HPLC in the Analysis of Steroidal Compound Mixtures as Applied to Biocrudes

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Abstract

Potential petrocrops are a rich source of hydrocarbon-type steroidal and terpenoidal compounds and waxes. These compounds and waxes can either be tapped or extracted as biocrude from the petrocrops by using different solvents. Upon further processing, they can be converted into liquid fuels, thus providing an alternate source of energy for the future. The reported high-performance liquid chromatographic method for the analysis of steroids is highly versatile, sensitive to the structure of steroidal compounds that are present in biocrudes, and results in fast screening of plant species into potential petrocrops. This method has also been helpful for use in deciding the best solvent sequence for the extraction of biocrude from potential plants. With further exploitation, the method could even yield useful information on the tentative structure of unknown steroids from their respective retention time data.

Introduction

The ever-growing rate of petroleum consumption and the continuing depletion of petroleum reserves may not be able to sustain the future liquid fuel requirement. This has led to the search for alternative sources of hydrocarbons or similar chemical species. One important alternative is the possibility of deriving hydrocarbon-type substances from renewable plant materials (petrocrops) which can be converted into liquid fuels to augment and possibly replace petroleum-derived fuels.

These potential plant species, like other plants, contain compounds such as polyphenols, fats, lipids, proteins, resins, fatty acids, waxes, etc. In addition, they also contain hydrocarbon-type steroidal and terpenoidal (tetracyclic and pentacyclic) hydrocarbons which characterize them as potential petrocrops. These steroids and terpenoids, after extraction as biocrude, provide liquid and gaseous fuels when processed. A method was required, therefore, to analyze the biocrudes for steroids and terpenoids to enable fast screening of plant species into potential and nonpotential petrocrops. The steroidal compounds that are present in biocrudes of potential petrocrops are expected to be diverse in chemical nature and contain sterols, acetates, diacids, etc. High-performance liquid chromatography (HPLC) has been reported in literature for analyzing steroids and terpenoids (1–15). However, to our knowledge, there is no reference available on the analysis of such a complex mixture of steroids as those present in biocrudes. A method based on HPLC was developed as an analytical tool for analyzing all these components in a mixture in a single run.

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Experimental

The steroidal compounds were procured from the University of Calcutta's College of Science in Calcutta, India. These samples were dissolved in chloroform. Chromatographic studies were performed on a DuPont 850 LC unit with Zorbax columns (25 cm × 4.6-mm i.d.) and an ultraviolet detector at 210 nm (0.08 and 0.04 AUFS) (DuPont, Wilmington, DE). Isocratic conditions were employed. The temperature of the column compartment was kept at 20°C. Various solvents used for extraction, fractionation, and HPLC were laboratory reagents (LR) from Qualigens (Bombay, India). They were purified by distillation and passed over activated silica gel (Qualigens). The alumina used for fractionation of the plant extracts was Alcoa F-20 grade (Bio-Rad Laboratories, Hercules, CA).

HPLC analysis of steroids

Optimization of column conditions was required for resolving the different classes of steroids as well as individual structurally similar constituents within each class.

Initially, a mixture containing seven sterols (Table I) was separated isocratically by using isopropanol–acetonitrile mixtures as the mobile phase and octadecyl silane (Zorbax ODS) as the stationary phase. The best resolution was achieved by 10% isopropanol in acetonitrile. This was followed by the resolution of a mixture of lupeol acetate, glutenyl acetate, α- and β-amyrin acetates, and teraxerol acetate by elution with 4% isopropanol in acetonitrile (Table II). To select a single solvent composition for all the steroidal compounds, the mixtures of sterols (previously resolved with 10% isopropanol) and steryl acetates

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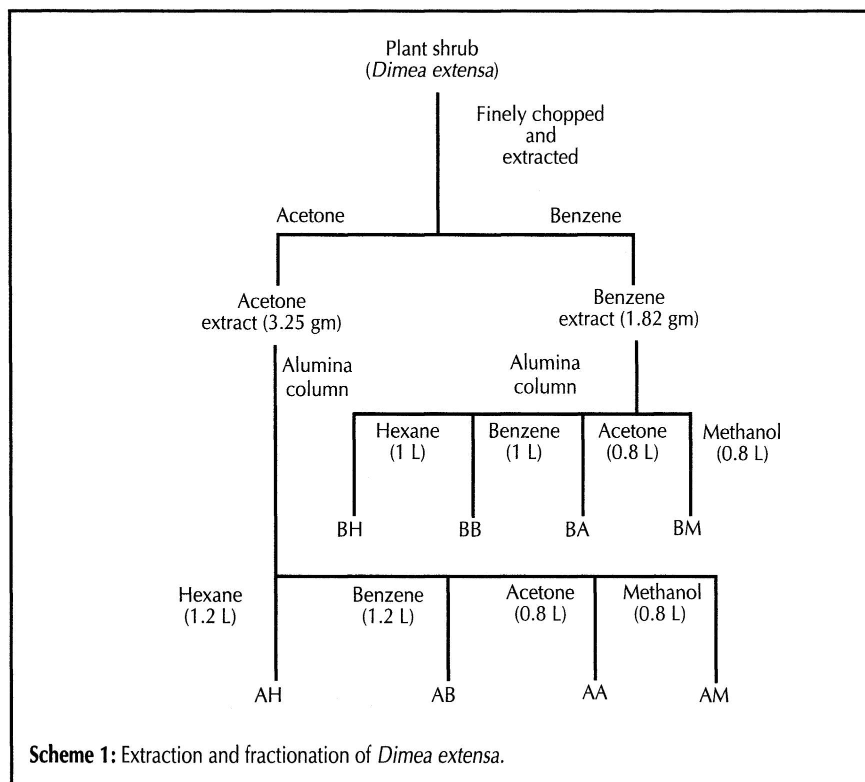

Scheme 1: Extraction and fractionation of *Dimea extensa.*

(resolved with 4% isopropanol) were mixed together and subjected to HPLC, using the latter composition of mobile phase and keeping other column conditions the same (Table III).

Having finalized the column conditions, another mixture of steroidal compounds that consisted of highly polar betulenic acid, ursolic acid, oleanolic acid, putranjivadione, friedelin, and betulin was also successfully resolved (retention time data in Table II), which suggests the versatility of the developed method.

This mixture of highly polar steroids was added to the previously analyzed mixture of sterols and acetates (mixture A), and the resulting mixture (mixture B) was again subjected to HPLC under the same conditions, except for the flow rate of the solvent, which was decreased from 1 cc to 0.9 cc for better resolution (Table III, Figure 1).

HPLC analysis of biocrude

A biocrude sample that was extracted from one of the potential petrocrops, *Dimea*

Table I. Effect of Mobile Phase Composition on Resolution of Sterols

Solvent (isopropanol–acetonitrile)		Retention time of sterols in mixture* (min)						
Solvent ratio	Flow rate (cc/min)	Lupeol	Cycloartenol	Teraxerol	Stigmasterol	Cholesterol	Campesterol	β-Sitosterol
7:13	1.0	11.5	14.0	14.0	14.0	14.9	15.7	17.80
7:13	0.6	20.4	24.4	24.4	24.4	26.2	27.8	30.00
1:4	2.0	10.4	13.3	13.9	13.9	15.1	16.4	18.90
1:9	2.0	16.2	21.5	22.6	24.1	26.2	29.3	34.35

* UV detector at 210 nm (0.08 AUFS)

Table II. Resolution of Sterol Mixtures and Sterol Acetates*

Sterols in mixture	Retention time (min)	Sterol acetates in mixture	Retention time (min)	Acids, diones, and ketones in mixture	Retention time (min)
Lupeol	38.8	Lupeol acetate	43.0	Betulenic acid	8.8
Teraxerol	45.4	Glutenyl acetate	52.6	Ursolic acid	9.6
Cycloartenol	52.4	β-Amyrin acetate	56.4	Oleanolic acid	10.8
Stigmasterol	65.8	Teraxerol acetate	58.6	Putranjivadione	11.6
Cholesterol	72.0	α-Amyrin acetate	64.0	Friedelin	13.1
Campesterol	80.8			Betulin	13.6
β-Sitosterol	95.2				

* Mobile phase: Isopropanol–acetonitrile (1:24); flow rate: 1 cc/min; UV detector at 210 nm (0.08 AUFS)

Table III. Resolution of Mixture of Sterols and Sterol Acetates*

Steroidal compounds in the mixture	Mixture A retention time (min)†	Mixture B retention time (min)‡
Betulenic acid		9.6
Ursolic acid		10.0
Oleanolic acid		11.6
Putranjivadione		12.4
Friedelin		13.6
Betulin		14.2
Lupeol	37.8	48.6
Lupeol acetate	43.0	55.2
Teraxerol	44.4	58.0
Cycloartenol	51.2	66.4
Glutenyl acetate	52.6	67.6
β-Amyrin acetate	56.4	71.6
Teraxerol acetate	58.6	75.2
α-Amyrin acetate	64.0	83.2
Stigmasterol	67.4	88.0
Cholesterol	70.0	90.4
Campesterol	78.4	99.2
β-Sitosterol	93.6	117.6

* Mobile phase composition: 4% isopropanol in acetonitrile.
† Sterols and acetates; flow rate of mobile phase: 1 cc/min.
‡ Sterols and acetates; acids, diketones; flow rate of mobile phase: 0.9 cc/min.

extensa, was analyzed for steroidal compounds under the optimized conditions above. A biocrude sample was extracted from the finely chopped and dried plant using acetone followed by extraction with benzene. Both the acetone and benzene extracts were subjected to analysis by HPLC under the above conditions. These two extracts were also subjected to fractionation by column chromatography over activated alumina by elution with hexane, benzene, actone, and methanol, successively (Scheme 1). The resulting four fractions from the two extracts were then analyzed by HPLC (Figure 2). Similarly, acetone and benzene extracts from other potential plants such as *Padilanthus tithimaloides*, *Cryptostegia grandiflora*, *Ficus elastica*, *Euphorbia lathyrus*, *Ficus carica*, *Calotropis gigantea*, and *Euphorbia nerifolia* were also subjected to fractionation over an alumina column as above, and the fractions were analyzed by HPLC.

Results and Discussion

With the developed method, mixtures of steroids were successfully resolved by using reversed-phase HPLC with a Zorbax ODS column, C_{18} as the stationary phase, and 4% isopropanol mixed with acetonitrile as the mobile phase. The optimum temperature and flow rate were 20°C and 0.9 cc/min. Under these conditions, individual components from each class of steroids (sterols, ketones, diketones, acids, etc.) were resolved.

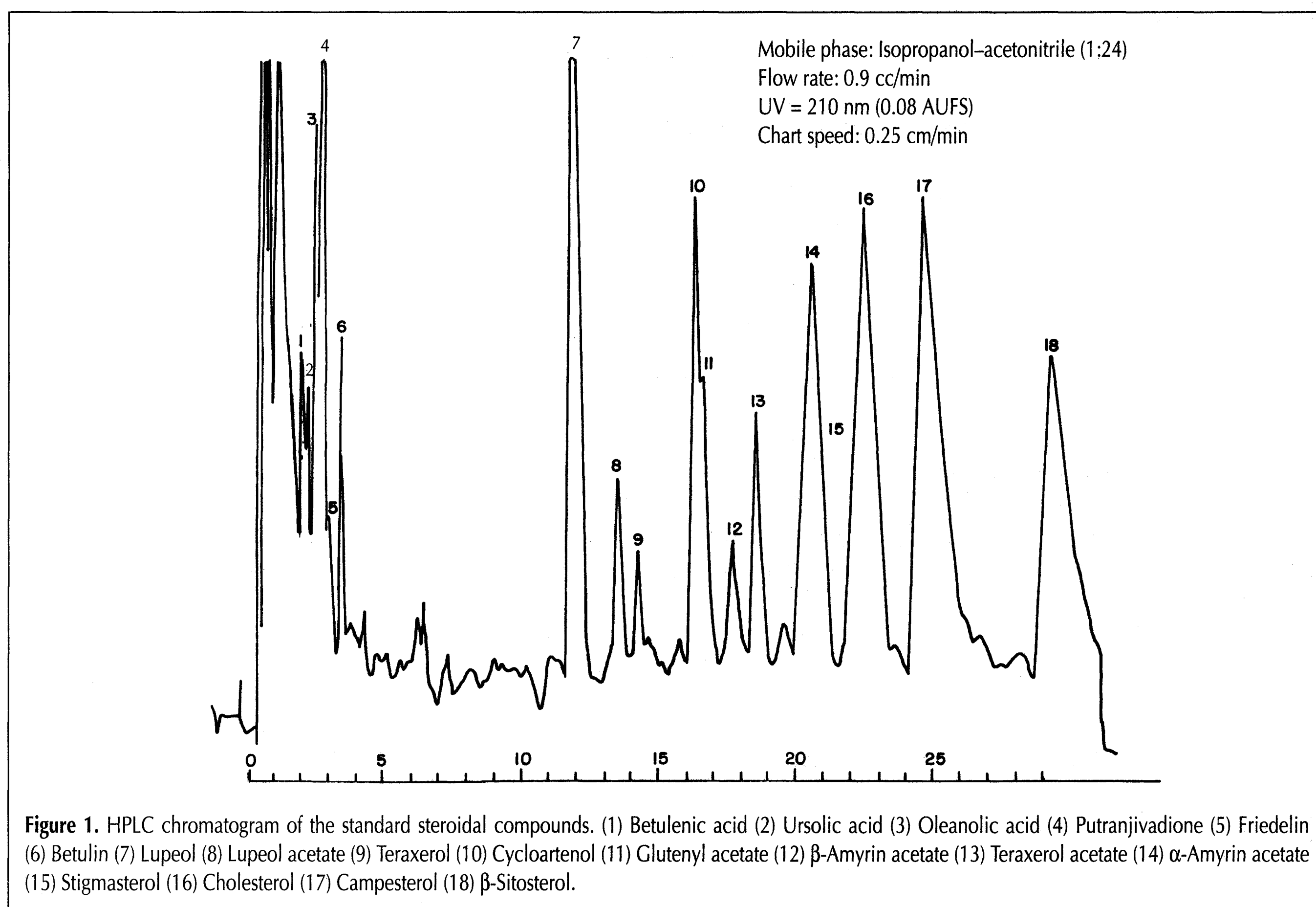

Figure 1. HPLC chromatogram of the standard steroidal compounds. (1) Betulenic acid (2) Ursolic acid (3) Oleanolic acid (4) Putranjivadione (5) Friedelin (6) Betulin (7) Lupeol (8) Lupeol acetate (9) Teraxerol (10) Cycloartenol (11) Glutenyl acetate (12) β-Amyrin acetate (13) Teraxerol acetate (14) α-Amyrin acetate (15) Stigmasterol (16) Cholesterol (17) Campesterol (18) β-Sitosterol.

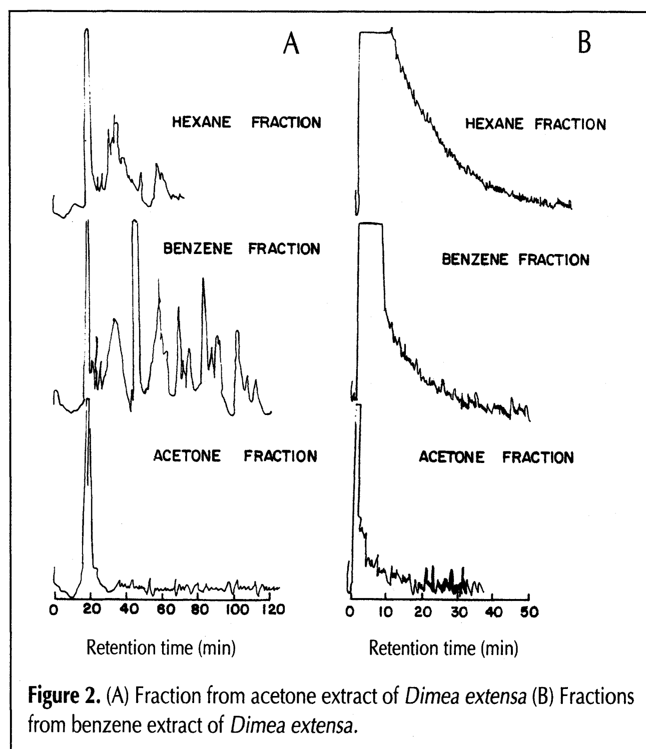

Figure 2. (A) Fraction from acetone extract of *Dimea extensa* (B) Fractions from benzene extract of *Dimea extensa*.

Standardization of column conditions

Standardization required the selection of a suitable column and the optimization of column temperature, composition, and flow rate to resolve all the steroids. This would not only resolve different classes of steroids but also the individual structurally similar constituents within each class.

Resolution was initially attempted with a mixture of sterols. The best resolution was achieved by using a Zorbax ODS column at 20°C and eluting with a mixture of isopropanol in acetonitrile (1:9). Table I shows the effect of solvent composition and flow rate on the resolution. The mixture of sterol acetates resolved best with 4% isopropanol in acetonitrile. The aim of the present study was to optimize a single solvent composition for all types of steroids. Therefore, the initial mixture of sterols was again subjected to HPLC by eluting it with this 4% mixture. The same solvent composition was used for resolving the mixture of steroidal acids and ketones. The resolution of components of individual classes was followed by resolving a mixture of sterols and sterol acetates (mixture A) and another mixture of sterols, sterol acetates, highly polar steroidal acids, ketones, and diketones (mixture B). The flow rate of the mobile phase had to be slightly reduced from 1 cc/min for individual classes (Table II) to 0.9 cc/min for the whole mixture (Table III).

Table IV. Various Structural Groups Present in Steroids for Correlation with their Retention Times

Steroid	Total no. of rings	No. of carbon atoms in rings D	E	Polar substituents at C_n	Double bonds
Betulenic acid	5	6	5	C_3–OH C_{17}–COOH	on the side chain
Ursolic acid	5	6	6	C_3–OH C_{17}–COOH	in ring C
Oleanolic acid	5	6	6	C_3–OH C_{17}–COOH	in ring C
Putranjivadione	5	6	6	C_3=O C_7=O	–
Friedelin	5	6	6	C_3=O	–
Betulin	5	6	6	C_3–OH C_{17}–CH_2OH	on the side chain
Lupeol	5	6	5	C_3–OH	on the side chain
Lupeol acetate	5	6	5	C_3–OAc	on the side chain
Teraxerol	5	6	6	C_3–OH	in ring D
Cycloartenol	5	6	–	C_3–OH	on the side chain
Glutenyl acetate	5	6	6	C_3–OAc	in ring B
β-Amyrin acetate	5	6	6	C_3–OAc	in ring C
Teraxerol acetate	5	6	6	C_3–OAc	in ring D
α-Amyrin acetate	5	6	6	C_3–OAc	in ring C
Stigmasterol	4	5	–	C_3–OH	on the side chain
Cholesterol	4	5	–	C_3–OH	in ring B
Campesterol	4	5	–	C_3–OH	in ring B
β-Sitosterol	4	5	–	C_3–OH	in ring B

Correlation between retention time and steroid structure

From the retention time of the authentic samples of steroids analyzed, it was observed that these compounds have a regular elution pattern according to their structure. This suggests separation according to the polarity and position of the functional group attached, the number of naphthenic rings in the molecule, the length of the side chain, etc. (Table IV, Figure 3). The following generalizations were made based on the different structural groups in the molecules (Figure 3, Table IV) and the corresponding retention times of the individual steroidal compounds (Table III). In general, it was observed that: (*a*) among the steroids containing different functional groups, the acids appeared first, followed by diones, ketones, diols, and sterols in that order; and (*b*) five-ring steroidal compounds appeared before four-ring compounds. Among the five-ring steroids belonging to one class, those containing a double bond in the side chain eluted before the ones with a double bond in the ring system.

Acids

Among the acids, those with a double bond in the side chain eluted first, followed by acids with *vic*-dimethyl on ring E in the molecule, followed then by acids with *gem*-dimethyl on ring E in the molecule.

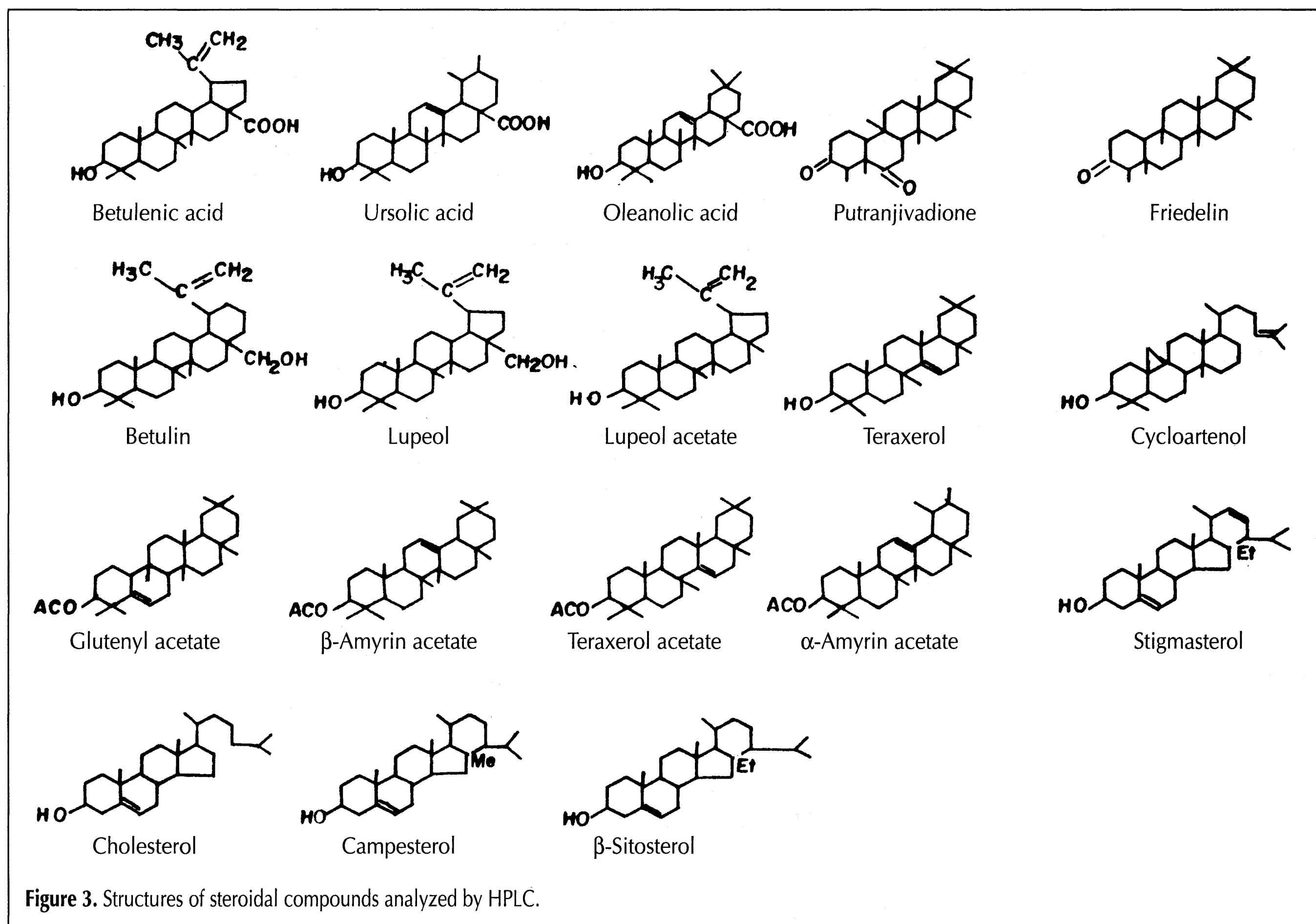

Figure 3. Structures of steroidal compounds analyzed by HPLC.

Acetates

Acetates appeared after their respective sterols. Among the acetates of similar structure, those with *gem*-dimethyl on ring E eluted first, followed by acetates with a double bond in ring B, followed then by acetates with a double bond in ring C, then those with a double bond in ring D, and finally, those with *vic*-dimethyl on ring E.

Sterols (Five Ring)

Among five-ring sterols, those with a five-membered ring E and a substituent eluted first, followed by those with a six-membered ring E and *gem*-dimethyl, followed then by those with a double bond in ring D.

Sterols (Four Ring)

Those with a C_3 ring on ring B eluted first, followed by those with a double bond in a C_8 side chain, then those with a double bond in ring B and another in the side chain, followed by those with a saturated alkyl chain, etc. Among the sterols with a double bond in ring B, those with the lowest carbon number side chain eluted first, followed by those with the next higher carbon number side chain.

The HPLC-based analytical method that was developed for the resolution of steroid mixtures is highly versatile and sensitive to steroid structures. The method resolves not only different classes of steroids but also the individual components of each class. In the case of unknown steroids, the retention time data could also yield useful information to help arrive at a tentative empirical structure (e.g., the number and size of rings, position of double bonds, polar groups such as OH, OAc, COOH, etc.) for the molecule.

The chromatogram (Figure 1) of the standard reference compounds can be divided into definite regions that are representative of particular classes of steroids (i.e., the region up to 10.4 min for acids; up to 16 min for dione, ketone, and diol; sterols and acetates appeared beyond 48 min). The region between 16 and 48 min could be further exploited for other structurally similar components. This demarcation of retention times was used while we looked for steroids in the biocrude from potential petrocrops.

The solvent sequence for the extraction of biocrude from plants (i.e., extraction with acetone followed by benzene) was selected under the assumption that acetone extraction would remove the unwanted chlorophyll and the highly polar oxygenated components, while subsequent extraction with benzene would yield the desired concentration of steroids and terpenoids. Although HPLC could not be used directly for analyzing these extracts because of their very complex nature, subsequent fractionation of these extracts by column chromatography yielded simplified fractions that could be analyzed by this method (Figure 2). The results that were obtained were contrary to this assumption (i.e., biocrude material, which is rich in steroids, was extracted in acetone, while benzene extracted mainly the polars). In some plants, there was practically no separation based on extraction by the two solvents; both

extracted similar components (suggested by the similarity of chromatograms of the fractions from the two extracts).

These studies led to a change in solvents for extraction (i.e., the extraction of a plant shrub with hexane followed by methanol). The respective extracts, when subjected to HPLC, suggested that all the biocrude was extracted by hexane, leaving behind mainly the polars in the methanol extracts. The hexane extracts were then subjected to cracking for the study of their conversion into fuels.

Conclusion

A method based on HPLC was developed for the analysis of steroids in highly complex mixtures. This very versatile method is sensitive to even the smallest details of the structure of steroidal molecules. The chromatogram suggests that there is scope for resolution of other types of steroids and terpenoids not included in the present studies. The method was successfully applied to the screening of potential petrocrops and finalization of the solvent sequence for the extraction of biocrudes from plants.

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