene; 7 = γ-carotene + fitoin; 8 = sterol esters; 9 = carotenoids; 10 = alkanes + alkenes; 11 = carotenoids, sterol esters, plasmalogenes; 12 = carotenoids, sterol esters, ethers; 13 = plasmalogenes (carotenoids, sterol esters, in trace amounts).

Compensation for the mobile phase background also permits a reduction in the requirements for solvent purification. In some cases a simple distillation from glass apparatus is sufficient.

Fractions for TLC analysis were evaporated under a flow of nitrogen, redissolved in 0.3 ml of chloroform and placed on a plate by the automatic TLC Autosampler II (Camag, Switzerland). Quantitation of identified fractions was performed using a densitometer Scanner II (Camag).

The first eighteen fractions corresponded to peaks with retention times from 24.89 to 36.65 min (Fig. 1) and thus can be attributed [according to spectrophotometric (Fig. 2) and TLC data] to hydrocarbons and carotenoids. By means of TLC analysis with standards, the above fractions were identified as hydrocarbons, including carotenoids, esterified vitamin A, plasmogenes and esterified sterols. The plates were visualized first by use of iodine vapour, then with phosphomolybdic acid, as illustrated in Fig. 3. When TLC analysis of carotenoids is performed, the position of the above mentioned fractions can be determined without additional visualization of the plates (Fig. 3).

Fractions with retention times of 42.47–53.41 min compromise free sterols and waxes. The maximum free sterols content is found in the fractions with retention time 50.76 min.

Fractions with retention times of 53–61.89 min correspond to triglycerides, the saturated triglycerides having shorter retention times than the unsaturated ones.

Fractions with retention times of 62–66.35 min correspond to free fatty acids, mainly (according to GLC data) myristic acid (80% of the total acid content) and much smaller amounts of palmitic, palmitoleic, linolic, oleic and stearic (each about 4%), eicosapentenoic (2%) and docosahexenoic (2%) acids.

Fractions with retention times of $\geqslant 68.1$ min, according to TLC data, comprised phospholipids eluted from the column in the following sequence: inositol-phosphatides, phosphatidic acid, lecithin, phosphatidylethanolamine, lyso-products. The main product was lecithin.

Thus, the preferred multistep gradient of mobile phase composition and the column dimensions used permitted the separation and reliable identification of about twenty lipid components with different polarities, a result far in advance of all data previously published on separations of lipophilic components of complex mixtures[22].

CONCLUSIONS

It is possible to obtain a very detailed separation on a single HPLC column with results which are comparable with those obtainable by the fractional open-column technique. Detection is achieved by means of common detectors (UV, fixed wavelength) due to the presence of unsaturated compounds. In assessing the significance of the results, it should be kept in mind that only a number of analytical methods (including HPTLC, GLC, MS, spectroscopy) can provide such qualitative and quantitative data. Detailed methods have been presented for the investigation of total lipophilic fraction contents.

The results were analyzed from the viewpoints of the usefulness of different solvents, including those which show too large an absorbance in the far-UV region, *e.g.*, acetone. Methods have been developed specifically for lipid analysis. The special

advantage of such an approach is that it can be applied to the separation of non-polar (neutral) as well as polar (complex) lipids.

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