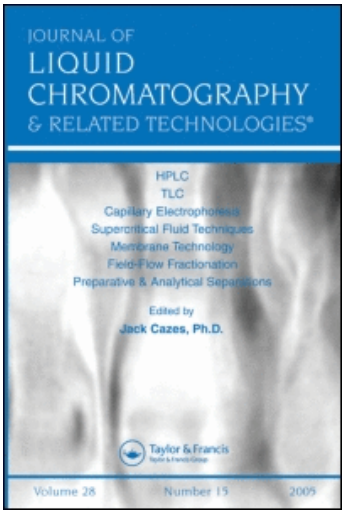


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# Separation of the Main Neutral Lipids into Classes and Species by PR-HPLC and UV Detection

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# SEPARATION OF THE MAIN NEUTRAL LIPIDS INTO CLASSES AND SPECIES BY PR-HPLC AND UV DETECTION

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ABSTRACT

A reversed phase high performance liquid chromatographic method is described that allows separation and estimation of the main neutral lipid classes as well as several species of each class.

By the represented method waxes, hydrocarbons, fatty acids and their methyl esters, sterols and their esters, free glycerylethers, fatty alcohols, vitamin E and mono-, di- and triglycerides are separated into classes and into class species within 55min.

A stepped gradient elution with methanol/water, acetonitrile/methanol, acetonitrile/tetrahydrofuran and isopropanol/acetonitrile was performed onto a reverse phase C18 HPLC column and the effluent was monitored by UV detection at 206 nm.

Application of the method in a plant extract and in a vegetable oil sample is represented.

## INTRODUCTION

Both normal (NP-HPLC) and reversed phase (RP-HPLC) high performance liquid chromatographic (HPLC) methods have been used for the separation of neutral and polar lipids from various plants and animal tissues. NP-HPLC is used for the separation of the lipid mixtures into classes while molecular species of each class are usually separated by the RP-HPLC mode.

In the last two decades several NP-HPLC systems have been reported for the separation of neutral lipid classes (1-9) or for both neutral and polar lipid classes (11-19) by using silica based columns of different types. Also, different detection techniques have been applied with the above NP-HPLC systems. For the analyses of mixtures containing the most lipid classes the ultraviolet (UV) detection was usually used in the 205-215nm region, in combination with gradient elution (4, 5, 16) or flow programming and isocratic elution (11), while UV-diode array detection at 190-350nm (9) or 200-400nm (13) was also used with gradient elution. Refractive index (RI) detectors, have been used for the analyses of less number of lipid classes (3,6-8) because they are compatible only with isocratic elution, while infrared (IR) detectors, compatible with gradient elution but requiring compatible solvents, have been rarely employed (2,7).

Lipid mixtures consisted of neutral (1) and phospholipid (19) or both phospholipid and glycolipid (10, 12, 14, 15, 17, 18) classes have been analyzed and quantitated in low concentrations by the flame ionization detection (FID) (1,10, 18) and the light scattering detection (LSD) (12, 14, 15, 17, 19) modes, which considered as "mass" or "universals" without limitations in gradient and solvents.

For the subsequent separation of each individual lipid class into species the reverse phase mode is required. Many RP-HPLC techniques have been developed for the separation of neutral and polar lipid species of an individual class. Such techniques have been separately reported for: waxes (W) (20, 21), long chain hydrocarbons (RH) (22, 23), fatty acids (FA) and their methylesters (FAME) (24, 25), sterols (ST) and their esters (ST.E) (26-33), glycerylether (GE) esters and derivatives (34-38), monoglycerides (MG) and diglycerides (DG) (34-40), fatty alcohols (F.Al.) (41-44) and triglycerides (TG) (33, 45-53).

Vitamin E (VIT. E) species, α-, β-, γ- and δ-tocopherols, have also been analyzed by RP-HPLC systems by UV, at 215 and 280 nm, (53) or fluorometric (FL) (33) detection. The detection of the other referred neutral lipid species analyzed by RP-HPLC was performed by using UV, RI, FL, FID and LSD techniques which are partially reviewed in the Results and Discussion section.

To our knowledge no method has been appeared, till now, describing the simultaneous separation of lipids into classes and species except of an our recent report referred to the separation of synthetic antioxidants, tocopherols and TG in vegetable oils (53) and α-tocopherol, free and esterified cholesterols and TG in human lipoproteins (33) by RP-HPLC and UV/UV-diode array (53) and UV/FL (33) detection.

In the present report a relative easy to run method is described which allows the simultaneous separation of a complex mixture of lipids into classes and species with estimation by UV detection especially useful in preliminary investigation and classification of specific complex natural mixtures and which can be easily applied with no highly sophisticated laboratory equipments.

Several neutral lipid standards as well as a lipid extract from nettle leaves and roots and a soya bean oil sample were analyzed by the represented method. Also, HPLC and detection techniques of the referenced methods are partially reviewed.

## EXPERIMENTAL

### Materials

All reagents used were of analytical grade purchased from Merck (Darmstadt, G) while HPLC grade solvents were from Fluka (Buchs, CH) and the water was Nanopure grade. Standard lipids were purchased from Sigma (St. Louis, MO, USA), Merck and Serva (Heidelberg, G). The oil sample used was a commercial soyabean oil (Athens, GR). Nettle (Urtica dioica) was collected from countryside (Athens, Attica, GR).



### Standard and sample preparation

All standards were prepared as 5% solutions except TG, ST and vitamin E as 1% solutions, in chloroform/methanol (1:1). Soyabean oil was prepared as 5% solution in chloroform/methanol (9:1). Homogenized nettle leaves and roots (approximately 200g) were extracted (54) and neutral lipids were separated from chlorophylles and phospholipids by thin layer chromatography (TLC) using the solvent system petroleum ether/benzene/acetic acid (30:70:2) (55). Neutral lipids as overlapped bands were scrapped off from the TLC plate and extracted according to Bligh-Dyer (54). Counter-current distribution (56) was also used for removing polar lipids traces. The final solution was evaporated in flash evaporator and the remained total neutral lipids were redissolved in a small volume of chloroform/methanol (1:1) which was used for the HPLC.

Chromatography

The liquid chromatographic system consisted of a dual pump Jasco (Tokyo, Japan) model 880-PU HPLC, supplied with a 330µl loop Reodyne (P/N 7125-047) injector, connected to a Jasco model 875-UV detector and a Hewlett-Packard (Avondale, PA,U.S.A.) model HP-3396A integrator-plotter. A Nucleosil-300, C18 column, (7µ, 250x4mm I.D) from Analysentechnik (Mainz, G) was used. The flow rate was 1ml/min and the detection at 206nm (0.4 a.u.f.s) except vitamin E at 280nm (0.4 a.u.f.s). The solvent gradients used are shown in Figs. 1, 2 and 3 with the following eluting solvents: A, methanol/water (80:20); B, acetonitrile/methanol (60:40); C, acetonitrile/tetrahydrofuran (99.5:0.5) and D, isopropanol/acetonitrile (99:1). Volumes 2-200µl of groups of standards were injected each time. Each group contained the species of one lipid class and the 1% or 5% solutions of each group were injected as following:

Vitamin E, 2µl; FA., FAME and ST, 10µl; W, RH, DG, ST.E. and TG, 20µl; GE, 50µl; MG, 150µl and F.Al, 200µl.



## RESULTS AND DISCUSSION

The analysis of standards was performed into groups (Fig. 1.). Each group consisted of the species of one (or two) neutral lipid class(es) and the injected amount of each group was the optimum for the UV detection limit of the used instrumentation. Injection of a solution containing the total of the used standards was not used in order to avoid overloading the column.

Before the analysis of each group, each individual standard species was injected separately and the resulted main peak was collected and co-chromatographed on TLC plates with authentic standard in order to con-

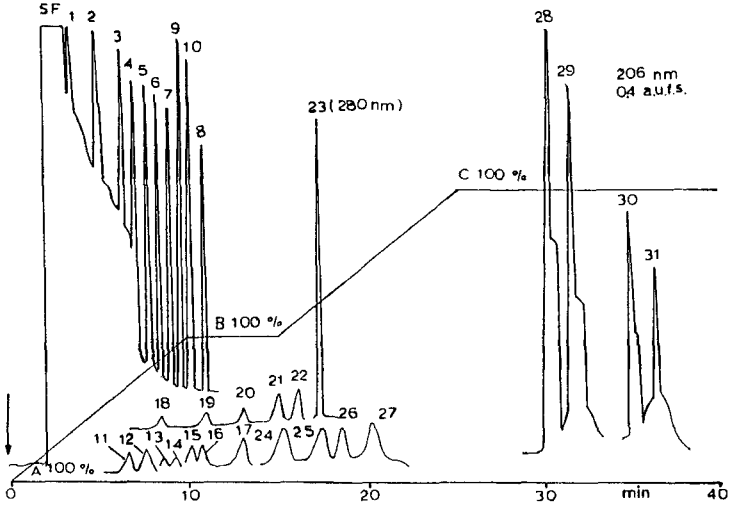


FIGURE 1. HPLC chromatogram(s) of neutral lipid standards. Conditions and solvents A, B and C in Experimental section. Gradient as indicated. Peak identification in Table 1. SF: solvent front.



firm the elution and retention time (RT). The RT's of the separately injected standards are summarized in Table 1.

Several mobil phase combinations were developed to achieve a quite satisfactory separation in a reasonable time. The final chosen solvent systems and gradients resulted in an effective separation within 40min (gradient with solvents A, B and C) or 55min (gradient with solvents A, B, C and D). A linear gradient from solvent A to solvent B in 10min, a hold for 5min in B and then a linear gradient to solvent C in 10min followed by a second hold in C for 15min resulted in a total separation within 40min. By introducing a third linear gradient step from solvent C (decreasing the hold step to 5min), to solvent D in another 10min and holding D for

TABLE 1

RT's of Individual Standards

| No | Classes | Species | RT(min) |
|---|---|---|---|
| 1 | W | Cetiolate | 3.50 |
| 2 | RH | Cycloheptane | 5.28 |
| 3 | RH | Cyclododecane | 6.85 |
| 4 | RH | Cyclodecatriene | 7.00 |
| 5 | FA | Linoleic acid | 7.51 |
| 6 | FA | Palmitoleic acid | 7.90 |
| 7 | FA | Oleic acid | 8.44 |
| 8 | FA | Arachidic acid | 10.40 |
| 9 | FAME | Methylpalmitate | 9.14 |
| 10 | FAME | Methylstearate | 9.80 |
| 11 | MG | α-Monopalmitine | 6.73 |
| 12 | MG | α-Monostearine | 8.05 |
| 13 | F.Al | Cetyl alcohol | 8.61 |
| 14 | F.Al | Behenyl alcohol | 9.63 |
| 15 | GE | Celachyl alcohol | 9.74 |
| 16 | GE | Chimyl alcohol | 11.80 |
| 17 | GE | Batyl alcohol | 13.20 |
| 18 | ST | Desmosterol | 8.69 |
| 19 | ST | Ergosterol | 11.30 |
| 20 | ST | Cholesterol | 13.00 |
| 21 | ST | Stigmasterol | 15.00 |
| 22 | ST | β-Sitosterol | 16.34 |
| 23 | VIT.E | Vitamin E | 17.00 |
| 24 | DG | Diolein | 15.62 |
| 25 | DG | (α,β)-Dipalmitine | 17.80 |
| 26 | DG | (α,γ)-Dipalmitine | 18.84 |
| 27 | DG | (α,β)-Distearine | 20.55 |
| 28 | TG | Trilinolein | 30.00 |
| 29 | TG | Triolein | 31.60 |
| 30 | ST.E | Cholesteryl linoleate | 34.80 |
| 31 | ST.E | Cholesteryl palmitate | 36.20 |

15min, a more distinct separation of TG can be achieved (Fig. 3). The first and the second hold of the gradients are also used for changing the solvent chambers from solvent A to C and from solvent B to D, respectively. These manipulations are required when a two pump delivery HPLC system is used. Tetrahydrofuran and aceto-

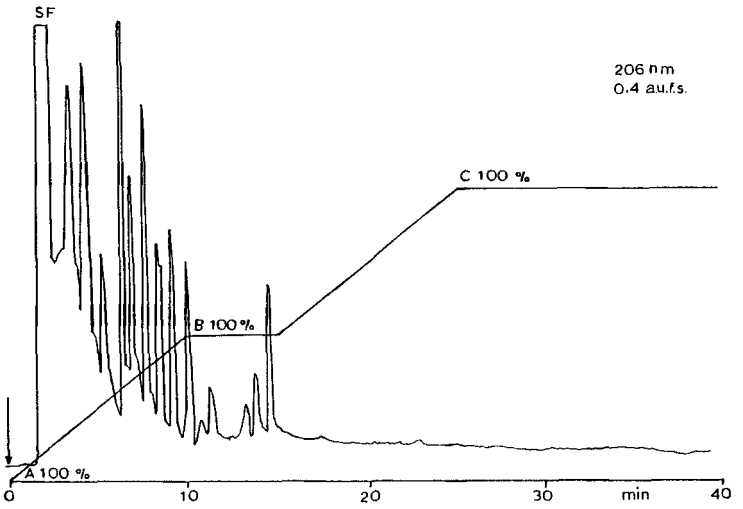


FIGURE 2. HPLC chromatogram of a neutral lipid fraction from nettle (Urtica dioica) leaves and roots extracts. Conditions as in Fig. 1 and in Experimental section. SF:solvent front.

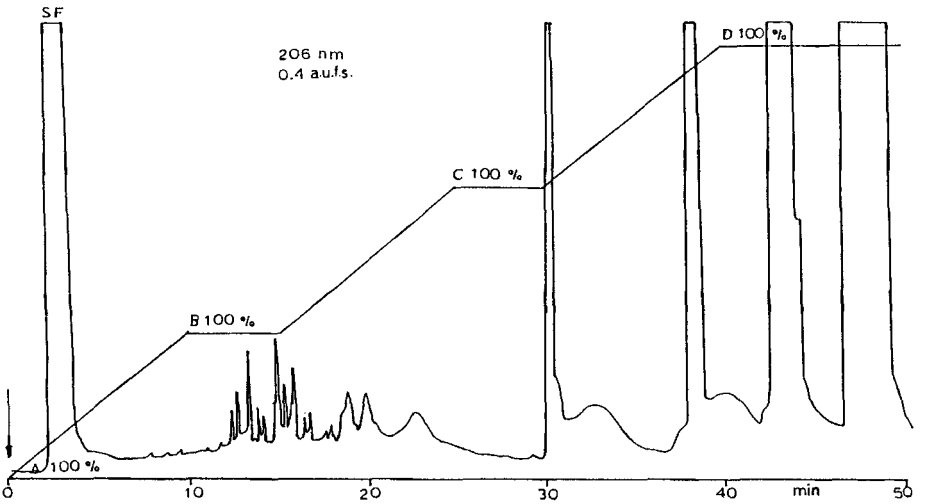


FIGURE 3. HPLC chromatogram of soyabean oil solution (5%). Conditions and solvents A, B, C and D in Experimental section. Gradient as indicated. SF:solvent front.

nitrile were used as minor constituents in solvents C and D, respectively for equilibration of the gradient and keep the baseline in low shift during the chromatographic run. An acid modifier (e. g. phosphoric acid) was not used in solvent A, for sharpening the peaks, because the method is recommended for further examination of the collected peaks in biological experiments (55). For the next injection a 15min equilibration step with solvent A is required. Cyclohexane or n-hexane can also be used for cleaning up the column for the possible nonpolar remained constituents of a natural source chromatographed sample. In the latter case a pre-equilibration step for 5min with solvent D is required.



In Fig. 1 are shown the separations of standard species of W, RH, FA, FAME, F.Al, ST, GE, VIT.E, MG, DG, TG and ST.E classes, which are summarized in Table 1. In the present method the used W and RH species comigrated almost with solvent front and even with low resolution and sensitivity the method offers an alternative choice of analysis, by UV detection, to the previous reported methods. In the previous methods W and RH species are usually quantitated by other techniques than UV detection because of their low absorptivity in the UV region. n-Alkane species have been separated by RP-HPLC using direct (22) or indirect (23) RI detection and wax-ester species have been quantitated by LC-GC coupled systems (20, 21) while RH classes (7, 13) and carotenoids (13) have been analyzed by NP-HPLC and RI (7) or UV-diode array detection (13).

The achieved separation of the used FA and FAME species by the present method, is comparable to the previous reported methods for FA and FAME with UV (24) or FL (25) detection. Free ST species from natural sources (oils and plants) have been separated by RP-HPLC within 30min, at 205 nm UV detection (26-29) with similar pat-

terns to the present method. The simultaneous analyses of cholesteryl esters and TG species have been previously performed by RP-HPLC and UV detection (33) and the individual peaks with good resolution were also represented superimposed. In the present method cholesteryl esters accumulated after the TG fraction but can be collected and further quantitated by other previous reported methods for plant and animal tissues (30, 31) and human plasma (32, 33). VIT.E standard was detected at 280nm and eluted as a single peak with RT similar to the RT of an our previous reported RP-HPLC method (53). Derivatized DG (34-40) and underivatized MG and DG (39) species have been analyzed using UV (35, 36, 38-40), FL (36, 37) or RI (39) detection with elution times comparable to the present method. Underivatized (8) and derivatized (34, 35) GE-ester subclasses have been analyzed by NP-HPLC with RI (8) or UV (34, 35) detection, while derivatized GE-ester species have been separated by RP-HPLC within 30min by UV (35, 36, 38) or FL (36) detection. In the present method is represented an analysis of the underivatized free GE species for the first time. Free F.Al have been previously analyzed by RP-HPLC and direct RI (41, 44) and indirect (42) detection. UV is not usually employed for the detection of F.Al e.g. there is a reference (43) for the effective detection of fatty diols at 200nm. In the present method although relative large amounts of the injected samples were required the detection of free F.Al was realized for the first time at 206nm. MG, DG, GE and F.Al can almost be detected by the used technique with almost appeared peaks even with large amounts of injected sample but the method has the advantage of using underivatized samples in the presence of other components from natural sources. Detailed separations of TG from plants and oils have been reported by RP-HPLC (45, 47, 49-53), silver-

ion HPLC (57-59) and NP-HPLC (60) by using UV (33, 45, 53, 60), RI (47, 50) and LSD or FID detection (49, 51, 52, 57-59). The TG patterns of the present method are comparable to those obtained by a method of semipreparative fractionation of TG, but having longer RT's (47). Finally phospholipids coeluted with solvent front can be collected and separated by HPLC in classes by UV detection (61) and further into class species by FL (62), RI (63) or UV (64) detection.

Applications of the described method are represented in Figs. 2 and 3. A lipid extract from nettle leaves and roots was separated as shown in Fig 2 by using the gradient with the solvents A, B and C. In Fig. 3 soyabean oil was analyzed by using the gradient with the solvents A, B, C and D. By increasing the sensitivity of the detection, several minor constituents of the oil eluted before TG's can be effectively detected. By introducing a fourth gradient step with the solvent D a further separation of TG's was achieved.

In the UV detection mode the detectability and the quantitative determination of each component is referred to its degree of unsaturation and/or to ester and carboxy configuration. These structures are absent or limited (with low extinction coefficients) in the W, RH, F.Al, GE, MG and DG molecules, but the relative large injected amounts shifts the absorptivity over the 200nm, near to the detectable area.

On the other hand UV detection, although requiring non UV-absorbing solvents in the detecting area, is compatible with gradient elution which is necessary for the effective separation of complex lipid mixtures. The recent commercially developed FID and LSD systems overcome the compatibility problems of the other detectors but they still have relative high purchasing, running and maintenance costs. For these reasons UV mode having

relative low cost and easy operation as compared to the resulted detecting informations, remains the most widespread detection technique. Even more UV detection could be the detection of choice when a preliminary separation of a complex natural mixture is required before a further detailed examination. These requirements meet the represented method by which all peaks from quite different in polarity components can be collected for further examination especially when the possible biological activity will be investigated (55).



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