Journal of Chromatography, 123 (1976) 225–230

CHROM. 9124

Note

Gel chromatographic separation of retinol, retinyl esters and other fat-soluble vitamins

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(Received January 21st, 1976)

Adequate analytical data are the pre-requisite for achieving a reliable nutritional evaluation of synthetic foods which closely resemble natural foods. Chromatographic procedures have therefore been applied to the determination of vitamin A levels. Adsorption on alumina has been the most widely used chromatographic method for the separation and purification of vitamin A compounds. Other adsorbents such as silica gel, magnesia and dicalcium phosphate have also been used but to a lesser extent. After Pharmacia (Uppsala, Sweden) had developed a hydroxypropyl derivative of Sephadex with a gel matrix which enabled the separation to be performed in both polar and non-polar solvents, gel chromatography was used extensively for the study of non-polar substances[1–3].

Vitamins such as retinol, tocopherols, calciferols, ubiquinone and carotenes have been separated[4–11] by gel chromatography on Sephadex LH-20. Other commercial materials such as Styragel and bio-Beads S have been applied[12] to the analyses of lipophilic substances. Pokorný and his co-workers[13] separated the components of the unsaponifiable fractions of fats and oils containing α-tocopherol, β-carotene and retinol on the S-832 gel (developed by the Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia).

Vitamin A occurs in nature usually in the form of esters with the palmitate being predominant. Pharmaceutical preparations of vitamin A may contain alcohol or acetate. Since, both the absorptions and the biological activities of the individual vitamin forms differ[14,15], these forms must be distinguished in a study of the nutritional value of foodstuffs.

In this paper we report the separation of retinol, retinyl esters and other lipophilic vitamins by gel chromatography.

EXPERIMENTAL

Materials

Retinyl acetate, retinyl palmitate, ergocalciferol and cholecalciferol were purchased from Hoffmann La Roche, Basle, Switzerland, α- and β-carotene from Mann Labs., New York, N.Y., U.S.A. and α-tocopheryl acetate from E. Merck, Darmstadt, G.F.R. Standard preparations of retinol and α-tocopherol were obtained

by saponification of the corresponding esters. Their purities were tested by thin-layer chromatography (TLC) on alumina[16] and by UV spectroscopy.

Methods

The unsaponifiable fraction from commercial "Hera" margarine (fortified with 5000 I.U. of vitamin A and 40 I.U. of vitamin E per 250 g) was obtained using the light petroleum method[17]. The vitamins from pork liver were isolated both by the Czechoslovak Standard Method and by direct extraction with light petroleum without saponification[18].

Gel chromatography on Sephadex LH-20 with chloroform as elution agent was carried out in a glass column (60 × 2.5 cm) shielded from light at 18–20°. The flow-rate of the eluent was 1 ml/min. The elution of the vitamins was monitored by examing the ultraviolet, visible and infrared absorption of the eluates. For comparison, gel chromatography in five columns (0.8 × 120 cm) connected in series and packed with the S-832 gel (developed in Czechoslovakia) was carried out at room temperature using tetrahydrofuran elution. The flow-rate of the eluent was 35 ml/h. Detection was by means of a flow UV-analyzer (Development Works, Czechoslovak Academy of Sciences, Prague) with monochromatic light of wavelength 254 nm.

RESULTS AND DISCUSSION

The results of the gel chromatographic analyses of the standard preparations are summarized in Table I. Figs. 1 and 2 show the separation of retinol and its esters. It can be seen that complete separation of both esters (acetate and palmitate) was obtained only on S-832 gel, and that partial overlapping of the ester peaks occurred with Sephadex LH-20 columns under our experimental conditions. On the other hand, mixtures of retinol and retinyl acetate or palmitate were well separated on Sephadex LH-20, whereas the elution volumes of these substances were so similar on the S-832 gel that their separation did not take place. The elution sequence corresponds to the decreasing molecular masses of the eluted compounds. The good resolution of the retinol and retinyl ester peaks is due partly to the fact that substances with hydroxyl groups are retarded on Sephadex LH-20 columns during chloroform

TABLE I

GEL CHROMATOGRAPHY OF STANDARDS

Compound	V_e *(ml)*	
	Sephadex LH-20	*S-832*
Retinol	154	194
Retinyl acetate	98	197
Retinyl palmitate	88	167
α-Tocopherol	118	176
α-Tocopheryl acetate	91	177
Ergocalciferol	130	185
Cholecalciferol	125	185
α-Carotene	85	172
β-Carotene	85	172

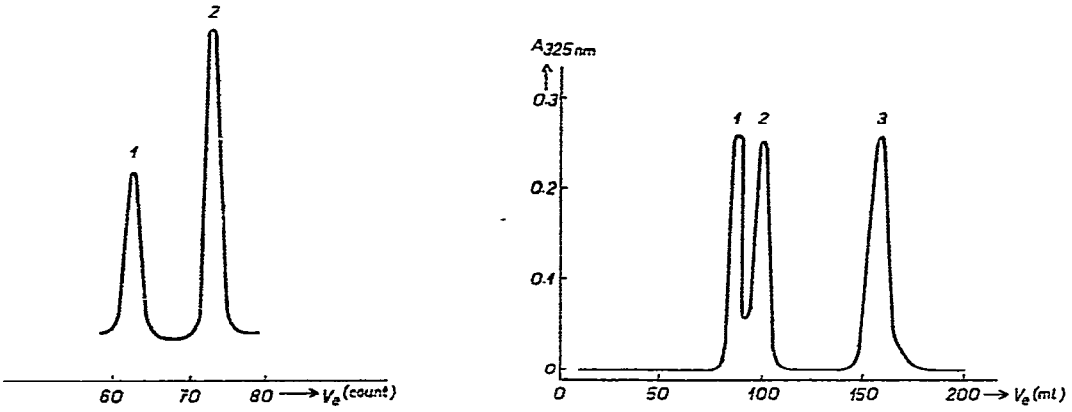

Fig. 1. Gel chromatogram of a standard mixture (S-832 gel, tetrahydrofuran elution, absorbance recorded with a UV detector at 254 nm, 1 count = 2.7 ml). 1 = Retinyl palmitate; 2 = retinyl acetate.

Fig. 2. Gel chromatogram of a standard mixture (Sephadex LH-20, chloroform elution, absorbance recorded at 325 nm). 1 = Retinyl palmitate; 2 = retinyl acetate; 3 = retinol.

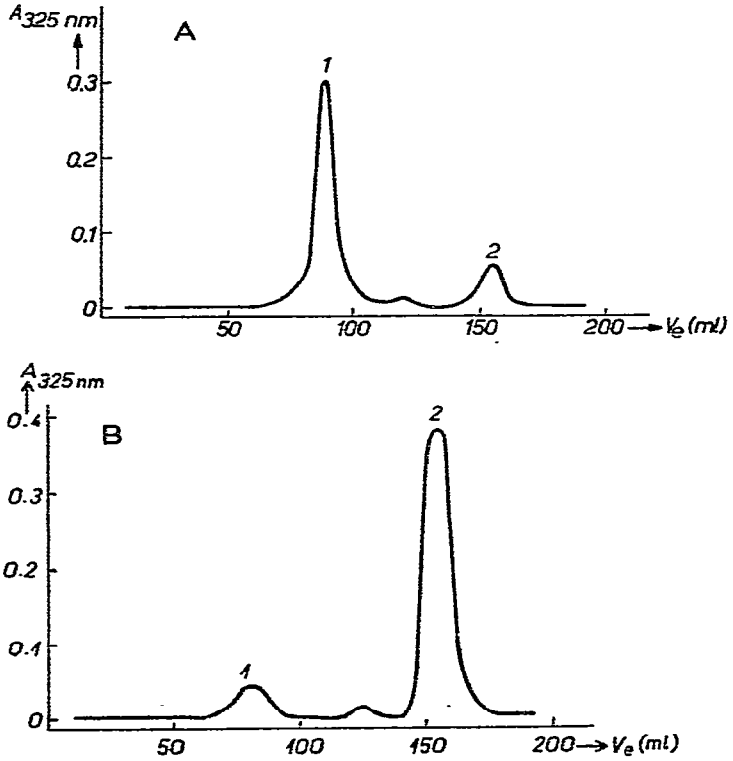

Fig. 3. Gel chromatogram of pork liver extract (Sephadex LH-20, chloroform elution, absorbance recorded at 325 nm). (A) Direct extract (1 = retinyl palmitate, 2 = retinol); (B) unsaponifiable fraction (1 = unidentified compound, 2 = retinol).

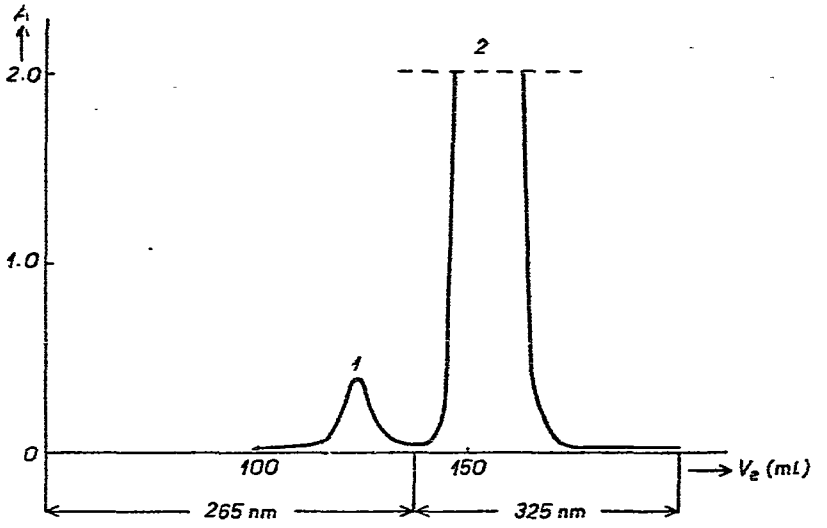

Fig. 4. Gel chromatogram of a standard mixture (Sephadex LH-20, chloroform elution, absorbance recorded at 265 and 325 nm). 1 = Ergocalciferol; 2 = retinol.

elution[12]. The chromatography on S-832 gel is less favourable because the hydroxyl groups of retinol are strongly associated with the solvent tetrahydrofuran[19,20]. The resulting associated compounds having higher molecular masses are characterized by much lower elution volumes than that of free retinol.

The gel chromatogram of vitamins from pork liver (Fig. 3A) shows the separation of retinol and retinyl palmitate in biological material. In agreement with literature[21,22], retinol and retinyl palmitate were found in the direct extract of pork liver. The release of retinol from its esters is revealed by the growth of peak 2 on the chromatogram of the unsaponifiable fraction (Fig. 3B).

Retinol, like calciferols, accumulates in the unsaponifiable fractions and causes interference with the spectrophotometric determination of calciferols. The colorimetric determination of calciferols is also affected by the presence of retinol. The similarity of the physical and chemical properties of these substances causes a distortion of the results. Gel chromatography in combination with adsorption and ion-exchange chromatography were applied to the separation of these substances by Ueda and coworkers[8–10]. We have succeeded in separating retinol from ergocalciferol (Fig. 4) on Sephadex LH-20. The mass ratio of retinol to ergocalciferol was *ca.* 10:1. Chromatography on S-832 gel was not suitable for this purpose under our experimental conditions.

Retinol, tocopherols and carotenes occur simultaneously in a series of materials. Their separation can be achieved by different chromatographic procedures. Gel chromatography on Sephadex LH-20 was described by Ueda *et al.*[7]. Fig. 5 shows our separation of a mixture of retinol, α-tocopherol and β-carotene on Sephadex LH-20 under the conditions mentioned above. The results in Table I suggest that the pairs retinol and α-tocopherol and retinol and carotenes are well separated on both gels, but that Sephadex LH-20 is more suitable for the separation of α-tocopherol and carotenes.

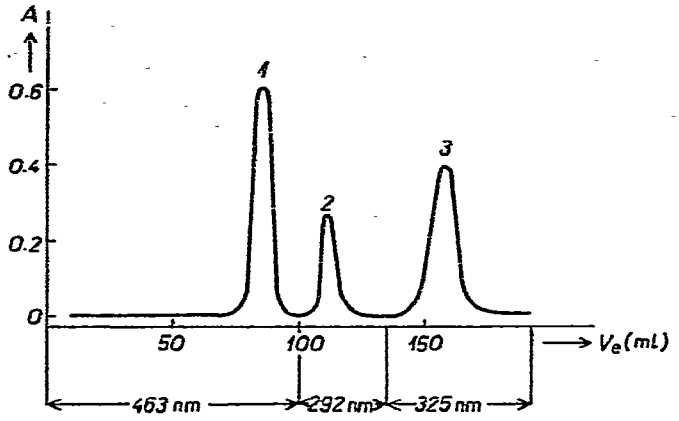

Fig. 5. Gel chromatogram of a standard mixture (Sephadex LH-20, chloroform elution, absorbance recorded at 292, 325 and 463 nm). 1 = β-Carotene; 2 = α-tocopherol; 3 = retinol.

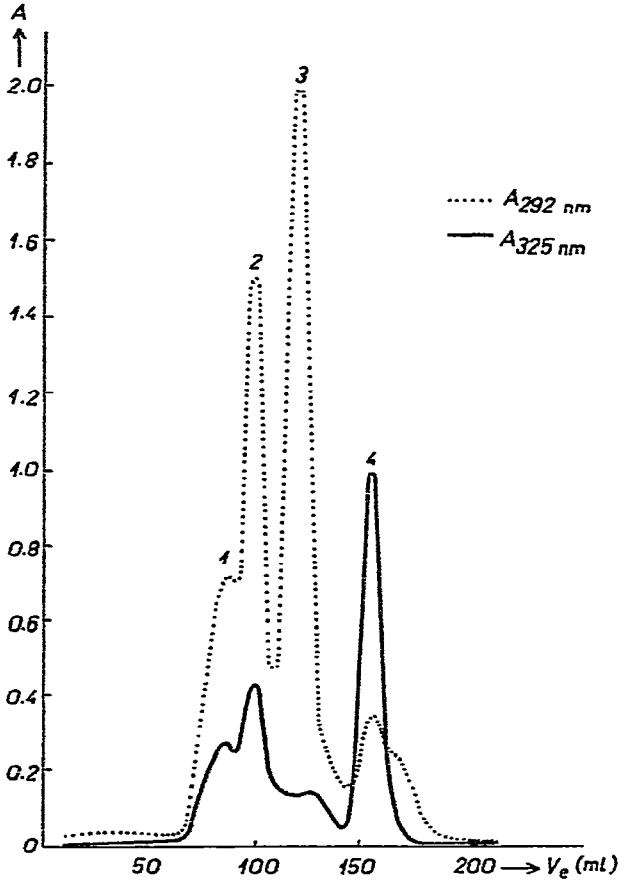

Fig. 6. Gel chromatogram of unsaponifiable fraction of "Hera" margarine (Sephadex LH-20, chloroform elution, absorbance recorded at 292 and 325 nm). 1 = Carotenes; 2 = unidentified compound; 3 = α-tocopherol; 4 = retinol.

Fig. 6 shows the separation of the unsaponifiable fraction of "Hera" margarine by gel chromatography on Sephadex LH-20. On the base of its elution volume and UV spectra, Peak 1 was assigned to α- or β-carotene (which are not separated). The substance corresponding to peak 2 is characterized by strong absorption in the UV region at 275 nm in chloroform. The infrared spectrum had an absorption band at *ca.* 1700 cm^{-1} corresponding to the presence of carbonyl groups in the molecule. Peak 3 was assigned to α-topopherol, peak 4 to retinol.

Our experiments thus show that gel chromatography on Sephadex LH-20 permits a good separation of biologically active forms of vitamin A, both in standard solutions and in biological materials. Satisfactory separation of retinol, α-tocopherol, calciferols and carotenes may also be achieved.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. J. Čoupek and Dr. S. Pokorný, Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, for the chromatographic analyses on S-832 gel.

REFERENCES

1 M. Joustra, B. Söderqvist and L. Fischer, *J. Chromatogr.*, 28 (1967) 21.
2 E. Nyström and J. Sjövall, *Anal. Biochem.*, 12 (1965) 235.
3 J. Ellingboe, E. Nyström and J. Sjövall, *J. Lipid Res.*, 11 (1970) 266.
4 Y. L. Ito, M. Zile, H. Ahrens and H. F. DeLuca, *J. Lipid Res.*, 15 (1974) 517.
5 M. N. Merzlyak, I. I. Ivanov and B. N. Tarusov, *Stud. Biophys.*, 30 (1972) 29; *C.A.*, 78 (1973) 25952k.
6 E. Nyström and J. Sjövall, *J. Chromatogr.*, 24 (1966) 212.
7 F. Ueda, T. Makito and Y. Toyohira, *Vitamins*, 40 (1969) 84.
8 F. Ueda, T. Makito and A. Kazama, *Vitamins*, 42 (1970) 99.
9 F. Ueda, T. Makito and A. Kazama, *Vitamins*, 42 (1970) 107.
10 F. Ueda, T. Makito, A. Kazama and K. Watanabe, *J. Vitaminol.*, 17 (1971) 142.
11 J. G. Bell, *Chem. Ind. (London)*, (1971) 201.
12 L. Fischer, *An Introduction to Gel Chromatography*, North-Holland, Amsterdam, 1969, p. 200.
13 S. Pokorný, J. Čoupek, N. T. Luân and J. Pokorný, *J. Chromatogr.*, 84 (1973) 319.
14 K. H. Westermann, *Int. J. Vitamin Res.*, 42 (1972) 104.
15 J. Fragner, *Vitamine, Chemie und Biochemie*, Vol. 1, Fischer, Jena, 2nd ed., 1964, p. 295.
16 J. Davídek and J. Blattná, *J. Chromatogr.*, 7 (1962) 204.
17 Czechoslovak Standard Method, ČSN 560053 (1971).
18 E. Knobloch, *Physikalisch-chemische Vitaminbestimmungsmethoden*, Akademie-Verlag, Berlin, 1963, p. 142.
19 D. Berek and D. Bakoš, *J. Chromatogr.*, 91 (1974) 237.
20 H. F. Hallam, in M. Davies (Editor), *Infrared Spectroscopy and Molecular Structure*, Elsevier, New York, 1963, p. 405.
21 J. Glover, T. W. Goodwin and R. A. Morton, *Biochem. J.*, 41 (1947) 97.
22 N. I. Krinski and J. Ganguly, *J. Biol. Chem.*, 202 (1953) 227.