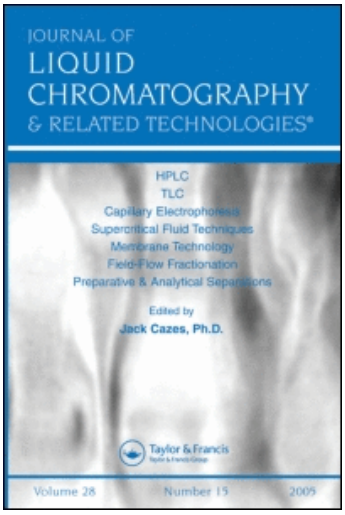

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Selected Uses of HPLC for the Separation of Natural Products

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SELECTED USES OF HPLC FOR THE SEPARATION OF NATURAL PRODUCTS

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INTRODUCTION

High pressure liquid chromatography (HPLC) has proven to be one of the most useful techniques available to the chemist for separating complex mixtures of organic substances. The range of compounds that have been successfully separated by HPLC continues to expand at an extremely rapid rate. Within the last five years, HPLC has been applied to increasingly complex separations in the field of natural products chemistry. This article reviews a number of separations in selected areas of natural products chemistry. While it is not an exhaustive survey of the literature, it does indicate the variety of separations already achieved and hopefully will suggest additional areas of utility.

Retinoids and Vitamin A Derivatives

For several years, research in our laboratories has been directed toward understanding various aspects of the vision process. During the course of this work, it has been necessary to synthesize derivatives and isomers of retinal (Vitamin A aldehyde) and to separate the various mixtures of isomers encountered during the synthesis. HPLC has been extensively used for these separations, some of the more interesting of which will be described in this review.

Vitamin A and its various derivatives are known to be quite labile, isomerizing and/or decomposing upon exposure to light or warming. HPLC offers many advantages over open column chromatography or thin layer chromatography (TLC) for isomer separations; these include speed, efficiency and low chance of contact with air and light during the separation process.

The first separations of retinal isomers conducted in our laboratory were performed using a Corasil-II (Waters) column with ether-hexane elution (1). Using this system, the five isomers of retinal (13-cis, 9,13-dicis, 11-cis, 9-cis and all-trans) could be separated without recycling (Fig. 1).

Using HPLC in the recycle mode, it has been possible to separate various isomers of sterically modified retinal analogs (2). By use of Corasil-II and a solvent system of hexane containing from 1 to 6% ether, it was possible to make a separation of 13-cis-, 9,13-dicis-, 11-cis-, 9-cis- and all-trans-14-methyl retinal, using the recycle mode (Fig. 2).

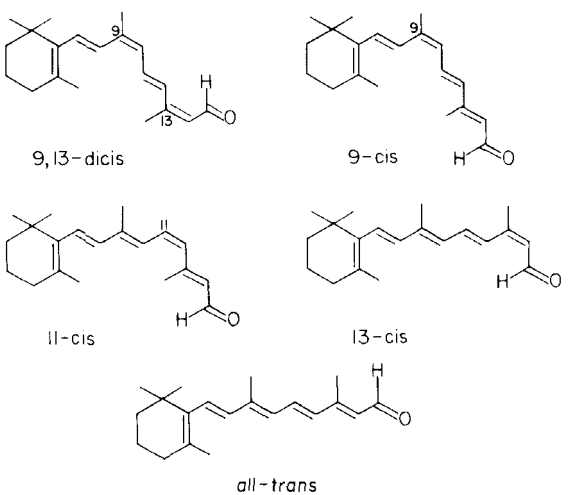


FIGURE 1. Structures of retinals.

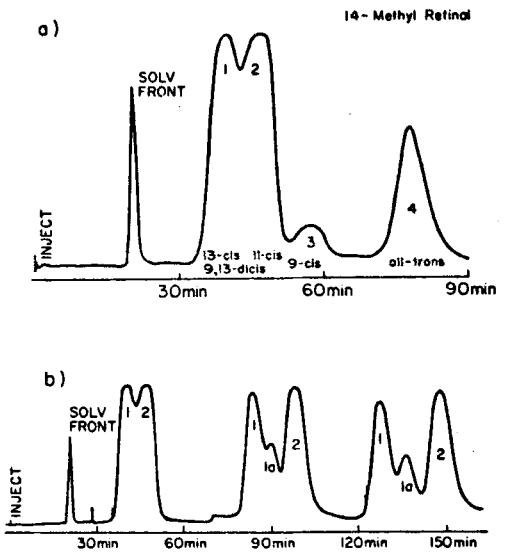


FIGURE 2. High pressure liquid chromatography of (a) irradiated all-trans-14-methylretinal, Corasil II, 3 ft x 3 (prep); 3 ml/min; 1.5% ether in hexane; (b) recycling of bands 1 and 2: (1) 13-cis-; (1a) 9,13-dicis-; (2) 11-cis-; (3) 9-cis-; and (4) all-trans-14-methylretinal.

With the advent of microparticulate silica columns it has been possible, for the most part, to eliminate the recycle step, which is time-consuming and does not always produce the best separations. Using columns and solvent systems such as Zorbax-SIL (12% ether in hexane) (3) and 10μm-Partisil (methylene chloride-acetic acid, 99.5:0.5) (4), the various retinoid isomers could be separated.

In our work with natural and artificial visual pigments, it has been crucial to identify and quantify the retinal moiety (chromophore) in the particular isomeric form in which it is bound to the apoprotein opsin. The procedure involves detachment of the

chromophore and characterization of the retinals by HPLC on silica gel columns (5,6,7). As far as resolution is concerned, silica gel is satisfactory. However, the disadvantage is the rapid deterioration of expensive columns since the hydrophobic opsins invariably require lipids or detergents for solubilization. A method for the HPLC determination of these retinals obtained from opsins has recently been developed in our laboratory (8). This method is based on the usage of a bonded phase column packing with nitrile groups; the column is compatible with both normal and reverse phase usage, an essential feature of this process. In summary, the process involves obtaining the retinal isomer contaminated with detergent. This mixture is applied to a μ-Bondapak-CN (Waters) column which is operated in the normal phase sense (1% ether in hexane), see Fig. 3.

If necessary, baseline resolution of the all-trans and 9-cis retinals can be produced by increasing the column length. This procedure permits quantification of the ratio of retinal isomers by dividing the integrated areas of the HPLC peaks by the respective ε values of the chromophores. The HPLC column is usable several times without removal of detergent. For column regeneration, the detergent was eluted by switching from normal to reverse phase mode and washing successively with chloroform, methanol and water followed by methanol and chloroform and finally, re-equilibration with ether-hexane. This regeneration is not possible with silica gel columns dues to incompatibility with water.

Normal phase HPLC (μ-Porasil, Waters, 10% ether in hexane) has also been used to separate all-trans-11,12-dihydroretinal from its other geometric isomers (Fig. 4).

Reverse phase HPLC has proven useful for separation of the more polar derivatives of retinal. Use of a Vydac 10μm ODS reverse phase column gave fast separation of all-trans and 13-cis retinoic acid (Vitamin A acid) in various food products. In comparison, a Zorbax column was reported to give better separation, but the separation time was longer (9).

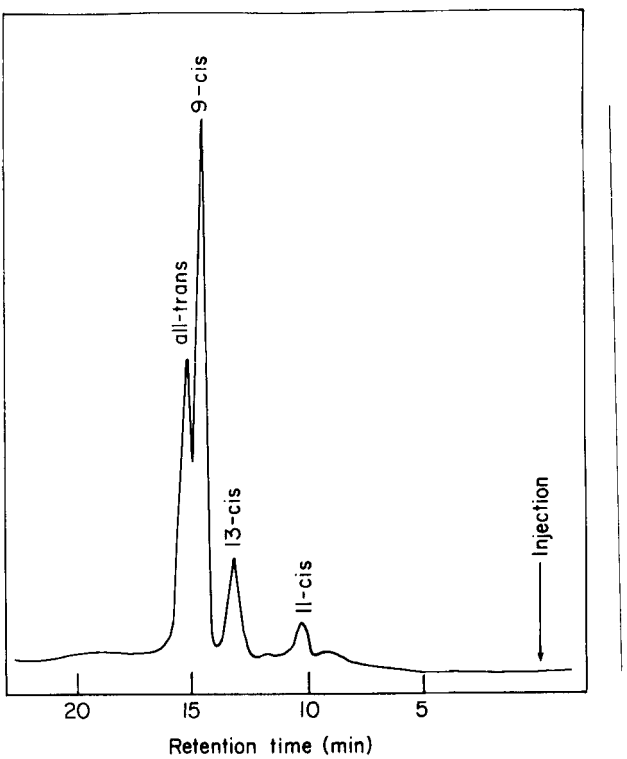


FIGURE 3. Elution of 11-cis, 13-cis, 9-cis and all-trans retinals on a μ-Bondapak-CN column, 4 mm i.d. x 30 cm; flow rate 1.0 ml/min; pressure 600 psi; solvent, 1% ether in hexane; Waters Model 6000 pump, Schoeffel variable u.v. detector, λ = 350 nm. Samples were injected under dim red light.

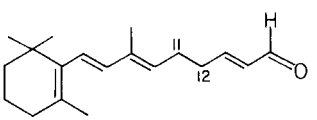


FIGURE 4. 11,12-dihydroretinal.

The need for a good quantitative assay for polar retinoids has recently developed. Retinoic acid has been found to prevent development of epithelial cancer in certain instances (10). Thus, all-trans retinoic acid, a natural metabolite of retinal, has been useful in the prevention of epithelial cancer but unfortunately it is toxic to humans. The isomeric 13-cis retinoic acid is known to be less toxic than all-trans but few metabolic data were known for this compound. A quantitative assay for 13-cis and all-trans retinoic acid in plasma was therefore developed (11).

The method involves lyophilization of the plasma sample, extraction of the residue with methanol and separation of the retinoids on a Partisil-10-ODS-2 (Whatman) column using acetonitrile-10% ammonium acetate (65:35) as the mobile phase. Under these conditions the two retinoic acid isomers were completely resolved. It was possible to detect as little as 0.05μg of either compound by this method (using ultraviolet detection). Similar results were obtained using a 5μm-Spherisorb-ODS (Spectra-Physics) column and acetonitrile-1% ammonium acetate (60:40) as the mobile phase. With this system, a mixture of retinoic acid, retinol, retinal and retinyl acetate could be completely separated. The small amount of ammonium acetate was added to suppress broadening of the retinoic acid peak (12). The advantage of a reverse phase column for these analyses is that it is water-insensitive, allowing an organic solvent extract of tissue to be directly applied to the column.

In a related study, mixtures of retinoic acid, retinol and retinyl palmitate and mixtures of all-trans and 13-cis retinoic acid were separated by a μ-Bondapak-C_{18} column using methanol-0.01M aqueous ammonium acetate (75:25) (13). Complete separation was achieved with 90-95% material recovery from the column. This system allowed detection of as little as 1 ng of retinoic acid.

On a μ-Porasil column, the same authors demonstrated that the methyl esters of retinoic acid were fully resolved using a

THF-hexane (7.5:92.5) solvent system. Recovery from the column was better than 95% (13).

A serious drawback in HPLC is the fact that although it is ideal for micro-scale separations, the maxinum quantity that can be handled by a single injection is usually less than 100 mg. In our laboratories, we have applied "prep scale" HPLC to selected difficult separations in the retinoid field in the range of 10 mg to 15 g by usage of commercial and homemade columns (14).

The C_{18} ketone (15) is a key intermediate in the synthesis of vitamin A, retinal and various retinoids. The all-trans and 9-cis isomers, 1 and 2 in Fig. 5, are obtained by synthesis from β-ionone. Separation of 1 and 2, which is not possible by classical methods, can be readily achieved by prep HPLC (Fig. 5). In this example, an 820 mg mixture of the two isomers could be separated in less than 20 min, the shaded areas in Fig. 5 correspond to greater than 99% purity.

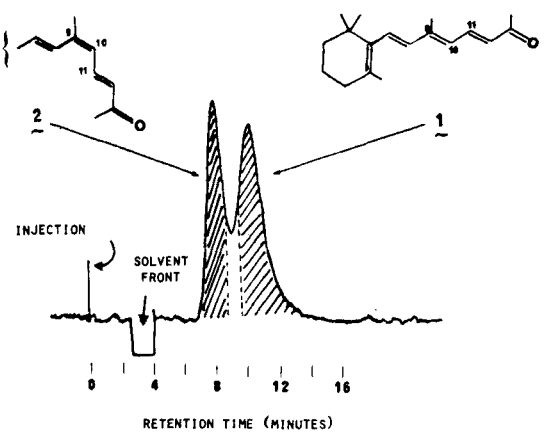


FIGURE 5. Separation of an 820 mg mixture of all-trans 1 and 9-cis 2 C-18 ketones on a Waters Prep LC-500, Refractive index detector. 30 x 5 cm silica gel column, solvent = 11% ether in hexane; flow rate = 250 ml/min.

In our vision studies, a major difficulty is in the securing of 10 mg of pure double bond isomers of retinoids for studies related to chromophore-protein (opsin) binding and visual pigment formation. The separation of retinals in the crucial 5-100 mg range, which are the average amounts obtainable in synthesis, could be achieved, however, by modifying a Waters Prep-LC 500 so that it could be equipped with a uv detector, since mixtures less than 100 mg cannot usually be seen by the refractive index detector. A complete separation of a 32 mg mixture of all-trans retinal 3 and its 13-cis isomer 4 was achieved in less than 30 min (Fig. 6) with quantitative recovery of the isomers. The inserts show analytical HPLC traces of the samples before and after prep-LC.

Xanthones and Flavones

Much work in recent years has been directed to isolation and structure elucidation of natural products having biological or medicinal activity. High pressure liquid chromatography has proved to be an effective tool for isolation, purification and

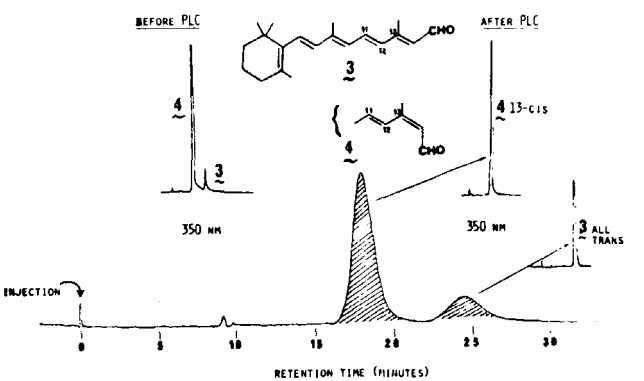


FIGURE 6. Waters Prep LC-500 modified with a JASCO UVIDEC-100 variable wavelength u.v. detector λ = 254, A = 2.56, 30 x 5 cm silica gel column, solvent = 8% ether in hexane, flow rate = 200 ml/min (analytical conditions: 30 cm x 4 mm μ-Porasil column, same solvent, flow rate = 2 ml/min, λ = 350).

analysis of naturally occurring substances with high molecular weights and/or sensitive functionalities.

Certain classes of natural products occur as isomeric mixtures, either as geometric or positional isomers. A particularly interesting group of compounds with this property is that of the xanthone glycosides, substances with interesting pharmacological activity (as antipsychotics, monoamine oxidase inhibitors and antituberculosis agents) (16).

Xanthone glycosides occur in nature as C-glycosides or O-glycosides (17). The O-glycosides contain a normal glycosidic linkage and differ from one another in their oxidation pattern, the relative number of free hydroxyl and methoxyl groups, and the nature and linkage site of the sugars.

These compounds are traditionally resolved on TLC or by column chromatography on polyamide; however, these techniques give poor resolution of isomeric glycosides.

The separation of various tetramethoxyxanthones (no sugar moiety) has been reported (18). The separation of five isomeric xanthones (Fig. 7) was attempted first using a μ-Porasil column, with no success. However, use of a μ-Bondapak-CN column with hexane-chloroform (17:7) as the eluant gave good separation. Xanthones having a free hydroxyl group instead of a methyl ether could

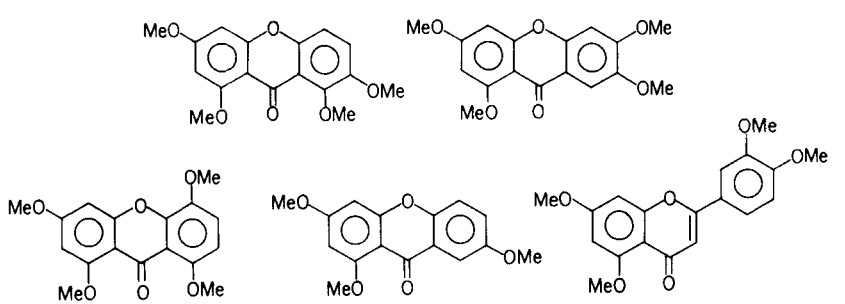
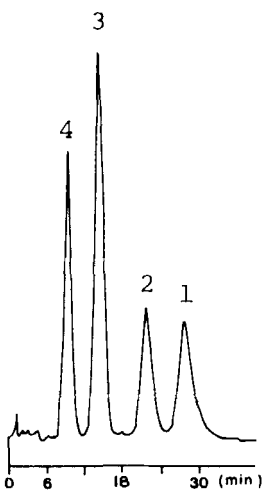


FIGURE 7. Structures of xanthones and a flavone.

be separated on a μ-Bondapak-CN column using isooctane-chloroform (3:17). These results represented a clear improvement over TLC separation methods, which gave poor results.

Attempts to use the previously described HPLC system for separation of more polar positional aglycone isomers or glycosides were unsuccessful (19). Various investigators had reported the use of reverse-phase columns for separations of structurally similar flavonoid glycosides (20-24), (vide infra) and a good separation system for isomeric xanthone glycosides has been developed (19).

Using a μ-Bondapak-C_{18} column with methanol-water (2:3) as the mobile phase, various xanthone glycosides could be separated, as shown in Fig. 8. By using methanol-water (45:55), baseline



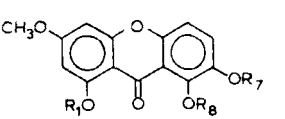


FIGURE 8. Separation of various xanthone glycosides (1 - 4 on μ-Bondapak-C-18. Mobile phase, methanol-water (2:3); flow rate 1.5 ml/min; pressure, 2200 psi; detection, u.v. at 260 nm. 1: R_1 = R_7 = H, R_8 = primeverosyl; swertianin-8-O-primeveroside. 2: R_1 = R_7 = H, R_8 = glucosyl; swertianin-8-O-glucoside. 3: R_1 = primeverosyl, R_7 = H, R_8 = CH_3; gentiacaulein-1-O-primeveroside. 4: R_1 = primeverosyl, R_7 = R_8 = CH_3; decussatin-1-O-primeveroside. [primeverose = 6-O-(β-D-xylopyranosyl)-β-D-glucopyranose].

separation of the two disaccharide isomers, 1 and 2 in Fig. 8, could be obtained. These compounds differ only in the positions of attachment of the hydrolysable sugars.

The separation of gentisin and isogentisin, two compounds of interest to chemotaxonomists, was performed on the μ-Bondapak-C_{18} column with 1:1 water-methanol as solvent (Fig. 9). Increasing the amount of water in the mobile phase gave better resolution, but the separation time was longer (Fig. 9b).

It appears that C_{18} reverse phase columns may be used to advantage for the separation of naturally occurring xanthone glycosides. As was mentioned above, this system has also been used for separation of flavone glycosides.

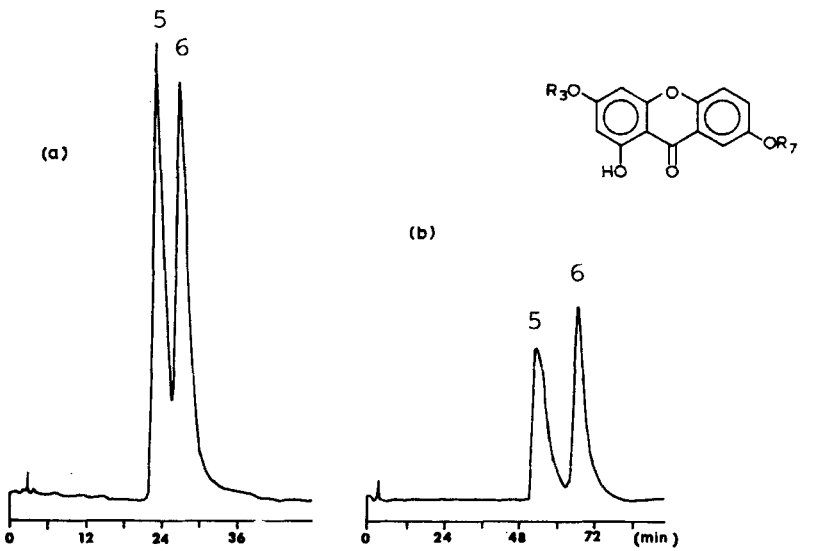


FIGURE 9. (a) Separation of isogentisin (5) and gentisin (6). Mobile phase, methanol-water (1:1); flow rate 1 ml/min. (b) As (a), except that the solvent is methanol-water (2:3). Pressure, 1600 psi. 5: R_3 = H, R_7 = CH_3; isogentisin. 6: R_3 = CH_3, R_7 = H; gentisin.

Partial resolution of isovitexin (Fig. 10a) and vitexin (Fig. 10b) was achieved on a Zorbax-ODS column using a gradient elution scheme with ethanol-water containing 0.1M phosphoric acid (25).

A slightly different system has given good separation results with the positional isomers of a flavanone, Fig. 11 (26). Using a LiChrosorb-NH_2 (Merck) column and gradient elution (acetonitrile-water, from 1:9 to 9:1 in 15 min), isomers a and b were completely resolved.

A practical application of the reverse-phase separation technique can be seen in an analysis of isoflavones in soybeans (27). In this example, the positional isomers genistein and 4',6,7-trihydroxyflavone (Fig. 12) were separated in water-acetonitrile (4:1) on a Partisil-10-ODS column.

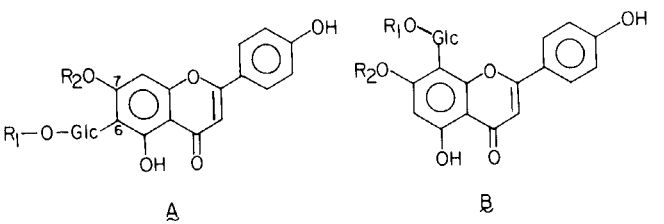


FIGURE 10. Isovitexin (A) and Vitexin (B).

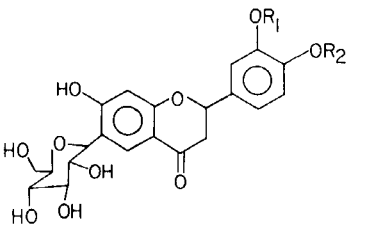


FIGURE 11. a: R_1 = β-D-glucosyl, R_2 = H; b: R_1 = H, R_2 = β-D-glucosyl.

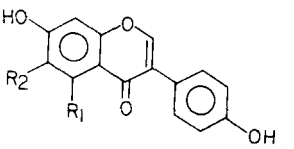


FIGURE 12. a: R_1 = OH, R_2 = H; genistein. b: R_1 = H, R_2 = OH; 4',6,7-trihydroxyisoflavone.

Pheromones and Pheromone Precursors

Many insect pheromones consist of structures having double bonds of specific geometry. The synthesis of these substances often results in mixtures of double bond isomers and, if the synthesis is to yield a useful product, a means of separating the isomers must be found.

A technique useful with these compounds involves use of silver ion impregnated HPLC columns (see also the section on prostaglandins in this article).

Some time ago, a method was developed for the separation of geometrical isomers of unsaturated acetates, aldehydes and hydrocarbons on silver nitrate coated silica gel HPLC columns (28). In this case it was noted that contamination of an insect sex pheromone with its double bond isomers would reduce or eliminate the activity of the pheromone. Essentially a purity exceeding 99.5% was needed for proper activity testing.

The separation system consisted of an Adsorbosil-2-ADN (Applied Science) column which had been treated with a 20% solution of silver nitrate. With benzene as eluant, complete separation of E and Z-9-dodecen-1-ol acetate and complete separation of a mixture of (E,E)-, (Z,E)-, and (Z,Z)-3, 13-octadecadien-1-ol acetate was obtained.

Other column packings, such as Porasil-A-60 (Waters), Biosil-A (Bio-Rad) and Partisil-20 (Reeve-Angel) were treated with silver nitrate and all gave similar results.

Using a silver-loaded Nucleosil-10SA (Macherey, Nagel) column cooled to 7°C, a mixture of tetradecan-1-ol acetate, trans-9-tetradecen-1-ol acetate, cis-11-tetradecen-1-ol acetate, trans-4-cis-7-tridecadien-1-ol acetate and cis-4-trans-7-cis-10-tridecatrien-1-ol acetate was separated. In this case the eluant was methanol (29).

Under identical conditions, a series of methyl esters including trans-9-octadecenoate, methyl cis-9-octadecenoate and methyl cis-9-cis-12-octadecadienoate were separated. Methyl-cis-6-cis-9-cis-12-octadecatrienoate was retained on the column.

It seems in this case that the lowered column temperature helps to improve the resolution. Trienes were so strongly π-bound to the column that they gave extremely broad bands; in some cases they did not elute at all.

The use of preparative scale HPLC has been helpful in the field of pheromone synthesis (14). During the course of synthesis of an insect pheromone it became necessary to separate a mixture of cis and trans-10 (Fig. 13). Use of the only commercially available cartridge (silica gel) for the Waters Prep-LC 500 instrument gave poor separation (Fig. 13a) of this mixture. A new cartridge was prepared by emptying a commercial one, impregnating its silica gel particles with 10% silver nitrate, refilling by dry-packing, and finally refitting the cartridge with the end frits. The success of this modification is illustrated in Fig. 13b in which a far better separation is achieved in significantly less time with a shorter column length.

Sesquiterpenes and Triterpenes

Pentacyclic triterpenes from a commercial extract of Euphorbaeca species were separated with a silver-ion loaded silica column (30). Lupeol (exo $\Delta^{28(29)}$ double bond) was separated from α- and β-amyrin (trisubstituted endo double bond) (Fig. 14).

Standard methods of gas chromatographic analysis failed to separate mixtures of germanicol and β-amyrin or lupeol and α-amyrin (Fig. 14). Chromatography on a Zorbax ODS column with 10%

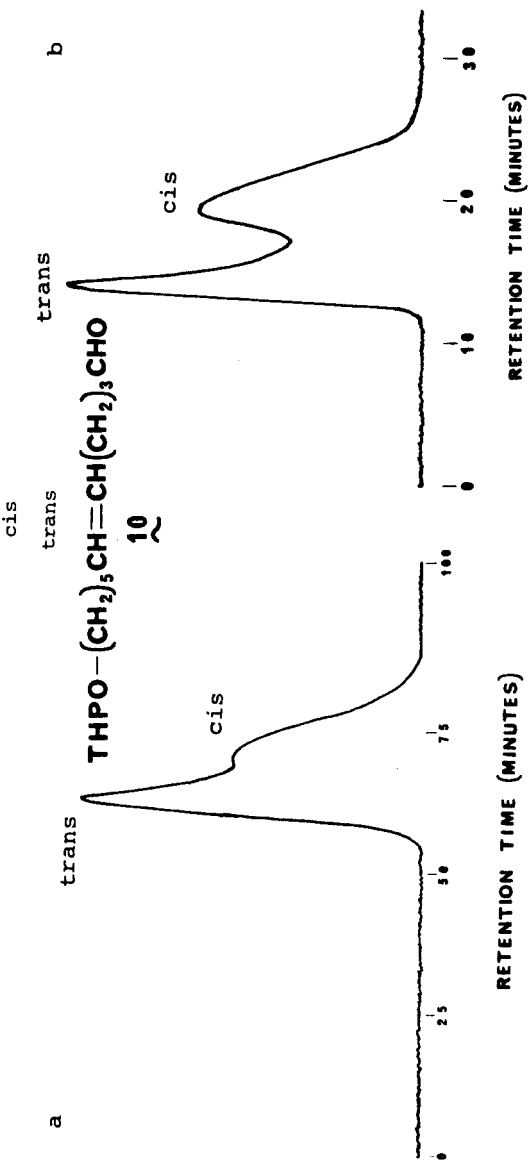


FIGURE 13. Separation of a 450 mg mixture of cis and trans 10, RI detector; solvent = 11% ether in hexane. (a) Two 30 cm x 5 cm silica gel columns; flow rate = 150 ml/min. (b) One prepared 10% $AgNO_3$ on silica gel column; flow rate = 100 ml/min.

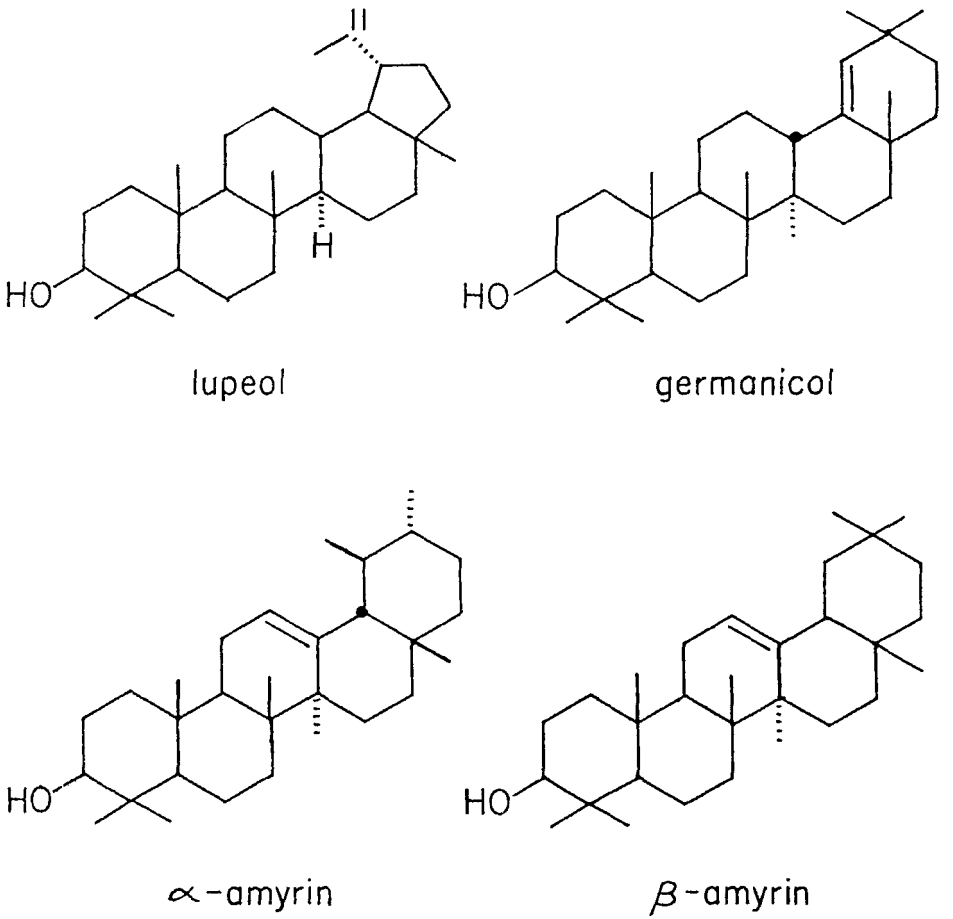


FIGURE 14. Structures of pentacyclic triterpenes.

phosphoric acid in methanol gave fairly good resolution of mixtures of these triterpenes (plus some others) (31).

The first example of the direct isolation of pure compounds from a crude plant extract by reverse-phase prep. LC has recently been reported by our group (32). In our search for insect antifeedant compounds, the crude aqueous methanol extract of fresh Schkuhria pinnata (Compositae) was found to possess significant biological activity. The closely related components of this crude polar material were inseparable by silica gel chromatography. However, a separation could be achieved by employing analytical reverse-phase HPLC (μ-Bondapak-C_{18}, methanol-water, 55:45). Accordingly, a 6.6 g sample of the crude extract was injected onto a Jobin-Yvon Chromatospac Prep 500 machine packed with 1 Kg of C_{18} stationary phase (33) (Fig. 15). In less than one hour, the major fractions were eluted, collected and evaporated to give gram quantities of pure compounds. Spectroscopic and chemical studies on the bioactive fractions (2 and 3) have shown them to be trans-1,10-cis-4,5-germacradienolides (Fig. 16). These are the first reported examples of sesquiterpene lactone compounds from the genus Schkuhria.

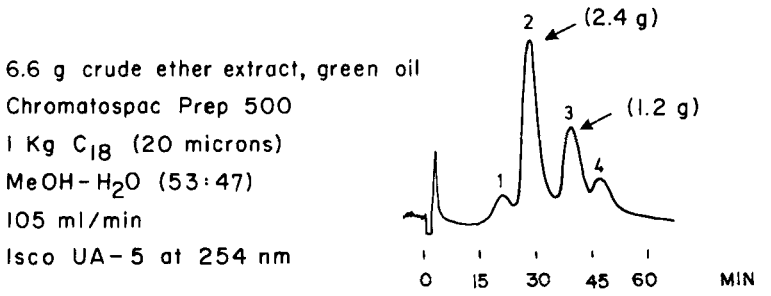
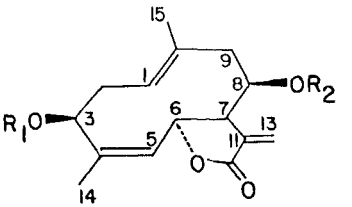


FIGURE 15. Prep LC of crude Schkuhria pinnata extract



	R_1	R_2
2	$OC-\underset{\|}{C}H-\underset{\|}{C}H-CH_3$ (O, OH, CH_3)	$OC-\underset{\|}{C}=CH(CH_2OH)$ (O, CH_2OH)
3	$OCOCH_3$	H
4	H	H

FIGURE 16. Structures of compounds 2, 3 and 4 from Schkuhria pinnata.

Prostaglandins

Prostaglandins are a group of structurally similar compounds found in mammalian tissues which display a large number of physiological effects. These compounds have been under active investigation for many years and their potential utility as therapeutic agents has led to a number of synthetic studies. Often in the syntheses, epimeric mixtures are produced. Traditional methods of separating epimeric mixtures of prostaglandins have relied on TLC methods, but these have generally been inadequate for complete resolution. In 1973, HPLC was used to separate epimeric mixtures of prostaglandins (PGA_1 and PGB_1) and the separations were compared with TLC (30). HPLC was found to be superior, and this result initiated other HPLC trials which have eventually led to the formulation of quite good separation techniques. Morozowich subjected PGA_2 and PGB_2 to chromatography on a triethylaminoethyl cellulose ion exchange column (using 0.05M tromethamine acetate at pH 7.2) and obtained almost complete resolution (34).

Various prostaglandins have been separated on a pellicular silica support and the degree of epimerization of $PGF_{2\alpha}$ has been determined (35).

Quantitative determination of prostaglandins in biological systems is of some importance to pharmaceutical investigators. Using reverse-phase HPLC (μ-Bondapak-C_{18} with 1:1 acetonitrile-water), the p-nitrophenyl esters of $PGF_{2\alpha}$, PGE_2, PGD_2 and 15-methyl-PGB_2 could be completely resolved (Fig. 17) (36). In addition, PGA_2 and PGB_2 could be partially resolved under these conditions.

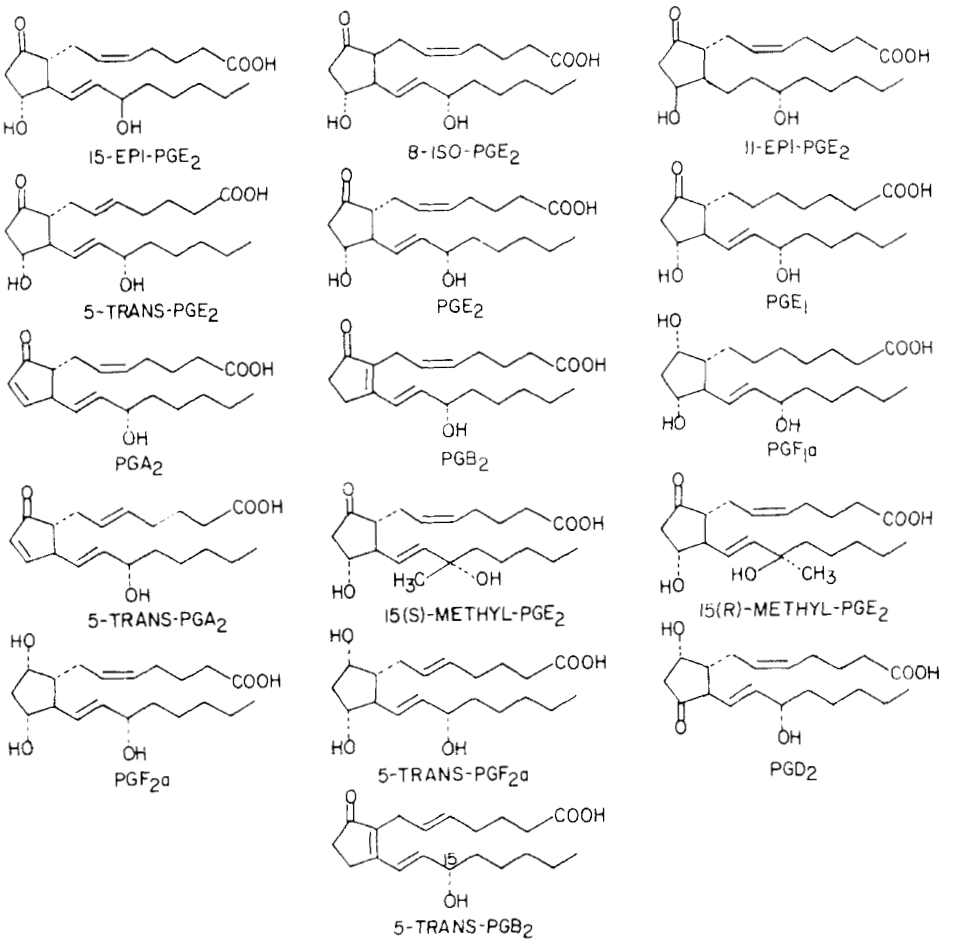


FIGURE 17. Structures of prostaglandins.

The use of silver-ion loaded HPLC columns has produced some good separations of isomeric prostaglandins.

Extracts of the soft coral Plexaura homomalla have been used as a source of PGA_2. These extracts are often contaminated with significant amounts of 5,6-trans-PGA_2. Weber relied on the tendency of silver ions to complex with double bonds as a method of separating these prostaglandin isomers (37). Using an ODS Permaphase (duPont) column and eluting with 0.5 M silver perchlorate in methanol-water (80:20), the methyl esters of PGA_2 and trans-PGA_2 could be separated. Mixtures of cis and trans PGB_2 could not, however, be separated. This is perhaps due to the hindered approach of silver to the double bond for steric reasons.

Using a Vydac strong cation exchange resin which had been equilibrated with 0.6M aqueous silver nitrate solution, dried and dry packed in an HPLC column, it was possible to separate the p-nitromethyl esters of PGA_2, PGB_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ (38). This method has been used as the basis of a quantitative assay for trace amounts of 5-trans-PGE_2 in PGE_2 (both as the p-nitrophenacyl esters) and is capable of detecting as little as 0.2% of the trans compound.

Using a silver-ion loaded microparticulate cation exchange column, Merritt and Bronson were able to get better separations of isomeric prostaglandins than were obtained with the Vydac support (39). In this example, a prepacked Partisil SCX (Reeve-Angel) column was loaded with silver ion by pumping aqueous 1M silver nitrate solution through the column. Elution of a mixture of prostaglandins with 0.06% dioxane in acetonitrile produced the separation shown in Fig. 18. Using this system, 5-trans and 5-cis-PGE_2, which could not be separated on microparticulate silica (40), was separated. Also, the silver-ion loaded column seems to give better results than reversed-phase HPLC. Fitzpatrick used reverse-phase HPLC to assay $PGF_{2\alpha}$, PGE_2 and PGD_2 (as the p-bromophenacyl esters) (36). He was unable, however, to resolve mixtures of PGA_2 and PGE_2, compounds which could be separated with the silver-ion loaded column.

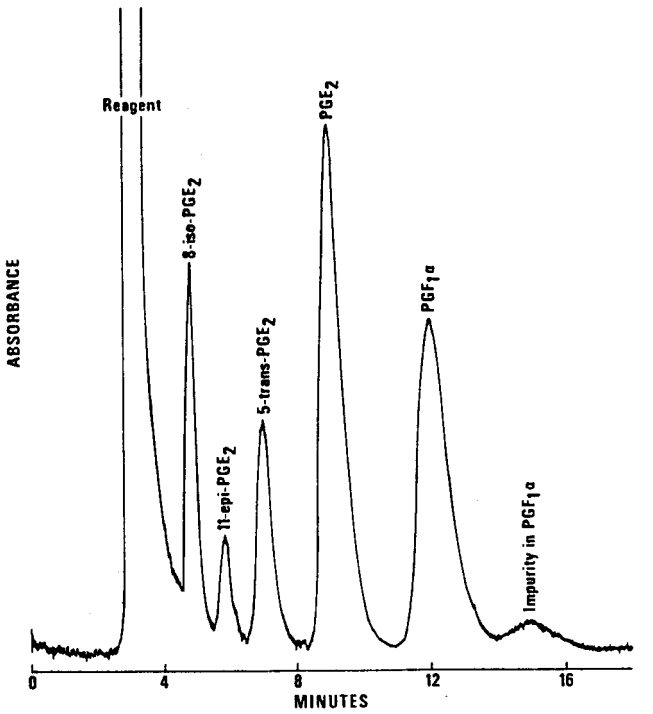


FIGURE 18. HPLC of p-nitrophenacyl esters of a mixture of 8-iso-PGE_2, 11-epi-PGE_2, 5-trans-PGE_2, PGE_2 and $PGF_{1\alpha}$ on a 25 cm silver ion loaded Partisil-SCX column with 0.06% acetonitrile in dioxane as the mobile phase.

Alkaloids

Alkaloids, in general, are nitrogen-containing bases which occur naturally in plants. These compounds usually show physiological activity and individual alkaloids are usually restricted to a few specific genera and families of plants (41).

Many structurally similar alkaloids may occur in a given plant, and the separation of these sometimes complex mixtures is a serious problem. In this section, the use of HPLC to separate mixtures of alkaloids of several different types will be examined.

The ergot alkaloids are produced primarily by a fungus, Claviceps purpurea, which grows most commonly on rye. The effects of these compounds on those that consume rye bread contaminated with this fungus are well known (42).

Recently, workers in the ergot alkaloid field have attempted to apply HPLC to the separation of mixtures of these compounds. Both normal phase (43,44) and reverse phase (45-47) HPLC have been used, but with limited success.

A more recent investigation (48) using LiChrosorb-Sil-60 and eluting with hexane-chloroform-ethanol (40:40:10) or chloroform-methanol (95:5) showed ergocornine, ergocryptine and ergocristine eluted together and were well separated from their corresponding stereoisomers ergocorninine, ergocryptinine and ergocristinine, which also eluted as a group.

To separate the individual alkaloids, a C_{18} reverse phase packing proved to be the most effective. Silica RP-18, obtained from the University of Saarbrucken, Saarbrucken, GFR, was used as the column packing and elution was accomplished with an acetonitrile-0.01M ammonium carbonate (2:3) solution. With this system it was possible to completely separate the following mixture: lysergic acid, ergometrinine, ergocornine, ergocryptine, ergocristine, ergotaminine, ergocorninine and ergocryptinine. Especially significant is the separation of the ergocryptine-ergocristine pair, which had proved difficult in previous attempts. Using the Saarbrucken RP-18 packing, α and β-ergocryptine isomers could also be partially resolved.

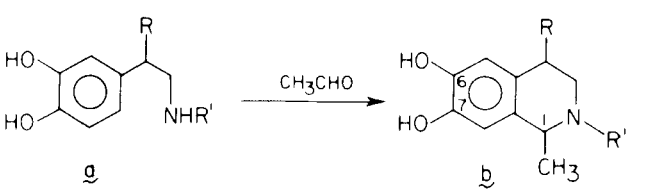
Other workers (49) have reported a separation of ergot alkaloids using a MicroPak-NH_2 column with an ether-ethanol gradient elution solvent system [(79:2) for 15 min, then increasing the ethanol concentration by 4%/min for 2 min. The remainder of the analysis is at 71:29 ether-ethanol]. It was possible to separate isosetoclavine, lysergine, setoclavine, lysergine, agroclavine and erginine (elute together), pyroclavine, festuclavine, ergine and elymoclavine (elute together) and chanoclavine. A pair of stereoisomers (setoclavine and isosetoclavine) were well separated under

these conditions. However, agroclavine and lysergine (which are double bond positional isomers) were only partially resolved.

An interesting theory concerning the mechanism of alcohol addiction has been advanced (50-53). Alcohol consumption raises the levels of aldehydes in the body. Catecholamines can react *in vivo* with these aldehydes to form tetrahydroisoquinoline alkaloids, which may in turn act as false neurotransmitters. The theory proposes that acetaldehyde will form alkaloids which may in some way be responsible for the addictive properties of alcohol (Fig. 19).

In conjunction with this theory, experiments were designed to separate model alkaloids and detect their presence in the HPLC effluent by an electrochemical detector (reported to be 2-3 times more sensitive than a uv detector) (54). The separation was accomplished using a pellicular Vydac-SCX column and eluting with 0.1 M citric acid-0.2 M disodium hydrogen phosphate-water (4.8:3.2:2). Electrochemical detection was provided by a 3 mm carbon paste electrode maintained at +0.7V potential vs. Ag/AgCl as the reference electrode. This method is sensitive to parts-per-billion levels of the alkaloids in body fluids and tissues.

In a related alkaloid system, three tetrahydroisoquinoline *Cactaceae* alkaloids were separated on silica columns, either LiChrosorb-SI-60 or μ-Porasil. With acetonitrile-conc. ammonia (96:4) as the mobile phase, it was possible to separate salsoline (6-hydroxy-7-methoxy-1-methyl-), isosalsoline (7-hydroxy-6-methoxy-1-methyl-) and arizonine (8-hydroxy-7-methoxy-1-methyl-tetrahydroisoquinoline) (Fig. 20).



a b

FIGURE 19. a, a phenethylamine; b, a tetrahydroisoquinoline alkaloid.

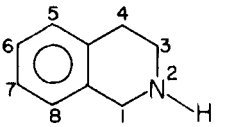


FIGURE 20. Basic ring structure for salsoline, isosalsoline and arizonine.

The separation of opium alkaloids has received recent attention. A mixture of 21 derivatives of morphine or codeine using ion-pair HPLC with n-heptane sulfonate as the counter ion was separated on a reverse-phase (μ-Bondapak-C_{18}) column (55).

Attempts at separating opium alkaloids with μ-Porasil using a variety of solvent systems were unsatisfactory. Use of μ-Bondapak-C_{18} with methanol-0.1N ammonium hydrogen carbonate solution resulted in poor separation and peak tailing. Ion pair chromatography worked quite well with methanol-water (60:40) (56). By varying the methanol to water ratio, retention times could be changed. Use of an elution gradient was useful in reducing the elution time. In addition, the alkaloids could be injected as salts or free bases with no change in retention time.

An analytical technique for the quantative determination of morphine, codeine and thebaine has been reported (57). By placing two μ-Bondapak-C_{18} columns in series and eluting with 25% acetonitrile in water, it was possible to obtain baseline resolution of some components of a crude gum opium extract. The separation of morphine, codeine, thebaine, papaverine and noscapine was thus achieved.

Alkaloids of Catharanthus roseus (Vinca rosea) are of great medical importance. For example, vinblastine and vincristine are potent antineoplastic drugs. A method to separate closely related alkaloids, based on HPLC techniques, was developed to circumvent difficulties encountered in conventional separation methods (TLC and column chromatography) (58).

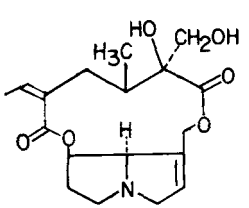
The separation of a mixture of 26 alkaloids was attempted using a LiChrosorb-RP-8 reverse-phase column and acetonitrile-0.01 M ammonium carbonate solution (47:53). In a typical separation it was possible to completely resolve lochnerine, vindoline, catharanthine and vinblastine while ajmalicine and vincristine were a partially resolved pair and tetrahydroalotonine, leurosine and desacetoxyvinblastine were partially resolved.

The plant Senecio vulgaris is common in certain areas of the world where cattle and sheep graze. It contains relatively large amounts of pyrrolizidine alkaloids and is quite toxic to animals which eat it. HPLC has proved to be a useful tool for the rapid analysis of alkaloid content of this plant (59).

Good results for the separation of three pyrrolizidine alkaloids, retrorsine, seneciphylline and senecionine, were obtained by two different methods (Fig. 21).

The first method involves a gradient elution sequence on a μ-Bondapak-CN (Waters) column. The solvent is initially 13% THF and 87% 0.01M ammonium carbonate (pH 7). The THF concentration was increased along a linear gradient from 13 to 26% for 30 min.

In the second method (isocratic), excellent results were also obtained. Using the same column and eluting with a mixture of 16% THF and 84% 0.01M ammonium carbonate (pH 7), the alkaloids were completely separated. The isocratic method has the advantage of speed, in that the column does not require a re-equilibration period after an analysis, as does the gradient method.



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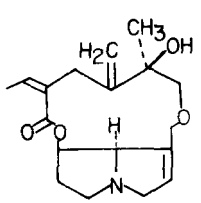

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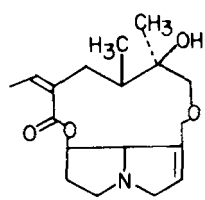


3

FIGURE 21. Pyrrolizidine alkaloids: 1, retrorsine; 2, seneciphylline; 3, senecionine.

A final comment can be made on the use of preparative scale HPLC for the fractionation of crude plant extracts to obtain alkaloids and other substituents in relatively pure form (60).

The dried bark of the well-known Indian and African medicinal plant Fagara chalybea Engl. (Rutaceae) was extracted with hexane. TLC showed this extract to contain several compounds of varying polarity. After a prefiltration on silica gel (hexane-ether, 1:1), the filtrate was submitted to preparative chromatography using a Waters Prep-LC 500. Using ether-hexane (1:9) compounds 1 and 2 (Fig. 22) were obtained in 6 min. After peak 2, the solvent system was switched to the more polar ethyl acetate-hexane (1:4) mixture, and the remainder of the material was collected in one fraction. This fraction was concentrated, and a second stage prep

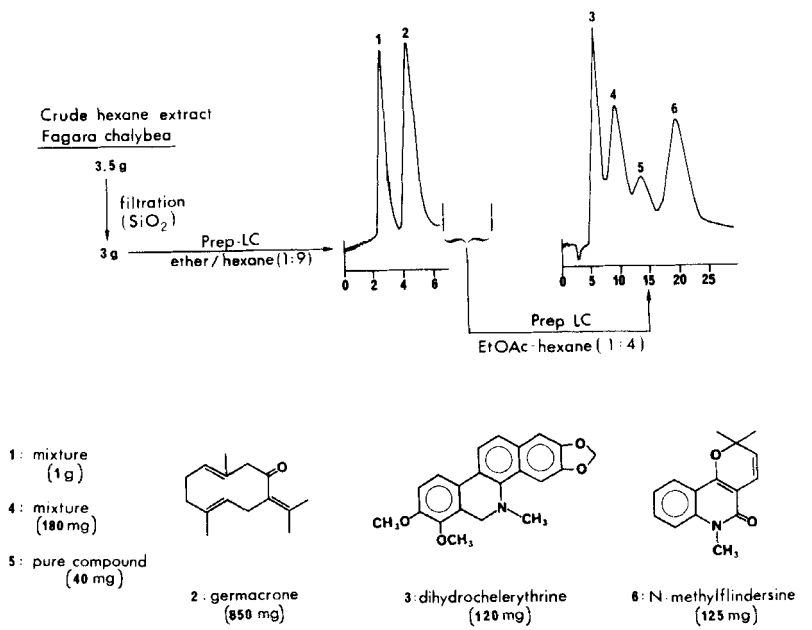


FIGURE 22. Treatment of a crude extract of Fagara chalybea by Prep-LC to yield pure compounds.

LC with the above solvent system was carried out. This resulted in the separation of components 3-6 in 25 min. As monitored by TLC, fractions 2, 3, 5 and 6 were pure. Spectroscopic studies of the pure compounds led to the following structures: 2, germacrone; 3, the alkaloid dehydrochelerythrine; and 6, N-methylflindersine, a new alkaloid.

A separation of the same scale by conventional column chromatography not only took two weeks but also required usage of repeated chromatographies under different conditions, including gradient elution. The prep LC method thus results in a great reduction of time, and as a consequence the risk of sample deterioration on the column is also minimized. For example, in the case of column chromatography, no compound corresponding to peak 5 was obtained. This compound, which has not yet been identified, is unstable and readily oxidized to a more polar substance.

Peptides

Some work has been done recently to find effective separation methods for small peptides. In an examination of several columns (61), including phenyl-Corasil (reverse phase, Waters), Poragel-PN(37-75 μm) and Hydrogel IV gel filtration medium (37-75 μm) using acetonitrile-water mixtures, various effects were noted. None of these columns showed good efficiency for separating peptides. Hydrogel was found to be generally unsuitable. The plate number for phenyl-Corasil and Poragel-PN was about four times greater than for Poragel-PS. Peaks from phenyl-Corasil separations showed tailing; Poragel-PN produced asymmetric peaks (probably from overloading). Residual silanol groups in phenyl-Corasil and functional groups in the Poragels have an influence on retention behavior of the various peptides.

Other reverse-phase HPLC columns have given better results for peptide separations. A LiChrosorb (5 μm)-RP-18 column, when used with gradient elution starting with 0.5 M perchloric acid (pH 0.2) and gradually changing to acetonitrile, combined with an elevated column temperature (70°C), gave excellent separation of a number of small peptides (62).

An examination of a number of small peptides (mostly dipeptides, two tetra-, one penta, one hepta- and one octapeptide) on a Nucleosil-5-C-18 (Macherey, Nagel) column gave very good separation (63). A gradient elution scheme was used, starting with 0.05 M potassium dihydrogen phosphate solution at pH 2 and finishing with pure methanol. The gradient time was 60 min and the column temperature was 31°C.

Results of this investigation imply that the C-terminal amino acid of a dipeptide is important in determining retention time. In this system, it was not possible to separate the Tyr-Gly/Leu-Gly pair or the Gly-Tyr/Gly-Leu pair. However, the following could be separated: Val-Gly, Gly-Val, Phe-Gly (partial), Gly-Phe, Ala-Ala-Tyr-Ala-Ala, Gly-Trp, Phe-Tyr, Leu-Trp-Met-Arg, Met-Glu-His-Phe-Arg-Trp-Gly, Leu-Leu-Val-Tyr and Renin inhibitor (an octapeptide).

A separation of some specifically labelled oxytocin derivatives has been reported (64). Using two μ-Bondapak C_{18} columns in series and eluting with 0.1 M ammonium acetate solution (pH 4)-acetonitrile (82:18) it was possible to obtain baseline resolution of the following oxytocins: [1-hemi-DL-[α-^{2}H]cystine]oxytocin, [6-hemi-DL[α-^{2}H]cystine]oxytocin, [2-DL-[α-^{2}H]tyrosine]oxytocin and [8-DL-[2-^{13}C]leucine]oxytocin.

Carbohydrates

Separation of sugars by column chromatography has proved to be extremely difficult. The three techniques used for these separations are adsorption, partition and ion exchange column chromatography. In recent years, HPLC has been used with good results in the areas of monosaccharides, nucleosides and nucleotides.

A large group of partially and completely substituted carbohydrates has been separated using a microparticulate silica gel column with various solvents. The compounds were glycosides, isopropylidine and benzylidene derivatives, methylated and acetylated carbohydrates (65,66).

An elaborate system for analysis of the isomerization products of carbohydrates has been reported (67). The system utilizes anion exchange resin columns (10-20 μm) with gradient elution to effect separations. Detection is by means of a solution of orcinol in sulfuric acid.

A further use of anion exchange for the separation of many sugars is described in reference 68.

Cation exchange resins have been used to separate a mixture of D-galactose, D-fructose, D-ribose, D-xylose and L-rhamnose. Elution was with acetone-isopropyl alcohol (3:1) (69).

A method of analysis for mono, di and oligosaccharides has been developed (70). Using a μ-Bondapak-Carbohydrate (Waters) column with an acetonitrile-water (85:15) mobile phase, the following carbohydrates were fully or partially resolved: rhamnose, xylose, arabinose, partial separation of mannose, glucose and galactose (all monosaccharides), and glucose, sucrose, maltose and lactose (partial separation). Also separated were β-(1→4) linked glucose oligomers: cellobiose, cellotriose, cellotetraose and cellopentaose.

An HPLC separation for the determination of anomeric forms of streptozocin in a drug preparation has been performed using a reverse phase column (Fig. 23) (71). With a μ-Bondapak-C_{18} column and 0.1 M acetic acid in water-methanol (97:3, pH 4) it was possible to resolve the anomers of streptozocin in a fashion that allowed for performance of a quantatative assay.

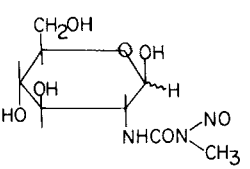


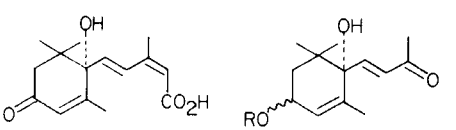
FIGURE 23. Streptozocin.

Resolution of Optical Isomers by HPLC

The most generally useful method for using HPLC to effect an optical resolution involves formation of a derivative of a racemic compound such that a pair of diastereomers may then be resolved by HPLC. The advantage of the HPLC method over the fractional crystallization method is that a baseline HPLC separation ensures 100% resolution. This general method has been applied several times in several laboratories and some of the results will be described below.

For the determination of the absolute configuration of natural (+)-abscisic acid (Fig. 24a), it was necessary to resolve the intermediate cis-diol (Fig. 24b) into its optically active enantiomers (72). To do this, the (+)-MTPA ester derivative (Fig. 24c) was prepared (73) and the diastereomers were separated by HPLC using Porasil-T and isopropyl alcohol-hexane (1:99) as eluant. The column was used in a recycle mode and after five passes (27 hours) the diastereomers were baseline separated. This method has also been applied to determining the absolute stereochemistry of blumenol A and blumenol B (74).

A similar approach was taken to determine the optical purity of chiral amines by HPLC (75). Reaction of chiral amines with optically pure O-methyl mandelyl chloride yielded diastereomeric amides, which were separated on an HPLC column of Merck silica gel. Elution was with ethyl acetate-hexane (1:1). Separation of the diastereomers was also achieved on a Merckosorb-SI-60 column with isooctane-ethyl acetate (1:1).



1 2,3

FIGURE 24. 1: Abscisic acid; 2, R = H; 3, R = (+)-α-methoxy-α-trifluoromethylphenyl acetate.

It was possible to directly determine R/S enantiomer ratios of citronellic acid and related compounds by HPLC (76). Racemic citronellic acid was converted to diastereomeric amides with (R)-(+)-α-methyl-p-nitrobenzylamine. Separation of the amides was accomplished on two Partisil-10 columns in series, using as the mobile phase THF-heptane (20:80).

Recently we have used the MPTA ester method to resolve enantiomers of periplanone-B, the sex excitant pheromone of the American cockroach, Periplaneta americana (77), Fig. 25a. By use of two μ-Porasil columns in series with 2% ether in hexane as eluant, it was possible to completely resolve the diastereomeric MTPA esters of periplanol-B (Fig. 25b). The separation is shown in Fig. 26. After removal of the MTPA group by basic hydrolysis, oxidation of the alcohol functionality gave both (+) and (-) enantiomers of periplanone-B.

The advent of microparticulate silica gel columns has greatly facilitated these separations. The HPLC separation of abscisic acid-MTPA diastereomers took 27 hours in recycle mode whereas separation of periplanol-MTPA diastereomers took less than 40 min on μ-Porasil.

Miscellaneous Separations

a. Fatty Acids. A number of separations of saturated and unsaturated fatty acids, fatty acid methyl esters, lipids and triglycerides have been reported (78-82). Fatty acid separation usually relies on use of a reverse-phase column and methanol-water elution. Occasionally, elevated column temperatures improve resolution.

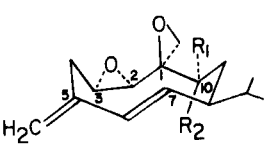


FIGURE 25. (a), R_1, R_2 = O; periplanone-B. (b), R_1 = O-MTPA, R_2 = H.

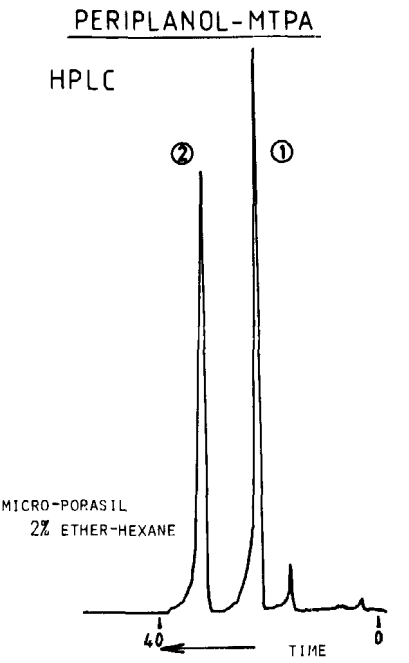


FIGURE 26. HPLC trace of the diastereomeric MTPA esters of periplanol-B. Two μ-Porasil columns in series, u.v. detection at 230 nm. Solvent, 2% ether in hexane. Flow rate, 1 ml/min.

An interesting development in fatty acid separation is the use of the Waters Radial Compression Separation System which uses 10 cm reverse phase ODS silica cartridges which have been radially compressed in a pressure chamber. Using this system with a water-methanol gradient (60:40 initially, finally 100% acetonitrile in 30 min) a large number of fatty acid p-bromophenacyl esters were completely separated (Fig. 27). Total separation time was 45 min (83).

b. Antibiotics. A μ-Bondapak-NH_2 column with various concentrations of acetic acid-methanol-acetonitrile-water (typically 1.4:2.8:10:85.5) is effective in separating cephalosporin derivatives (84). Ampicillin has been separated from epicillin on a LiChrosorb-RP8 column by water-methanol elution (85).

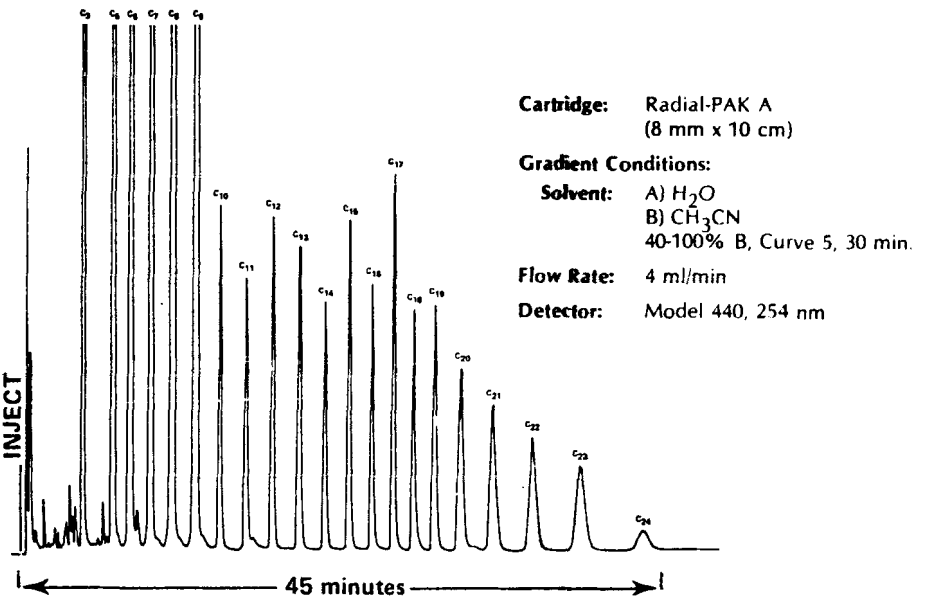


FIGURE 27. A mixture of p-bromophenacyl esters of fatty acids (indicated by chain length) which was separated in 45 min using one Waters Radial-PAK A cartridge.

A series of diastereomeric 7-ureidoacetamidocephalosporins (Fig. 28) was separated on μ-Bondapak-C_{18} using 0.01 M diammonium hydrogen phosphate in methanol as eluant (86).

c. Natural Products. Pteridines, dihydropteridines and tetrahydropteridines were separated by HPLC on Partisil-SCX or Zipax-SCX strong cation exchanger columns. By using a citrate buffer mobile phase (pH 6) with 5% added dioxane, tailing was eliminated and good resolution was obtained (87).

Both normal and reverse-phase techniques have been used for isolation of natural prenylquinones (ubiquinones) (88,89).

A mixture of ubiquinones-7, -8, -9 and -10 was separated on μ-Bondapak-C_{18} using gradient elution from 9:1 methanol-water to 100% methanol (Fig. 29). In similar manner, mixtures of ficaprenols-10, -11 and -12 were resolved.

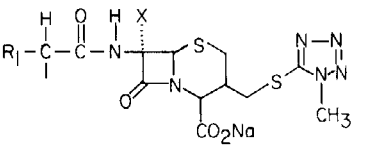


FIGURE 28. A representative 7-ureidoacetamidocephalosporin (L and D isomers of the arylglycine side-chain).

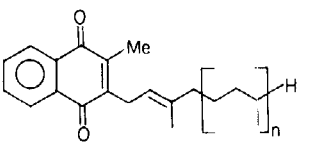


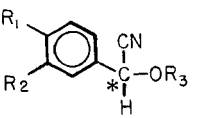
FIGURE 29. Ubiquinone-n.

With LiChrosorb-SI-60 and dioxane-hexane (1:99), various mixtures of prenylquinones could be separated from prenyllipids (β-carotene, phytol, chlorophylls) with good results in most cases.

A mixture of chlorophylls a, b, a', b' and pheophytin a was completely resolved with gradient elution (acetone-hexane, 8:92 to 12:88) on a silica gel column constructed from a teflon tube and SS-05 (0.5 μm) silica gel (90,91). This separation was useful for developing an assay for chlorophyll a which was sensitive to as little as 10^{-10} g of this compound.

Bile acids have traditionally been separated by gas chromatography, but often the resolution was not good. Recent application of HPLC to this problem led to better results. The major conjugated bile acids of human bile have been resolved using a two stage elution sequence with Corasil-II or μ-Porasil columns. An alkaline solvent system (isopropyl alcohol-ethyl acetate-water-7N ammonium hydroxide; 260:600:50:3) was used to separate the bile acids into groups, e.g. tauro-dihydroxy derivatives, taurocholate, glyco-dihydroxy derivatives and glycocholate.

TABLE I



Entry	Compound	R_1	R_2	R_3	*
1.	Sambunigrin	H	H	Glc	(S)
2.	Prunasin	H	H	Glc	(R)
3.	Dhurrin	OH	H	Glc	(S)
4.	Taxiphyllin	OH	H	Glc	(R)
5.	Zierin	H	OH	Glc	(S)
6.	Holocalin	H	OH	Glc	(R)
7.	Neoamygdalin	H	H	gentiobiose	(S)
8.	Amygdalin	H	H	gentiobiose	(R)

The fraction containing glyco-dihydroxy conjugates was separated by rechromatography using acetonitrile-acetic acid (40:1) and the tauro-dihydroxy derivatives were partially resolved by rechromatography with acetonitrile-acetic acid-formic acid (97%)-water (100:2:1:2) (92).

Reverse phase chromatography has also been used for separation of bile acids (μ-Bondapak-NH_2 or μ-Bondapak-C_{18}) (93).

A group of epimeric benzaldehyde cyanohydrin glycosides has been separated by normal phase HPLC using a μ-Porasil column and ethylacetate-methanol (97:3) elution (see Table 1). Note that the isomeric pairs (1,2) and (7,8) were successfully resolved (94).

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