notes on methodology

Chromatography of unusual lipids on thin layers of magnesium oxide

H. P. KAUFMANN, H. K. MANGOLD, and K. D. MUKHERJEE

Bundesanstalt für Fettforschung, Münster i. Westf., Germany

SUMMARY The chromatographic behavior of some minor components of natural lipids was studied using layers of magnesium oxide and of commercial adsorbents containing magnesium oxide. The lipids investigated included wax esters, cholesteryl esters, diester waxes, and esters, ethers and ether-esters of ethanediol and glycerol. Pronounced differences were found in the patterns of separation of certain lipids on magnesium oxide as compared to silica gel and Florisil. The procedures described afford means for the detection of unusual lipids in natural materials, and their isolation.

SUPPLEMENTARY KEY WORDS adsorbents · silica gel · Florisil · detection of minor lipids · wax esters · cholesteryl esters · diol lipids · diester waxes · sterols · long-chain alcohols · alkoxylipids

IT IS WELL KNOWN that in adsorption chromatography the sequence of elution of compounds differing in the types and numbers of functional groups depends to some extent on the nature of the adsorbent (1, 2). In the lipid field, silica gel is the adsorbent used most commonly, and hence the chromatographic behavior of various lipid classes on this adsorbent is well established (3–6). However, certain separations have not been accomplished using silica gel. This has prompted us to investigate the potential of other adsorbents.

We have found that magnesium oxide is especially suitable for the separation of unusual lipids. Magnesium oxide has been employed previously as such (7, 8) or in admixture with other adsorbents (9–12) for the fractionation of alicyclic lipids. To the best of our knowledge, only two reports have appeared on its use in the fractionation of aliphatic lipids (8, 13). However, in the latter studies it has been stated that magnesium oxide does not offer any advantage over silica gel.

The present communication records the application of thin layers of magnesium oxide, and of mixed adsorbents containing magnesium oxide, in the fractionation of classes of lipids which cannot be resolved satisfactorily by thin-layer chromatography on silica gel. Specifically, experimental conditions are described for the separation of wax esters from cholesteryl esters, diesters of ethanediol from triglycerides, and sterols from long-chain alcohols and diglycerides. Furthermore, the chromatographic behavior of alkyl and alk-1-enyl ethers of ethanediol and glycerol, and their acyl derivatives, on layers of magnesium oxide as well as commercial adsorbents containing magnesium oxide is compared with that on layers of Silica Gel G and Florisil.

Reference Substances. Refined soybean oil and rapeseed oil were purchased locally. Diesters of ethanediol were prepared by esterifying ethanediol with the total fatty acids derived from soybean and rapeseed oils, respectively. Wax esters, cholesteryl esters, diester waxes, and partial glycerides were prepared following established procedures (14). Syntheses of long-chain alkyl and dialkyl ethers of ethanediol (15) and glycerol (16, 17), trialkyl ethers of glycerol (17) as well as of ether-esters of ethanediol and glycerol (15), and of alk-1-enyl ether-esters of ethanediol (18) were described previously. Synthetic alk-1-enyl ether-esters of glycerol (19) were gifts of Dr. R. Gigg, London, England.

Adsorbents. Three different charges of pure magnesium oxide (article no. 28306, Serva Entwicklungslabor, Heidelberg, Germany) were used. According to the supplier, the distribution of particle sizes was 64%, 75%, and 80%, respectively, between 35 and 100 μ. Magnesium oxide for thin-layer chromatography (article no. 5864, E. Merck A.G., Darmstadt, Germany) was used for comparison.

Anasil B (containing 10% plaster of Paris as binder) and Anasil S (without binder) (Analabs Inc., North Haven, Conn.) are preparations of silica gel that are known to contain 15% magnesium oxide.

Silica Gel G (E. Merck A.G.) was used without further treatment.

Florisil (article no. 21528, Serva Entwicklungslabor) was pulverized in a ball mill to yield a product that could be spread as a thin layer.

Preparation of Layers. Batches of five glass plates, 20 × 20 cm, were coated with adsorbent layers, 0.25 mm in thickness, unless stated otherwise, using a commercial applicator (Shandon Scientific Co., London, England).

Magnesium oxide, 30 g, was slurried with 55 ml of water. After coating with this slurry, the plates were allowed to stand for 15 min at room temperature, and the layers were activated at 95°C for 30 min.

Layers of Anasil B were prepared from slurries of 30 g of adsorbent with 65 ml of water; for Anasil S, 30 g of adsorbent was slurried with 70 ml of water. Layers of both Anasil B and Anasil S were activated at 130°C for 2 hr.

Layers of Silica Gel G were prepared from slurries of 30 g of adsorbent with 60 ml of water and activated at 130°C for 2 hr.

Florisil layers were made from slurries of 50 g of pulverized adsorbent and 67 ml of water. After partial drying and setting at room temperature for 30 min, the layers were activated at 100°C for 1.5 hr.

All of the adsorbent layers were used immediately after activation and subsequent cooling to room temperature.

Chromatography. Known amounts of lipids, dissolved in benzene, were spotted onto the adsorbent layers, and the plates were developed by the ascending technique in rectangular jars lined with filter paper. The solvent systems used are recorded in the legend of Fig. 1. After development, the chromatograms were heated for 10–15 min at 95–100°C to remove residual solvents.

Detection. On layers of magnesium oxide, lipid fractions usually were detected by spraying with a 0.02% aqueous solution of rhodamine 6G (20) and viewing the chromatogram in UV light (240 or 260 nm). The conventional procedures of charring with aqueous mineral acids were not suitable for layers of magnesium oxide, as they tended to crack and peel off during heating. This difficulty was largely overcome by adopting the following procedure: The chromatograms were sprayed with a freshly prepared 0.1% solution of potassium dichromate in concentrated sulfuric acid up to a point where the lipid fractions became just visible due to wetting of the layer. Subsequently the chromatograms were covered with a glass plate and placed in an oven at 95°C for 10 min. The covering plate was then removed and the chromatogram was heated at 210°C for 20 min.

On layers of Anasil B, Anasil S, and Silica Gel G, lipid fractions were detected by spraying with a saturated solution of potassium dichromate in 70% aqueous sulfuric acid and charring at 210°C for 20 min.

For the charring of lipids on layers of Florisil, a 0.1% solution of potassium dichromate in concentrated sulfuric acid was employed as spray reagent; charring was carried out at 210°C for 20 min.

Results. The separation patterns of various ester lipids on layers of magnesium oxide, and other adsorbents, are compared in Fig. 1.

It is obvious that wax esters and cholesteryl esters are separated more effectively on magnesium oxide than on any of the other adsorbents. Furthermore, on magnesium oxide as well as on the two types of Anasil, the diester wax, 1,2-dihexadecanoyloxy-hexadecane, is well resolved from wax esters and from ethanediol diesters, whereas on Silica Gel G and on Florisil these separations are not satisfactory. The differences in the distribution of particle sizes in the various lots of magnesium oxide have hardly any effect on the separation of the lipids studied. The chromatographic behavior of lipids on the two types of Anasil is essentially identical.

Fig. 1 shows that on layers of magnesium oxide ethanediol diesters migrate ahead of triglycerides, and that the two lipid classes are clearly separated. Similar resolution is achieved on layers of Anasil B and Anasil S. This point is of particular interest, as small amounts of esters of ethanediol and other diols reportedly occur in nature, together with esters of glycerol (21, 22). It is known that adsorption chromatography on silica gel does not resolve the esters of ethanediol from those of glycerol (21, 23). We have found that by using thin-layer chromatography on magnesium oxide, it is possible to detect as little as 0.001% of ethanediol diesters in mixture with triglycerides.

Model mixtures of compounds representing these two classes of lipids have been separated recently by gel permeation chromatography on columns of Sephadex LH-20 (24).

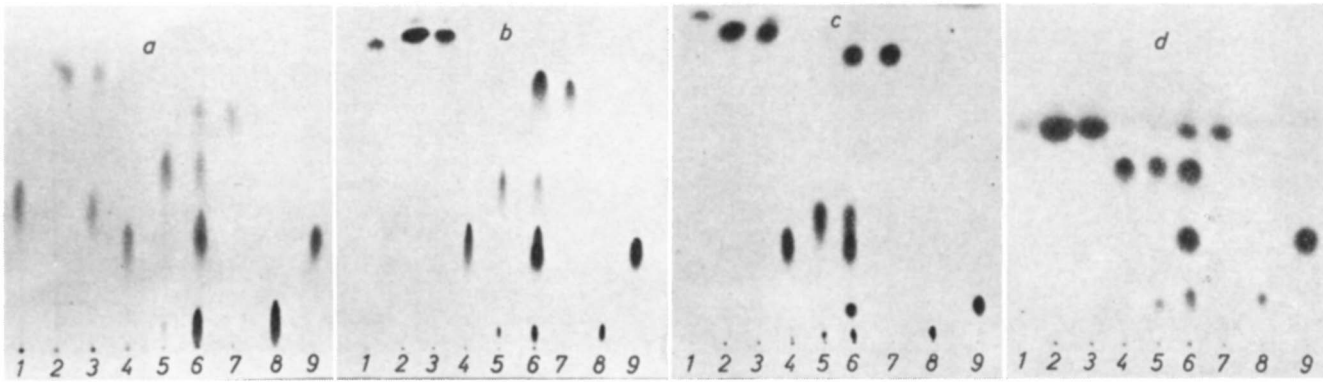

FIG. 1. Chromatographic behavior of ester lipids. Adsorbents: *a*, magnesium oxide; *b*, Anasil B; *c*, Silica Gel G; *d*, Florisil. Solvents: *a* and *d*, hexane–diethyl ether–ethyl acetate 60:40:1; *b* and *c*, hexane–diethyl ether 85:15 and 90:10, respectively. *1*, cholesteryl esters; *2*, wax esters; *3*, mixture of *1* and *2*; *4*, triglycerides of soybean oil; *5*, diesters of ethanediol derived from the fatty acids of soybean oil; *6*, mixture of *4*, *5*, *7*, *8*, and *9*; *7*, diester waxes (diesters of long-chain 1,2-alkanediols); *8*, diglycerides; *9*, long-chain *prim.* alcohols. Amount of each substance, 20 μg. Detection by charring.

It is known that mixtures of long-chain alcohols, diglycerides, and sterols cannot be resolved by adsorption chromatography on silica gel (25, 26) nor by gel permeation chromatography on Sephadex LH-20 (27). We have found that on layers of Silica Gel G as well as Florisil using hexane–diethyl ether 60:40 as developing solvent, long-chain alcohols can be separated as a class from sterols, e.g., cholesterol and β-sitosterol. Using the same solvent system on Silica Gel G, long-chain alcohols are not resolved from diglycerides, whereas on Florisil, diglycerides and sterols migrate together. Thus, a mixture containing these three lipid classes is not separated completely on either of the two adsorbents. In contrast, a distinct separation of these three lipid classes is achieved on layers of magnesium oxide using hexane–diethyl ether–ethyl acetate 50:50:1 as developing solvent; long-chain alcohols migrate ahead of sterols and the latter ahead of diglycerides. It should be noted, however, that on magnesium oxide, using the solvent systems described, long-chain alcohols are not resolved from triglycerides.

It is evident from the foregoing discussion that the order of migration rates of the various ester lipids on layers of magnesium oxide is similar to that observed on Silica Gel G. In contrast, the order of elution of long-chain ethers of ethanediol and glycerol on magnesium oxide is different from that observed on Silica Gel G. Thus, on layers of magnesium oxide, with hexane–diethyl ether–ethyl acetate 90:10:1 as solvent, diethers of ethanediol migrate ahead of the triethers of glycerol, and alkyl ethers of ethanediol migrate ahead of 1,2-dialkyl ethers of glycerol. The same is true for the ether-esters of ethanediol and glycerol when chromatographed with hexane–diethyl ether–ethyl acetate 70:30:1. On layers of Silica Gel G, with hexane–diethyl ether 90:10 as solvent, the aforementioned ethanediol-derived alkoxylipids migrate behind the corresponding glycerol-derived compounds.

Alk-1-enyl diacyl glycerols and alkyl diacyl glycerols, which are well resolved on layers of Silica Gel G, migrate together on magnesium oxide, and the same is true for the corresponding diol lipids.

Anasil B is as suitable as Silica Gel G for the resolution of alk-1-enyl ether-esters and alkyl ether-esters of ethanediol and glycerol. Thus, in the separation of alkoxylipids the properties of Anasil B are similar to those of Silica Gel G, whereas in the chromatography of ester lipids, as mentioned above, Anasil B and Anasil S resemble magnesium oxide.

Neutral lipid fractions resolved by thin-layer chromatography on magnesium oxide can be eluted from the adsorbent with water-saturated diethyl ether. The magnesium oxide can be dissolved in 2 N aqueous sulfuric acid, and the lipids, with the exception of lipids containing alk-1-enyl moieties, can be recovered with hexane or benzene. However, gravimetric analyses of eluted fractions are rather impractical, as the capacity of magnesium oxide is considerably less than that of silica gel. The amounts available are sufficient for colorimetric analyses, e.g., by the hydroxamic acid method (28).

The preparation of methyl esters for their further gas–liquid chromatographic analysis is possible by reacting the lipids with methanol–sulfuric acid in benzene (29) without prior elution from the magnesium oxide.

The skillful and conscientious assistance of Mrs. Irmgard Kiewitt is gratefully acknowledged. The authors are indebted to Dr. R. Gigg, London, England, for a generous gift of synthetic alk-1-enyl diacyl glycerols.

Manuscript received 4 December 1970; accepted 17 February 1971.

References

1. Brockmann, H. 1949. Chromatography of colorless substances and the relation between constitution and adsorption affinity. *Discuss. Faraday Soc.* **7:** 58–64.
2. Strain, H. H. 1948. Molecular structure and adsorption sequences of carotenoid pigments. *J. Amer. Chem. Soc.* **70:** 588–591.
3. Kaufmann, H. P., and Z. Makus. 1960. Die Dünnschicht-Chromatographie auf dem Fettgebiet. I: Trennung von Modell-Mischungen. *Fette Seifen Anstrichm.* **62:** 1014–1020.
4. Kaufmann, H. P., Z. Makus, and F. Deicke. 1961. Die Dünnschicht-Chromatographie auf dem Fettgebiet II: Trennung der Cholesterin-Fettsäureester. *Fette Seifen Anstrichm.* **63:** 235–238.
5. Mangold, H. K., and D. C. Malins. 1960. Fractionation of fats, oils, and waxes on thin layers of silicic acid. *J. Amer. Oil Chem. Soc.* **37:** 383–385.
6. Mangold, H. K., and R. Kammereck. 1962. New methods of analyzing industrial aliphatic lipids. *J. Amer. Oil Chem. Soc.* **39:** 201–206.
7. Graff, M. M., and E. L. Skau. 1943. Colored chromatograms with higher fatty acids. *Ind. Eng. Chem. Anal. Ed.* **15:** 340–341.
8. Kirchner, J. G., J. M. Miller, and G. J. Keller. 1951. Separation and identification of some terpenes by a new chromatographic technique. *Anal. Chem.* **23:** 420–425.
9. Johnson, D. F., R. D. Bennett, and E. Heftmann. 1963. Cholesterol in higher plants. *Science.* **140:** 198–199.
10. Bennett, R. D., and E. Heftmann. 1963. Devices for continuous development and sample application in preparative thin-layer chromatography. *J. Chromatogr.* **12:** 245–248.
11. Seher, A., and E. Homberg. 1968. Die Untersuchung von Sterin-Gemischen mit Hilfe der Dünnschicht-Chromatographie. *Fette Seifen Anstrichm.* **70:** 481–485.
12. Seher, A. 1969. Gemeinschaftsarbeiten der DGF, 52. Mitteilung. Neubearbeitung der "Einheitlichen Untersuchungsmethoden für die Fett- und Wachs-Industrie." XXXV: Analyse von Fettbegleitstoffen III. *Fette Seifen Anstrichm.* **71:** 833–837.
13. Kartnig, Th., and G. Mikula. 1969. Über die Verwendung von Magnesiumoxid als Sorptionsmittel in der Dünn-

schichtchromatographie von Pflanzeninhaltsstoffen. *Pharm. Zentralh.* **108:** 457–465.
14. Mahadevan, V., and W. O. Lundberg. 1962. Preparation of cholesterol esters of long-chain fatty acids and characterization of cholesteryl arachidonate. *J. Lipid Res.* **3:** 106–110.
15. Baumann, W. J., H. H. O. Schmid, H. W. Ulshöfer, and H. K. Mangold. 1967. Alkoxylipids. IV. Synthesis and characterization of naturally occurring ethers, esters and ether-esters of diols. *Biochim. Biophys. Acta.* **144:** 355–365.
16. Baumann, W. J., and H. K. Mangold. 1964. Reactions of aliphatic methanesulfonates. I. Syntheses of long-chain glyceryl-(1) ethers. *J. Org. Chem.* **29:** 3055–3057.
17. Baumann, W. J., and H. K. Mangold. 1966. Reactions of aliphatic methanesulfonates. II. Syntheses of long-chain di- and trialkyl glyceryl ethers. *J. Org. Chem.* **31:** 498–500.
18. Kramer, J. K. G., and H. K. Mangold. 1970. Preparation and characterization of neutral diol plasmalogens. *Chem. Phys. Lipids.* **4:** 332–344.
19. Gigg, J., and R. Gigg. 1968. The synthesis of neutral plasmalogens. *J. Chem. Soc. C.* 2030–2032.
20. Witter, R. F., G. V. Marinetti, A. Morrison, and L. Heicklin. 1957. Paper chromatography of phospholipids with solvent mixtures of ketones and acetic acid. *Arch. Biochem. Biophys.* **68:** 15–20.
21. Bergelson, L. D., V. A. Vaver, N. V. Prokazova, A. N. Ushakov, and G. A. Popkova. 1966. Diol lipids. *Biochim. Biophys. Acta.* **116:** 511–520.
22. Bergelson, L. D. 1969. Diol lipids. *Progr. Chem. Fats Other Lipids.* **10:** 239–286.
23. Carter, H. E., P. Johnson, D. W. Teets, and R. K. Yu. 1963. Isolation of ethylene glycol from the lipids of beef lung. *Biochem. Biophys. Res. Commun.* **13:** 156–161.
24. Calderon, M., and W. J. Baumann. 1970. Fractionation of neutral lipids on a lipophilic dextran gel. *Biochim. Biophys. Acta.* **210:** 7–14.
25. Takahashi, T., and H. H. O. Schmid. 1970. Long-chain alcohols in mammalian tissues. *Chem. Phys. Lipids.* **4:** 243–246.
26. Blank, M. L., and F. Snyder. 1970. Long-chain fatty alcohols in normal and neoplastic tissues. *Lipids.* **5:** 337–341.
27. Calderon, M., and W. J. Baumann. 1970. Gel permeation chromatography of neutral hydroxy lipids on Sephadex LH-20. *J. Lipid Res.* **11:** 167–169.
28. Snyder, F., and N. Stephens. 1959. A simplified spectrophotometric determination of ester groups in lipids. *Biochim. Biophys. Acta.* **34:** 244–245.
29. Chalvardjian, A. M. 1964. Fatty acids of brown and yellow fat in rats. *Biochem. J.* **90:** 518–521.