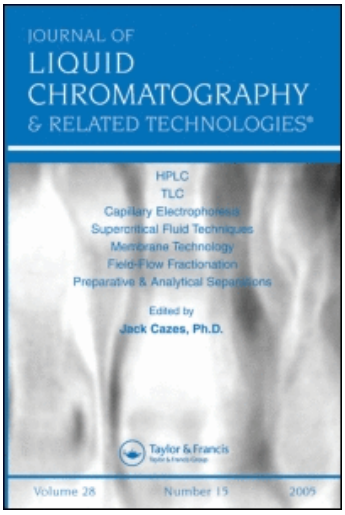

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Reversed-Phase High Performance Liquid Chromatography of Underivatized Fatty Acids by Fatty ACID Analysis Column

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REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF UNDERIVATIZED FATTY ACIDS BY FATTY ACID ANALYSIS COLUMN

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ABSTRACT

Underivatized fatty acids of mammalian source after group separation by thin layer chromatography were further separated among saturates and monounsaturates as a group, and polyunsaturates as another group by reversed phase μBondapak Fatty Acid Analysis Column. The mobile phases consisted of tetrahydrofuran-acetonitrile-water-acetic acid in ratios of 25:35:X:Y (v/v/v/v). When saturates (from C12 through C24) and monounsaturates (from C14:1 through C24:1) were analyzed, X=50 and Y=0.2 were the best combination; When polyunsaturates (from C18:2 through C22:6) were chromatographed, X=70 and Y=0.3 provided the best resolution. Saturates were monitored by refractive index (RI) at 4X and UV 213 nm. Monoenoic acids were scanned at UV 207nm. Polyenoic fatty acids were analyzed at UV 210 nm and 235 nm. The 235 nm was for detection of possible peroxidation of those polyenoic acids. The minimal detection levels for saturates, monoenes, and polyenes were about 20 μg, 2μg, and 0.2μg at 0.01 aufs.

INTRODUCTION

In most cases chromatographic analysis of fatty acids are accomplished indirectly by derivatization into methyl esters (1-6), phenacyl esters (7), p-bromophenacyl esters(8), and other esters (9-11). They then are analyzed by gas liquid chromatography(1,3), gas chromatography-mass spectrometry(2), capillary gas liquid chromatography(5), and a variety

of high performance liquid chromatography (HPLC) methods (1-8). However, these derivatization-dependent methods sometimes caused the modification of unsaturated fatty acids (12) and also risked the formation of artifacts (13). The only direct method for fatty acids analysis is by siliver nitrate impregnated argentation thin layer chromatography (1,14,15). Nonetheless, argentation chromatography has only limited use in routine analysis. Therefore non-esterification has been a prefered approach (16). Recent advances in HPLC have made it possible that free fatty acids can be separated and quantitated directly by HPLC (7,13,16-21). For example, reversed phase HPLC has been used for separation of volatile fatty acids (21), for skin long chain fatty acids (7) and for fatty acid composition analysis of margarines (20).

Fatty Acid Column has offered good results of direct fatty acid analysis (5,18,20). The present study was undertaken to improve the separation profile of commonly encountered free fatty acids by the Fatty Acid Analysis column after the fatty acids have been separated in groups by TLC (22).

MATERIALS AND METHODS

CHEMICALS.

Reference fatty acids were obtained either from Sigma Chemical Company, St Louis, MO. or from Matreya, Pleasant Gap, PA. They were used as received. Saturated fatty acids were dissolved in hydroperoxide-free tetrahydrofuran(THF), 10 mg per ml. Monounsaturates and polyunsaturates were dissolved in THF/acetonitrile(MeCN) (1:1), 1 mg/ml and 0.5 mg/ml. The polyene standards usually come in chloroform solution. Chloroform even in trace amount gives broaden void volume under the present HPLC conditions, therefore chloroform has to be dried out with nitrogen before lipid standards are dissolved in THF/MeCN (1:1) solution.

THIN LAYER CHROMATOGRAPHY.

Precoated thin layer plates of silica gel 20x20 cm, 250 μm thick, silica gel 60, F-254 were purchased from EM Science, Cincinnatti, OH. Uniplates of Analtech, Newark. DE. were also used in the experiments. Fatty acids of rat tissues after separated from total lipids by TLC in solvents hexane/diethyl ether/acetic acid (70:30:1, v/v/v) were further separated in groups by hexane/acetic acid/water (100:5:2.5) (22).

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.

Waters (Waters Chromatography Division of Millipore, Milford, MA.) HPLC equipments including pump model M6000A, injector model U6K, Fatty Acid Analysis column (3.9 mm x 30 cm), photodiode array detector model 990, APC 111 of NEC computer and plotter model 990 were sequentially streamlined. Effluent of mobile phase was monitored first by UV detector from 200 nm through 250 nm and then by RI at 4X of Waters Differential Refractometer model R401. UV responses at 207, 210, 213 and 235 nm were analyzed and printed. Spectral analysis of each absorption peak was performed. All these analyses were done using Waters' software Ver. 4.08, APC III 990 Plus, P/N 021765.

Mobile phase consisting of THF-MeCN-water-acetic acid was a modification of Manku (5), King et al (18) and Baile et al (20). The organic solvents were from Burdick-Jackson, Muskegon, MI. THF in quart is preferable to larger size for minimizing peroxide formation. Water was deionized and distilled. Prior to use water was boiled to purge trapped air and cooled to room temperature. Inadequate purge may cause RI baseline drifting downward. Mobile phases were designed according to the degree of unsaturation of fatty acids to be analyzed. Mobile phases were in the ratios of 25:35:50:0.2 (system I), 25:35:70:0.3 (system II), and 25:35:75:0.4 (system III). For saturates and monounsaturates the system I was used and the eluent was monitored at RI at 4X, 213 nm and 207 nm; for polyenoic fatty acids the system II was employed and scanned at 210 nm and 235 nm. For semipreparation of polyunsaturates mobile phase of 25:,35:75:0.4 (system III) may be used.

PROCESS OF TISSUES FOR FREE FATTY ACIDS.

Six Sprague-Dawley male rats of 250-300 g after on Purina Laboratory chow for a month were sacrificed for brain, liver, kidney, and heart. Tissues were processed for lipids according to Folch et al (23). Free fatty acids were group separated by TLC (22). The TLC method is briefly stated here. On a 20x20 cm silica gel F-254 plate streak 5-10 mg tissue free fatty acid sample. The plate is then developed in a solvent mixture hexane/acetic acid/water (100:5:2.5) for 5 times. The saturates and monounsaturates migrate to top zone and polyunsaturates to the lower zone. The recovered groups of free fatty acids were brought up to a concentration equivlent to 1 g fresh tissue in 0.05 ml of THF/MeCN (1:1) solution.

RESULTS AND DISCUSSION

Separation of saturated, 50 μg each, and monoenoic fatty acids, 12.5 μg each, from 12 carbon skeleton through 24 carbon skeleton was achieved on a Fatty Acid Analysis column by mobile phase System I. The chromatography of 12:0, 14:1, 14:0, 16:1, 16:0, 18:1, 18:0, 20:1, 20:0, 22:1, 22:0, 24:1, and 24:0 was completed in 100 min with a flow rate at 1 ml/min and pump pressure at 1,500 psi (Fig. 1). The separations were printed under three wavelengths 207, 210 and 213 nm. Saturates and monounsaturates of shorter chains were eluted earlier than longer chains. A given saturated fatty acid of x chain length was immediately followed by a monoenoic acid of x+2. They appeared as a pair, i.e. 12:0 and 14:1, 14:0 and 16:1, 16:0 and 18:1, etc. These pairs were reported inseparable previously using Fatty Acid column (17). Their esters had been referred as compounds of equivalent chain length (ECL) and have been partially separated by reversed phase HPLC (4).

In analyzing the spectra of those monounsaturates and saturates the monosaturates have maximal absorption at 204-207 nm (Fig. 2A), while saturates have absorption peaks from 210-215 (Fig. 2B). Polyenoic fatty acids, 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3 and 22:6n-3, 0.5 μg each, were selectively printed at the same three wavelengths (Fig. 3). Their spectra revealed very strong UV absorption from 209-212 nm (Fig. 2C)

The RI responses of saturates at 4X were stronger than at UV 213, while monounsaturates responded better at 207 nm than at RI 4X. These are illustrated in the section of tissue fatty acid HPLC analysis.

Tissues free fatty acids and some reference fatty acids are used to demonstrate the TLC group fatty acid separation method. In Fig. 4. the top group contains long chain saturates: 14:0, 16:0, 18:0, and long chain monosaturates: 16:1, 18:1, the lower contains 18:2, 18:3, 20:4, 20:5, and 22:6. There are separated fatty acid groups of kidney and liver in the 4 middle lanes. These were the pre-grouped fatty acids for HPLC analysis.

Rat brain free fatty acid, 25 μl equivalent to 0.5 g fresh tissue, was injected for saturates and monounsaturates by system I (25:35:50:02)(Fig. 5A). The elution profile of RI at 4X, and UV 207 nm and 213 nm indicated there were 16:1, 18:1. A further chromatography by system II (25:35:70:0.3) revealed 18:2, 20:4 and 22:6 (Fig. 6A). Rat heart free fatty acids were chromatographed as in Fig 5B, which showed the presence of 18:1 and 18:0 by mobile phase system I. There were also 18:3, 20:5, 18:2, 20:4, and 22:6

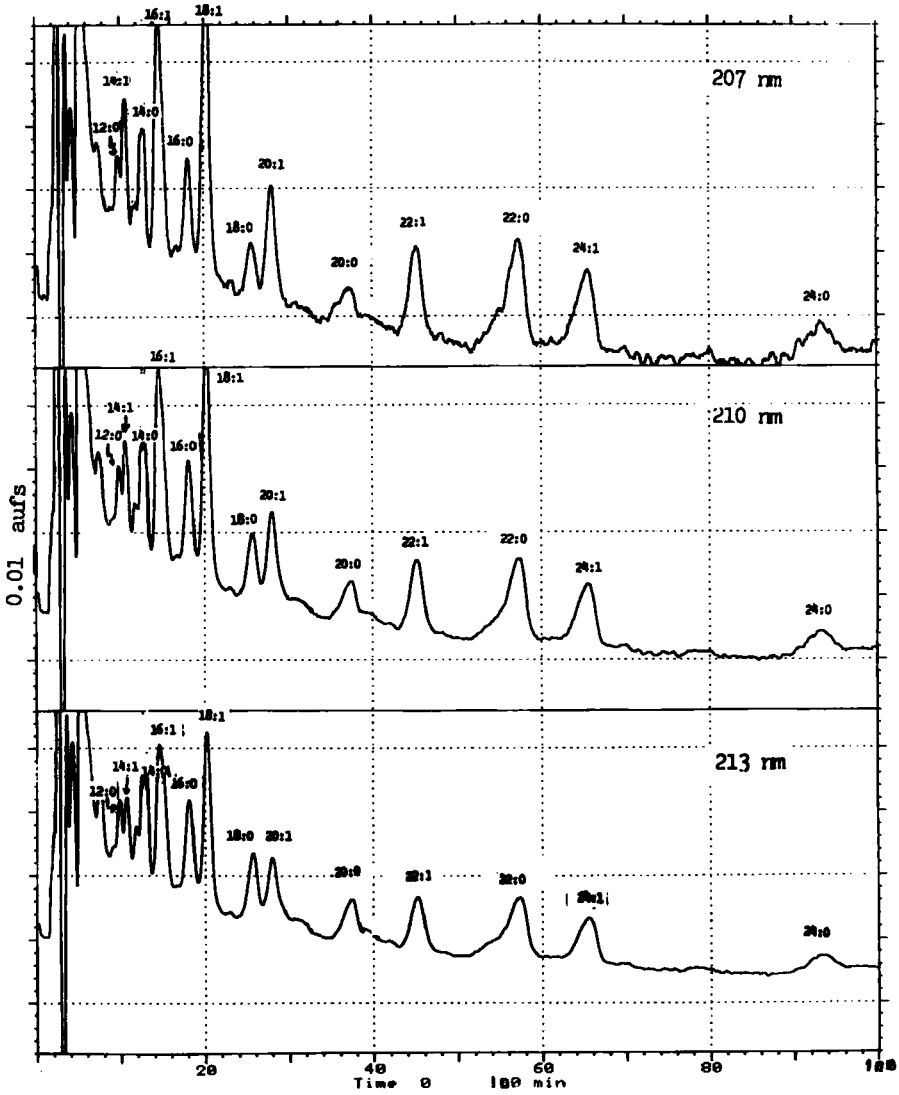

Fig.1. Chromatograms of saturated and monounsaturated fatty acids at wavelengths 207 nm, 210 nm and 213 nm on a Fatty Acid Analysis column using mobile phase system I, THF/MeCN/water/acetic acid (25:35:50:0.2) at a flow rate of 1 ml/min. Amounts injected: for saturates were 50 μg each, for monounsaturates 12.5 μg each.

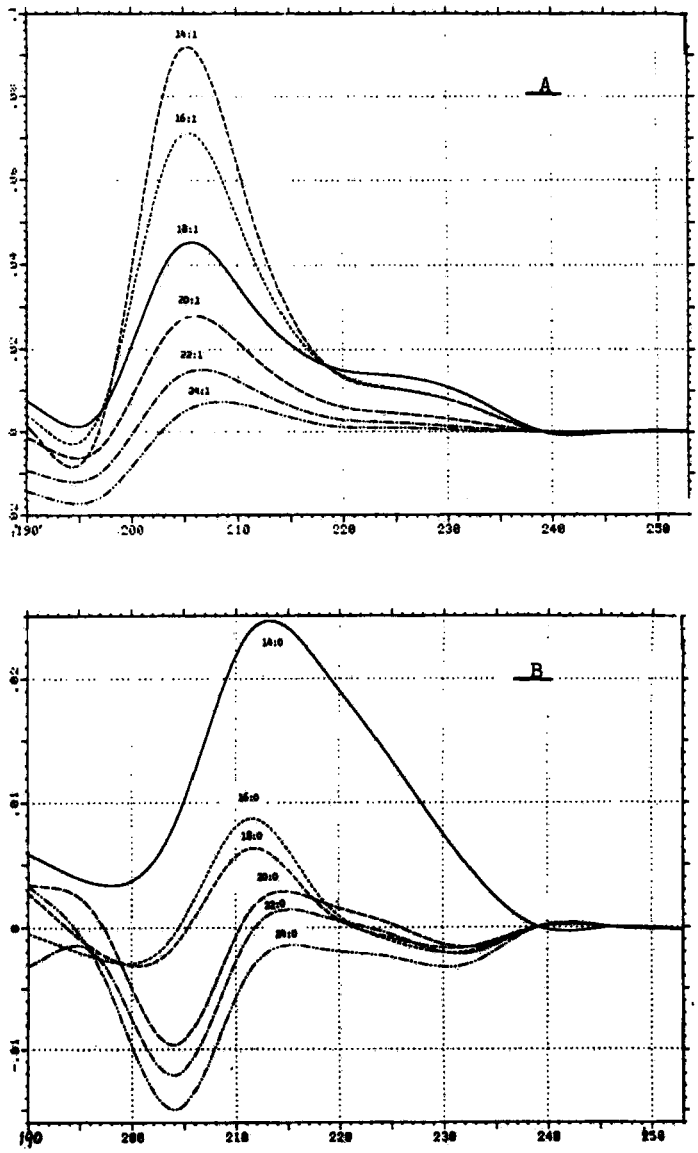

Fig.2. Spectral analyses of HPLC separated fatty acids. Panel A, saturated fatty acids; Panel B monounsaturated fatty acids; Panel C, polyunsaturated fatty acids.

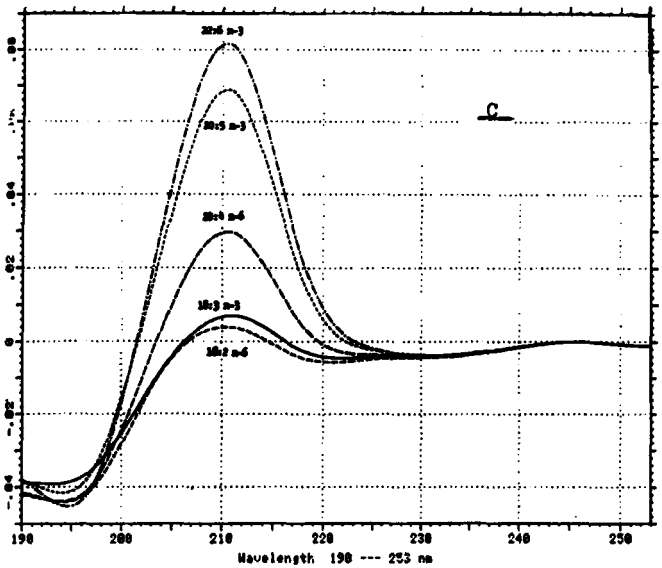

Fig. 2 (continued)

by system II (Fig. 6B). Rat kidney free fatty acids chromatographed by system I (Fig. 5C) identified the presence of 14:1, 14:0, 18:1, and 18:0. The polyenoic fatty acid group was separated by system II (Fig. 6C) into 18:3, 20:5, 18:2, and 20:4. Rat liver free fatty acids when chromatographed with system I(Fig. 5D) most evident peaks were 14:0, 16:1, and 18:1. There was no any longer chain fatty acid after 18:1. The nature of the liver free fatty acids was revealed by system II (25:35:70:0.3) (Fig. 6D). This chromatogram showed the prensence of 18:3, 20:5, 18:2, and 20:4.

In conclusion, The expense and bother of argentation TLC is avoided by use of the preferred, alternative ternary solvent system hexane-acetic acid-water (100:5:2.5, v/v/v), that effectively resolves saturated and monounsaturated fatty acids from PUFA and accords group separations suitable for subsaquent HPLC analysis. The combination of TLC and HPLC thus avoids the problems of overlap of some fatty acids experienced by others, for instance, 16:0/18:2 (5,18) and 16:1/18:3 (18, 20) on a HPLC chromatogram. The discriminately use of different mobile phases strategically made separation possible among saturates, monounsaturates, and polyunsaturates. The lower UV detection from 207 nm

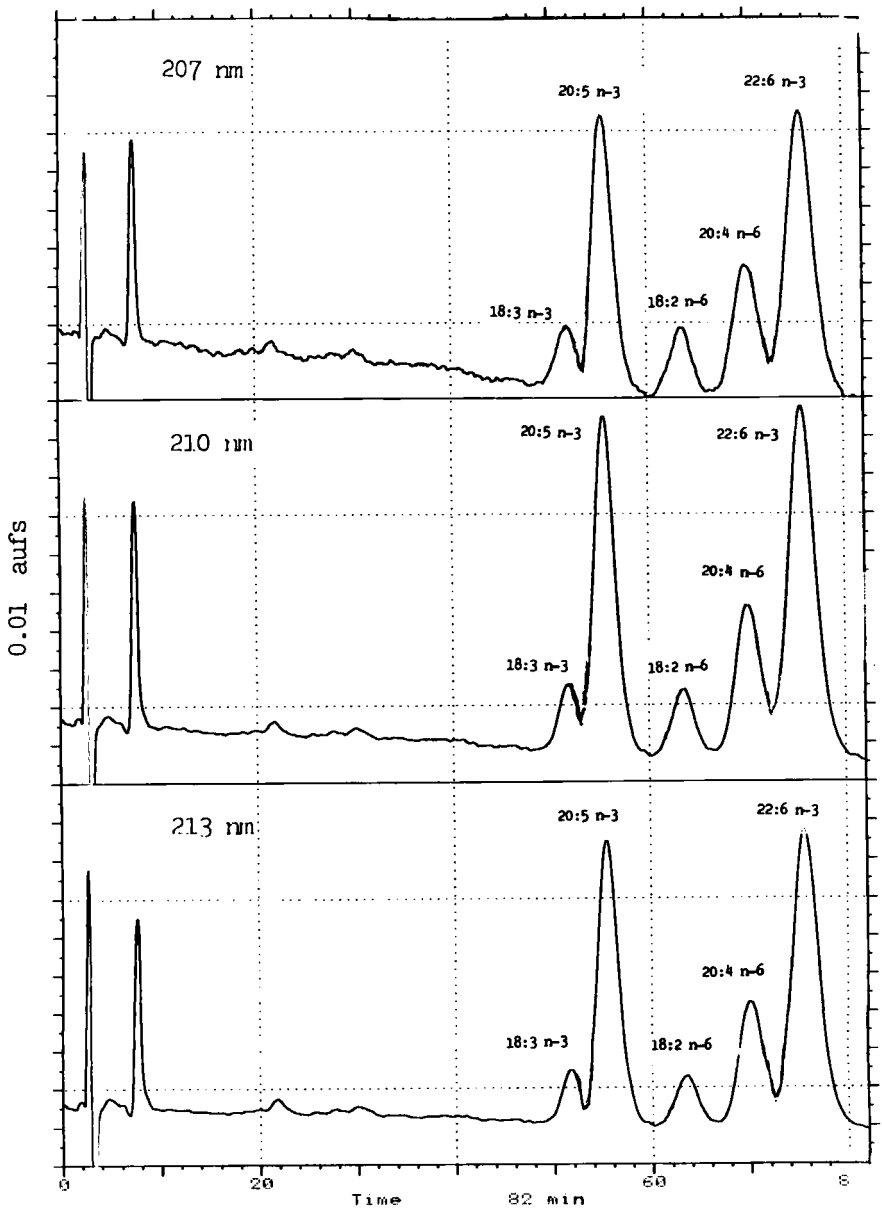

Fig.3. Chromatograms of polyunsaturated fatty acids at wavelengths of 207 nm, 210 nm and 213 nm on a Fatty Acid Analysis column using mobile phase system II, THF/MeCN/water/acetic acid (25:35:70:0.3) at a flow rate of 1 ml/min. Amounts injected: 0.5 ug each.

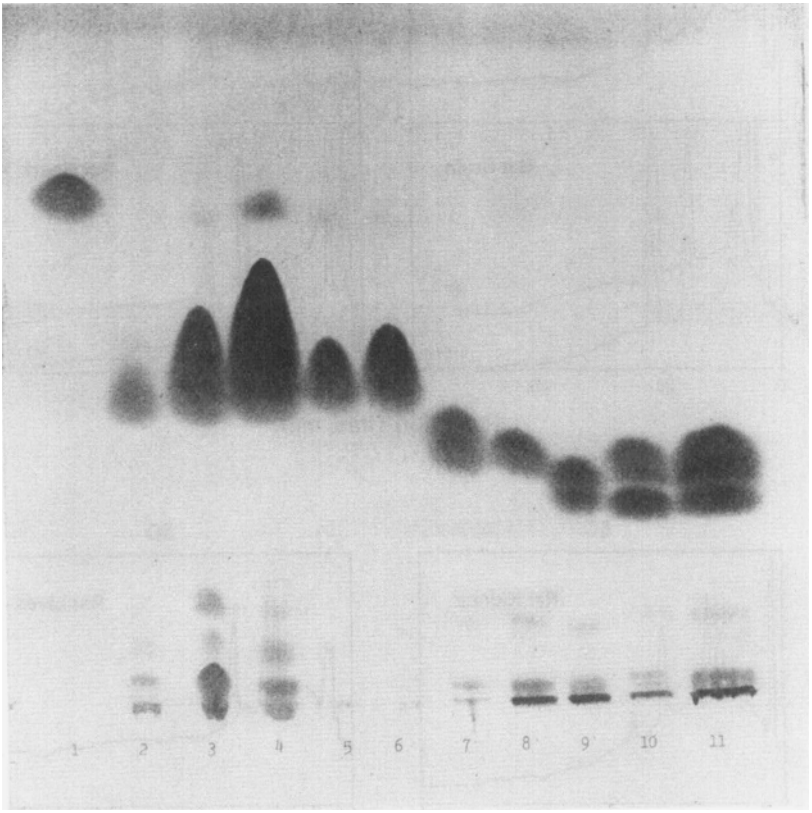

Fig.4. Reference fatty acids and rat tissue free fatty acid groups separation by thin layer chromatography. Solvents: hexane/acetic acid/water (100:5:2.5, v/v/), 5 times ascending chromatography. Thin layer plate: Analtech, 250 μm silica gel layer. detection by iodine vapor, 1. methyl oleate, 2. 14:0, 3. 16:0 and 16:1, 4. 18:0 and 18:1, 5. rat liver saturates and monounsaturates, 6. rat kidney saturates and monounsaturates, 7. rat liver polyunsaturates, 8. rat kidney polyunsaturates, 9. 20:4n-6, and 20:5n-3, 10. 18:3n-3, 22:4n-6, 22:6n-3, 11. mixture of 9 and 10. The reference lipids have been used for quite some time. Those lower spots may be degradation products.

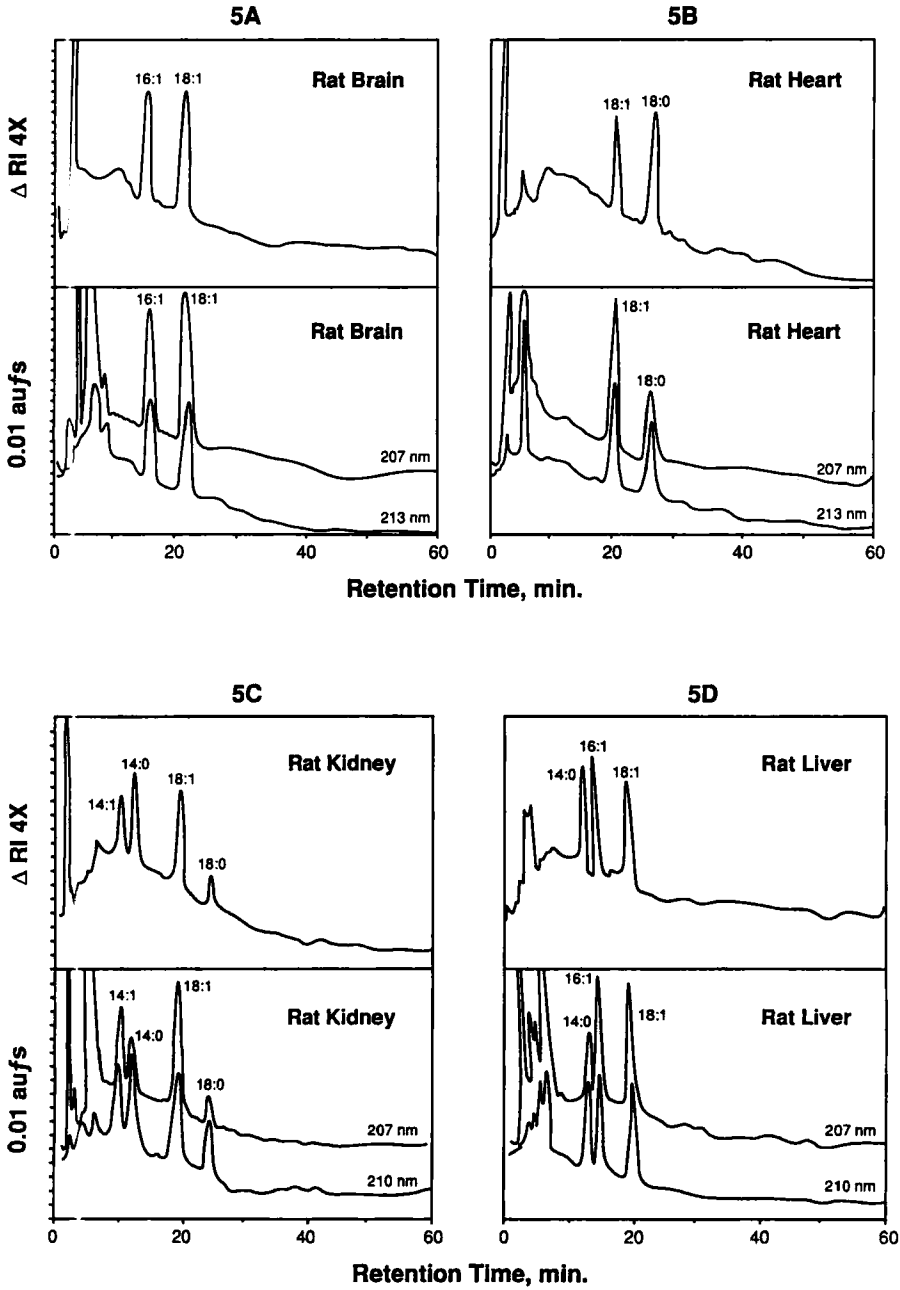

Fig.5. Rat tissues saturated and monounsaturated fatty acids by mobile phase system I, THF/MeCN/water/acetic acid (25:35:50:0.2), detection by RI at 4X and UV 207 and 213 nm. Fig. 5A (Rat Brain), Fig. 5B (Rat Heart), Fig. 5C (Rat Kidney), Fig. 5D (Rat Liver).

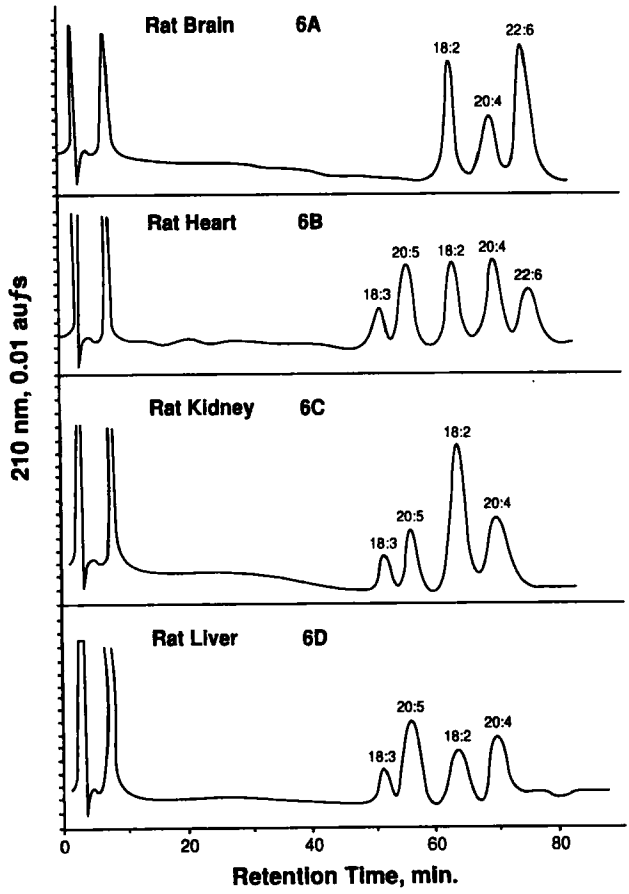

Fig.6. Rat tissues polyunsaturated fatty acids by mobile phase system II, THF/MeCN/water/acetic acid (25:35:70:0.3), detection by UV 210 nm. Fig. 6A (Rat Brain), Fig. 6B (Rat Heart), Fig. 6C (Rat Kidney), Fig. 6D (Rat Liver).

through 214 nm applied to specific degree of unsaturation increased absorption maxima. Refractive Index proved valuable and useful but could be cumbersome. If advantages of UV absorption can be fully explored, the refractive index may not be necessary.

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