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Note

Separation of lipids from Antarctic krill (*Euphausia superba* Dana) by isocratic high-performance liquid chromatography on silica using a flow-program

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The separation of lipids into classes by high-performance liquid chromatography (HPLC) on normal phase presents some problems, because of the wide range of polarities within the neutral and polar lipid classes and the difficulty of detecting the aliphatic compounds. Different classes may be detected by the absorbance near 200 nm due to their non-conjugated double bonds. The solvent system preferred for lipid separation by thin-layer chromatography (TLC) or column chromatography on silica, chloroform–methanol–glacial acetic acid, cannot be used in HPLC because of its opaqueness in this UV region.

Some attempts at lipid separation by HPLC[1] used chloroform-containing eluent systems and the "moving wire" detector[2] or a refractive index detector[3]. The introduction of chloroform-free eluents allowed a detection at 190–210 nm.

Hurst and Martin[4] separated lecithin in chocolate using acetonitrile–methanol–water (65:21:14) as eluent with detection at 210 nm. Other separations on normal phase columns with UV detection in the 200 nm region were described by Hax and Geurts van Kessel[5], eluting with *n*-hexane–propanol-2–water (6:8:0.75), the water content increasing from 0.75 to 1.75. Yandrasitz *et al.*[6] introduced NH_4Cl and sulphuric acid as mobile phase modifiers using the same system and a gradient as well. Nasner and Kraus[7,8] proposed a flow-program with a buffer-modified mobile phase instead of gradient elution. By this they achieved a separation of soy lecithin into phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidic acid (PA). In a similar manner, Patton *et al.*[9] separated polar lipids from rat liver applying *n*-hexane–propanol-2–phosphate buffer–ethanol–acetic acid (367:490:62:100:0.6); however, the time needed for separation was more than 120 min.

Besides sulphuric acid and phosphate or acetate buffers, also water, other mineral acids and mineral salts have been used as phase modifiers. Reviews on lipid separation by HPLC were recently given by Porter and Weenen[10] and Plattner[11]. A simultaneous and complete separation of total lipids into polar lipids and neutral lipid classes as free fatty acids, sterols and glycerides has not been achieved to our knowledge.

In this investigation a buffer-modified mobile phase was used together with a

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flow-program to separate the polar and neutral lipid classes on a 5-μm silica column in a single run. With this system a separation of Krill lipids into triglycerides, sterols, free fatty acids, PE and PC was possible in less than 10 min.

MATERIALS

Lipids were extracted from Antarctic krill (*Euphausia superba* Dana) caught during the second Antarctic expedition of the Federal Republic of Germany (1977–1978) according to Folch *et al.*[12]. The extracts were evaporated to dryness under a flow of nitrogen and redissolved in *n*-hexane–propanol-2 (1:1) to give a final concentration of 1 μg lipid per μl.

All solvents were reagent grade or better (E. Merck, Darmstadt, G.F.R.). Standard kits of lipids for TLC were obtained from Sigma (St. Louis, MO, U.S.A.). Solvents were saturated with helium and stored under a helium atmosphere after degassing ultrasonically.

METHODS

HPLC analyses were performed on a Milton Roy instrument (Milton Roy, Hasselroth, G.F.R.) consisting of a Constametric III pump, an UV Spectromonitor III and a chromatographic control module CCM 301 with integrated printer/plotter. A 12.5 cm × 4.6 mm Hyperchrome SC column (Bischoff Analysentechnik, Leonberg, G.F.R.) filled with Shandon Hypersil, 5 μm, was equipped with a 2-cm precolumn containing the same stationary phase.

Mobile phases used were *n*-hexane–propanol-2–0.01 *N* sulphuric acid (100:100:15) and *n*-hexane–propanol-2–0.1 *M* sodium acetate buffer (pH 4.66) (8:8:1). The eluents were used in recycling mode. The flow-programs were controlled and recorded by the CCM unit. The temperature was ambient, and the UV monitor was operated at a wavelength of 206 nm.

Amounts of 10 μl sample (10 μg lipid) were injected into a 20-μl loop (Rheodyne 7125). The components separated were identified after collection of fractions by two-dimensional TLC using standard substances[14].

RESULTS AND DISCUSSION

Separation

The separation of krill lipids into neutral lipids, triglycerides, free fatty acids and sterols (three incompletely resolved signals) and PE and PC is shown in Fig. 1, while Fig. 2 exhibits a separation into triglycerides, sterols, free fatty acids, PE and PC. The order of elution was according to the polarity of the lipid classes. In both chromatograms the same main classes of krill lipids appear, as reported by other authors[14,15] who used TLC for analysis.

The separation in Fig. 1 was achieved with *n*-hexane–propanol-2–0.01 *N* sulphuric acid, whereas the buffer-modified mobile phase was used in Fig. 2. From both figures it is evident that the sulphuric acid-containing mobile phase permits a separation as described earlier (*e.g.*, refs. 7 and 8); the application of the buffered solvent, which was proposed by Nasner and Kraus[7,8] for phospholipid separation, only re-

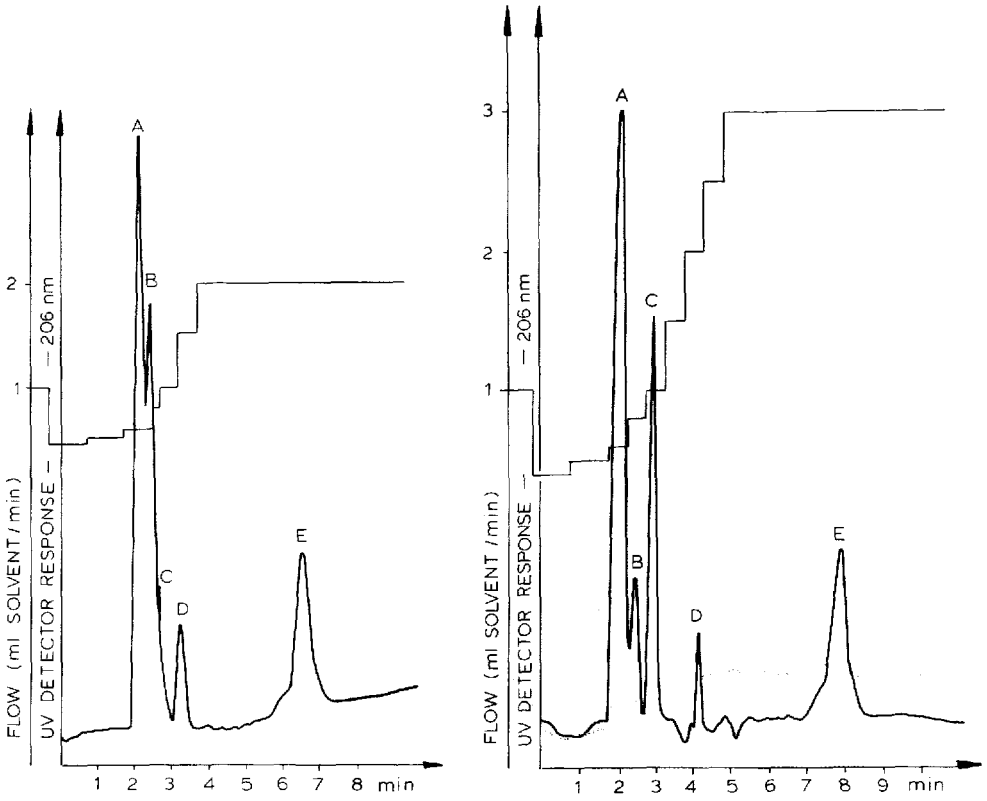

Fig. 1. Separation of krill lipids by HPLC on 5-μm silica. Eluent: *n*-hexane–propanol-2–0.01 *N* sulphuric acid (100:100:15). Flow-program from 0.4 ml/min to 2 ml/min. Peaks: A = triglycerides; B = free fatty acids; C = sterols (shoulder); D = phosphatidylethanolamine; E = phosphatidylcholine.

Fig. 2. Separation of krill lipids by HPLC on 5-μm silica. Eluent: *n*-hexane–propanol-2–0.1 *M* acetate buffer (8:8:1). Flow-program from 0.4 ml/min to 3 ml/min. Peaks: A = triglycerides; B = sterols + sterol esters; C = free fatty acids; D = phosphatidylethanolamine; E = phosphatidylcholine.

markably increases the efficiency of total lipid separation. The main advantage of the buffered mobile phase is the better resolution of the peaks derived from neutral lipid classes.

The resolution of the peaks is somewhat inferior using the sulphuric acid-containing eluent, but this system avoids the precipitation of buffer substances, which can lead to serious damage of the HPLC apparatus.

Most of the methods proposed using solvent gradient systems had the disadvantage of long analysis times, long reequilibration times and drifting baselines. Isocratic separation procedures using the flow-program (Figs. 1, 2) lead to short separation times, a constant baseline and no reequilibration is necessary.

The application of the flow-program did not improve the separation of the single lipid classes, but the total time of analysis was shortened. At a constant flow-rate of 0.5 ml/min the separation shown in Fig. 2 took approximately 50 min, which could be reduced to 8 min by applying the flow-program.

Detection

The detection of lipid substances at 206 nm is mainly due to the UV-absorption caused by isolated double bonds in the side-chains of glycerolipids and unsaturated sterols. For fish and other marine organisms like Antarctic krill, which contain large amounts of highly unsaturated fatty acids[13,14], this mode of detection is recommended. For lipid classes containing none or only a limited number of unsaturated fatty cacids, *e.g.*, lysophosphatidylethanolamine, detection at 206 nm is less sensitive.

CONCLUSION

The separation method described allowed a very fast ($\leqslant 8$ min) separation of neutral and polar lipid classes in a single run without using a solvent gradient. The main advantage is the short time of analysis compared with other methods like TLC or Chromarods, and the facile recovery of the individual classes of lipids for further investigations.

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REFERENCES

1 F. B. Jungalwala, R. J. Turel, J. E. Evans and R. H. McCleur, *Biochem. J.*, 155 (1976) 55.
2 K. Aitzetmüller, *J. Chromatogr.*, 139 (1977) 61.
3 J. S. Rhee and M. G. Shin, *J. Amer. Oil Chem. Soc.*, 59 (1982) 98.
4 W. J. Hurst and R. A. Martin, Jr., *J. Amer. Oil Chem. Soc.*, 57 (1980) 307.
5 W. M. A. Hax and W. S. M. Geurts van Kessel, *J. Chromatogr.*, 142 (1977) 735.
6 J. R. Yandrasitz, J. G. Berry and S. Segal, *J. Chromatogr.*, 225 (1981) 319.
7 A. Nasner and Lj. Kraus, *Fette, Seifen, Anstrichm.*, 83 (1981) 70.
8 A. Nasner and Lj. Kraus, *J. Chromatogr.*, 216 (1981) 389.
9 G. M. Patton, J. M. Fasulo and S. J. Robins, *J. Lipid Res.*, 23 (1982) 190.
10 N. A. Porter and H. Weenen, *Methods Enzymol.*, 72 (1981) 34.
11 R. D. Plattner, *Methods Enzymol.*, 72 (1981) 21.
12 J. Folch, M. Less and G. H. Sloane-Stanley, *J. Biol. Chem.*, 226 (1957) 497.
13 R. G. Ackmann, in J. J. Connell (Editor), *Advances in Fish Science and Technology*, Fishing News Book Ltd., Farnham, 1980, p. 86.
14 H. S. G. Fricke, *Master Thesis*, University of Hamburg, Hamburg, 1981.
15 A. Clarke, *J. Exp. Mar. Biol. Ecol.*, 43 (1980) 221.