*Journal of Chromatography*, 207 (1981) 457–463
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,518

## Note

# High-performance liquid chromatography of triterpenoids

JIANN-TSYH LIN, W. DAVID NES and ERICH HEFTMANN*

*Plant Physiology and Chemistry Research Unit, Western Regional Research Center, Science and Education Administration, U.S. Department of Agriculture, Berkeley, CA 94710 (U.S.A.)*

(Received November 24th, 1980)

While high-performance liquid chromatography (HPLC) has been applied extensively to steroids[1,2], HPLC of polycyclic triterpenoids has not been studied in detail, except for the soyasapogenols[3]. Gas–liquid chromatography (GLC) of tri- and tetrahydroxytriterpenoids frequently suffers from excessive retention times and sample degradation[4] and requires derivatization. Liquid column chromatography, having a greater load capacity than GLC, is more suitable for metabolic studies[5,6].

We have now developed a method for separating triterpenoids by HPLC without derivatization, which is based on both adsorption and reversed-phase partition chromatography. By combining these two HPLC systems, the identification of most of the metabolites of interest to us has become possible. In an analogous study of steroid metabolism in plants[7], we have used the combination of adsorption and reversed-phase partition chromatography to great advantage.

## EXPERIMENTAL*

The HPLC apparatus was assembled from commercially available components. The pump was of the single-piston reciprocating type, Altex Model 110A (Altex, Berkeley, CA, U.S.A.), and the sample injection valve was a Rheodyne Model 7125 (Rheodyne, Cotati, CA, U.S.A.) with a loop volume of 1 ml.

The adsorption column was a 250 × 4 mm I.D. stainless-steel chromatography tube (Altex), packed with Zorbax BP-SIL (7–8 $\mu$m; DuPont, Wilmington, DE, U.S.A.). The reversed-phase column had the same dimensions, but it was packed with Zorbax BP-ODS (7–8 $\mu$m; DuPont). The columns were packed in our laboratory. The packing method, detectors, recorder and solvents were as previously described[2]. The chromatographic conditions are given in the figure legends. A Hitachi Model 100-30 spectrophotometer (Hitachi, Tokyo, Japan) was used to determine the absorption maxima.

* Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

0021-9673/81/0000–0000/$02.50 

## RESULTS AND DISCUSSION

Our results are shown in Figs. 1–5. Because the triterpenoids containing carbon–carbon double bonds have a $\lambda_{max.}$ near 202 nm, we used that wavelength for the detection of unsaturated triterpenoids. Ketonic triterpenoids, such as friedelin and lupeone, and conjugated double bond triterpenoids, such as 24-dihydroagnosterol, can also be detected at 202 nm. Although saturated monohydroxytriterpenoids, such as friedelanol and tetrahymanol can be detected by the ultraviolet (UV) detector at 202 nm and by the refractive index (RI) detector, the sensitivities of both are low.

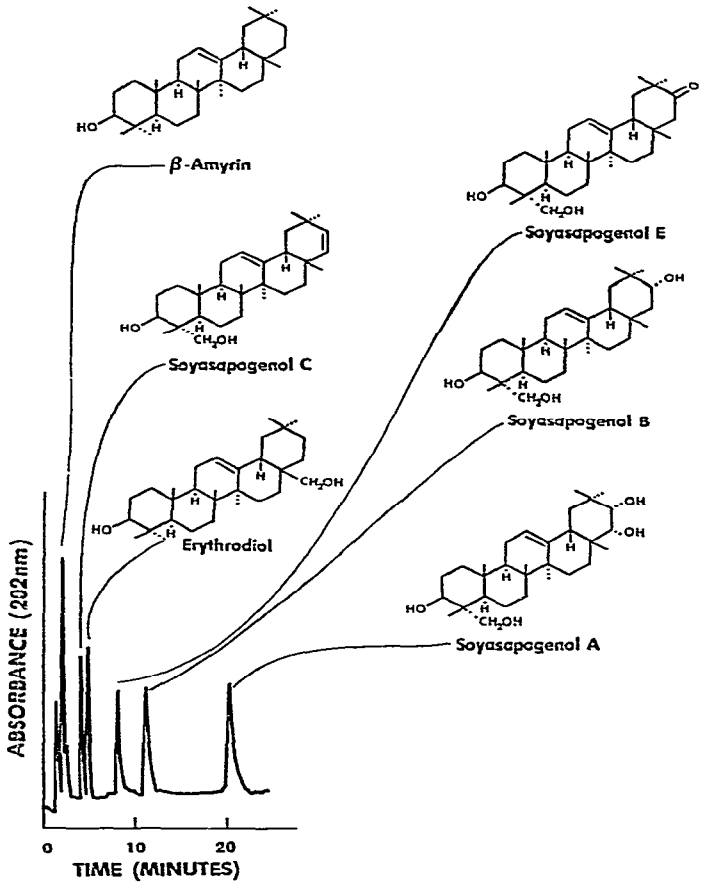


Fig. 1. Adsorption chromatogram of β-amyrin analogs. Between 2 μg (β-amyrin) and 5 μg (soyasapogenol A) of the triterpenoids, dissolved in about 200 μl of the eluent, were chromatographed on a column of Zorbax BP-SIL, 250 × 4 mm I.D. Eluent, ethanol–hexane (4:96); flow-rate, 2 ml/min; pressure, 500 p.s.i.; detector, 202 nm, range 0.1. Recorder speed, 12 cm/h, span 10 mV.

Adsorption chromatography with 4% ethanol in hexane as the eluent (Fig. 1) completely separated the β-amyrin analogues in the order of elution: β-amyrin, soyasapogenol C, erythrodiol, soyasapogenol E, soyasapogenol B, and soyasapogenol A. This is the same order as that previously obtained with a different adsorption

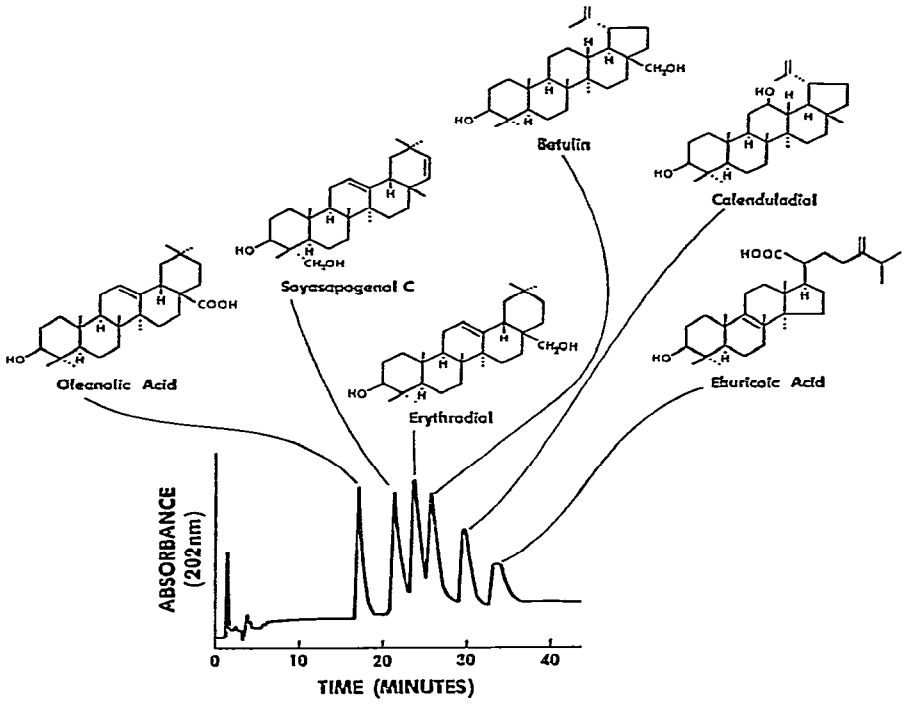


Fig. 2. Adsorption chromatogram of dihydroxy and monohydroxy monocarboxy triterpenoids. For conditions, see Fig. 1, except 5 $\mu$g of each triterpenoid and an eluent of ethanol–hexane (1:99) were used.

system[3]. It depends not only on the number of hydroxyl groups, but also on their locations. Thus, erythrodiol is more polar than soyasapogenol C, where the two hydroxyl groups are closer together. Other monohydroxy and monoketonic triterpenoids are eluted in the region of $\beta$-amyrin, but this group can be fractionated by the use of a lower alcohol concentration in the eluent (*cf.* Fig. 3). Similarly, other dihydroxytriterpenoids and monohydroxy carboxylic acids are eluted in the region of soyasapogenol C and erythrodiol, but this group can be fractionated by elution with an alcohol concentration intermediate between that used in Figs. 1 and 3 (*cf.* Fig. 2).

Fig. 2 shows the fractionation of dihydroxytriterpenoids and monohydroxy carboxylic acids. Oleanolic acid is now completely separated from soyasapogenol C and erythrodiol. The pair of position isomers, betulin and calenduladiol, is likewise completely resolved. Eburicoic acid is much more polar than oleanolic acid, which, having a shielded carboxyl group, is less polar than the dihydroxy compounds. Ursolic acid (Fig. 6), which differs from oleanolic acid only in the placement of one methyl group, cannot be separated from it in this system.

The least polar eluent fractionates the group of monoketonic and monohydroxytriterpenoids (Fig. 3). For adequate peaks with either the UV or the RI detector, 100 $\mu$g of the fully saturated triterpenoids had to be used. Euphol (Fig. 5) was eluted just slightly ahead of cycloartenol, but 24-dihydrolanosterol did not sepa-

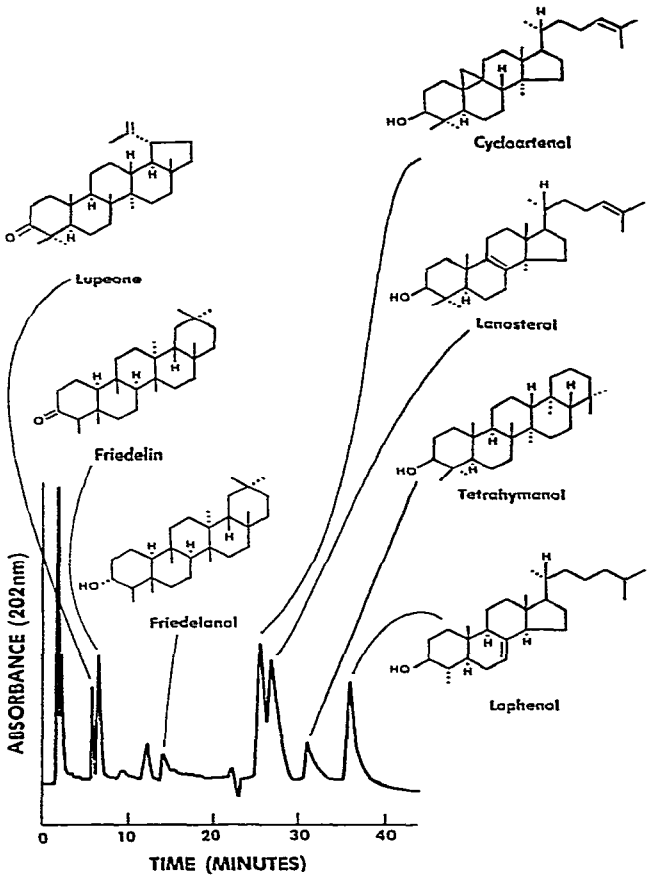


Fig. 3. Adsorption chromatogram of monoketonic and monohydroxytriterpenoids. For conditions, see Fig. 1, except 5 $\mu$g of each, lupeone, cycloartenol, lanosterol, and lophenol; 10 $\mu$g friedelin; and 100 $\mu$g of each, friedelanol and tetrahymanol; and an eluent of ethanol–hexane (0.15:99.85) were used and the range was 0.2.

rate from cycloartenol. Similarly, germanicol (Fig. 6), lupeol (Fig. 5), $\alpha$-amyrin (Fig. 6), $\beta$-amyrin (Fig. 1), and 24-dihydroagnosterol (Fig. 6) were eluted together with lanosterol. Thus, the ketones were eluted before the alcohols, but the resolution of the monohydroxytriterpenoid group was generally unsatisfactory.

For reversed-phase partition chromatography the triterpenoids were divided into a more polar and a less polar group. Fig. 4 shows the elution pattern of the more polar triterpenoids with 80% aqueous methanol containing a trace of formic acid to repress the ionization and trailing of the carboxylic acid. The peaks for eburicoic acid (retention time 83 min) and soyasapogenol C (retention time beyond 3 h) are not shown. The elution was not exactly in the reverse order of that in adsorption chromatography (Figs. 1 and 2). Thus, triterpenoids not separated by adsorption chromatography may separate in reversed-phase partition chromatography and *vice versa*. For instance, calenduladiol was only partially separated from soyasapogenol E and

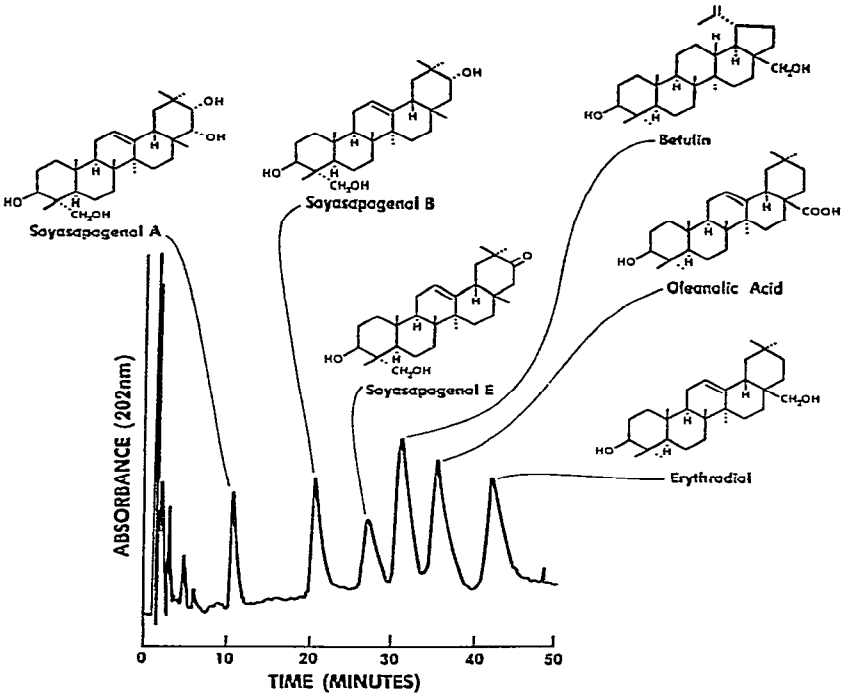


Fig. 4. Reversed-phase partition chromatogram of the more polar triterpenoids. Three $\mu$g of soyasapogenol A and 6 $\mu$g of each of the other triterpenoids, dissolved in 50 $\mu$l methanol were chromatographed on a column of Zorbax BP-ODS, 250 × 4 mm I.D. Eluent, methanol–water (80:20) containing 0.05% of 88% formic acid; flow-rate; 2 ml/min; pressure, 2000 p.s.i.; detector, 202 nm, range 0.1. Recorder speed 12 cm/h, span 10 mV.

betulin by reversed-phase partition chromatography (Fig. 4), but these compounds separated completely in the adsorption system (Fig. 2). Conversely, ursolic acid and oleanolic acid, which could not be separated by adsorption chromatography, were at least partially separated by reversed-phase partition chromatography. Ursolic acid was eluted just slightly behind oleanolic acid.

Reversed-phase partition chromatography was particularly useful for separating the 3-hydroxy-4,4-dimethyltriterpenoids (Fig. 5), which could not be well separated by adsorption chromatography (Fig. 3). On the other hand, friedelin, friedelanol, and tetrahymanol could not be adequately separated by the reversed-phase partition system (Fig. 5), but these triterpenoids were completely separated by adsorption chromatography (Fig. 3). Germanicol (Fig. 6) was eluted just slightly ahead of lanosterol by reversed-phase partition chromatography. Other triterpenoids not separated by reversed-phase partition chromatography were lupeone (Fig. 3) and euphol; $\beta$-amyrin (Fig. 1), 24-dihydroagnosterol (Fig. 6), and lanosterol; $\alpha$-amyrin (Fig. 6) and cycloartenol; and tetrahymanol (Fig. 3) and friedelanol (Fig. 3). Thus, in general, the two chromatographic principles complement each other, as shown previously[2].

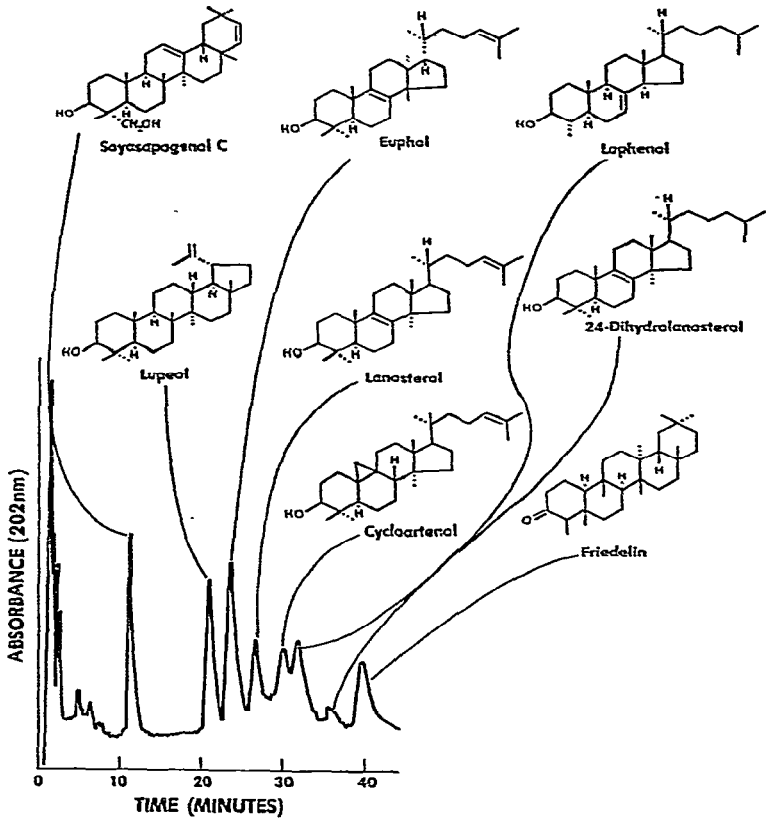


Fig. 5. Reversed-phase partition chromatogram of the less polar triterpenoids. For conditions see Fig. 4, except 15 $\mu$g of friedelin and 5 $\mu$g of each of the other triterpenoids, and an eluent of methanol–water (96:4) were used and the pressure was 1000 p.s.i.

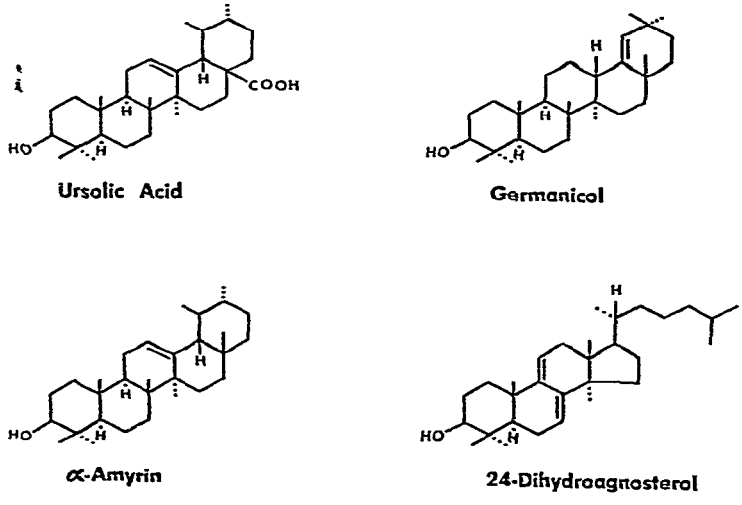


Fig. 6. Additional triterpenoid structures.

ACKNOWLEDGEMENTS

Gifts of reference compounds from W. R. Nes, H. W. Kircher, M. J. Thompson and A. N. Starratt are gratefully acknowledged.

This work was supported, in part, by Grant No. I-84-79 from the United States–Israel Agricultural Research and Development Fund (BARD).

REFERENCES

1 E. Heftmann and I. R. Hunter, *J. Chromatogr.*, 165 (1979) 283.
2 J.-T. Lin, E. Heftmann and I. R. Hunter, *J. Chromatogr.*, 190 (1980) 169.
3 E. Heftmann, R. E. Lundin, W. F. Haddon, I. Peri, U. Mor and A. Bondi, *J. Nat. Prod.*, 42 (1979) 410.
4 N. Ikekawa, *Meth. Enzymol.*, 15 (1969) 200.
5 I. Peri, U. Mor, E. Heftmann, A. Bondi and Y. Tencer, *Phytochemistry*, 18 (1979) 1961.
6 W. D. Nes, G. W. Patterson and G. A. Bean, *Plant Physiol.*, 66 (1980) 1008.
7 J. T. Lin and E. Heftmann, *Phytochemistry*, in press.