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N.m.r. lipid profiles of cells, tissues and body fluids

Neutral, non-acidic and acidic phospholipid analysis of Bond Elut chromatographic fractions

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Although the advantages of *in vitro* n.m.r. analysis of cellular lipids have been documented, this purely n.m.r. approach also has drawbacks. Rapid and quantitative separation of total lipids into neutral lipids, non-esterified fatty acids, non-acidic phospholipids and acidic phospholipids using Bond Elut ion-exchange columns as demonstrated here permitted a more quantitative and complete n.m.r. analysis of glycerides, cholesterol, saturated and unsaturated sphingolipids and ether lipids, as well as of diacylcholine and ethanolamine lipids. Acidic lipids were also analysed. The fatty acid compositions of the intact lipids in each of the four Bond Elut fractions were determined from the n.m.r. spectrum of each fraction.

INTRODUCTION

In vitro lipid assays are increasingly used as a more rapid and comprehensive technique for monitoring lipid compositions of cells, body fluids and tissues. The commonly used techniques such as t.l.c. [1], h.p.l.c. [2], ion-exchange chromatography [3] and gas chromatography [4] provide a simultaneous detection and quantification of several lipids in a given sample or lipid class [5–7]. However, most lipid assays are time-consuming, and usually more than a single step is needed. N.m.r., on the other hand, has provided a rapid and comprehensive lipid assay. The relative quantification of phospholipids by one-dimensional ^{31}P n.m.r. spectra [8,9], and assignments of the one-dimensional (1D) and two-dimensional (2D) proton n.m.r. spectra of lipids from cell and tissue extracts have been reported [10–12].

Although this n.m.r. approach to lipid analysis is promising, there are several drawbacks still to be overcome. By applying a simple ion-exchange chromatographic procedure using commercial Bond Elut columns [13,14] prior to the n.m.r. assay, lipids are separated into four individual fractions according to their polarity. Thus improved analysis of individual lipids in each fraction can be achieved. The recovery after such separation was found to be high and reproducible, and the n.m.r. analysis of each fraction gave improved results both quantitatively and qualitatively because of reduced overlap and separation of low-abundance lipids from each other.

EXPERIMENTAL

Materials

Bond Elut (NH_2-aminopropyl) solid-phase extraction columns were purchased from Analytichem International. Deuterated chloroform (99.8%) and perdeuteromethanol (99.8%) were purchased from Aldrich, h.p.l.c.-grade hexane was from May and Baker, and ammonium acetate was from Pharmacos Ltd. All other solvents were reagent grade and were purchased from BDH.

Tissue extraction and Bond Elut separation methods

Procedures for liver lipid extraction were as described previously [10,11], except that 1.0 g of tissue was used instead of 0.5 g.

Bond Elut separations were done in triplicate. All column elutions were achieved in 5 min under low-speed centrifugation conditions (500 *g*) and the columns were washed with 8 ml of h.p.l.c.-grade dry hexane prior to application of the lipid extracts. The extracted lipid residue was redissolved in 1.0 ml of chloroform; 0.20 ml of dissolved lipids was then applied to each Bond Elut column and another 0.20 ml was retained as a control, for the determination of recovery after separations.

According to their different polarities, lipids were separated into four individual fractions [13,15] by passing different eluants through the columns in the following order: (1) chloroform (eluted non-polar lipids and cholesterols), (2) diethyl ether with 2% acetic acid (eluted non-esterified fatty acids), (3) methanol (eluted non-acidic phospholipids) and (4) 0.05 M ammonium acetate in chloroform/methanol (4:1, v/v) plus 2% (v/v) 28% aqueous ammonium solution (eluted acidic phospholipids). A volume of 2×4.0 ml of eluants was used for all elutions.

The 0.20 ml unseparated lipid extract and all eluates were evaporated under a stream of nitrogen. The residues were then resuspended in 0.6 ml of $C^2H_3O^2H/C^2HCl_3$ (2:1, v/v) and transferred to 5 mm n.m.r. tubes. Samples were bubbled with nitrogen prior to recording the spectrum.

Proton n.m.r. spectroscopy

Spectra were recorded using a Bruker AM500 n.m.r. spectrometer. The conditions and parameters for recording 1D proton spectra were described previously [10,11] 2D homonuclear shift-correlated experiments (COSYHG) were performed with the following pulse sequences: (1) low-powered pulse with relaxation delay of 25 s, (2) 90° pulse, (3) evolution, (4) 90° pulse and (5) detection. The decoupler was on during D1 in order to presaturate the water signal. The 2D spectrum consisted of 2K data points, obtained from 512 free induction decays (FIDs) of 64 scans each,

Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PA, phosphatidic acid; LPC, lysophosphatidylcholine; GPC, glycerophosphocholine; PUFA, polyunsaturated fatty acids; 1D and 2D, one- and two-dimensional; COSY, two-dimensional correlation spectroscopy; HETCOR, heteronuclear correlation spectroscopy.

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with zero-filling in the F_1 dimension. The data were multiplied with a square sine-bell function in both dimensions prior to transformation.

Calculations and quantification of lipids

Linear baseline correction was applied to all proton 1D spectra prior to integral calculations. The relative concentrations of individual lipids were determined by measuring the integral at their characteristic chemical shift. Details for the identification of chemical shifts were described previously [10,11]. Integration of C^2HCl_3 was used as an internal standard for spectral normalization.

RESULTS AND DISCUSSION

Neutral and non-acidic phospholipids, and non-esterified fatty acids

The published n.m.r. profiles of liver [10], brain [11] and macrophages [12], carried out by 1D and 2D techniques, provided a rapid and quantitative assay of most of the major classes of lipids in cells. The spectra also yielded information on the fatty acid composition of the intact lipids. Eventually 2D n.m.r. analysis should yield even more information. There were, however, disadvantages to this purely n.m.r. approach to lipid analysis, including the following: (1) fatty acid analysis was only of total lipid and not of each class of lipid; (2) some low-abundance lipids, even in 2D n.m.r. spectra, were difficult to identify and quantify; (3) acidic and highly acidic lipids, such as phosphatidylserine (PS), cardiolipin, phosphatidylglycerol (PG), phosphatidic acid (PA) and the highly phosphorylated inositol lipids were not quantitatively analysed; (4) glycolipids were not included; and (5) saturated and unsaturated sphingolipids and ether lipids were not distinguished and quantified.

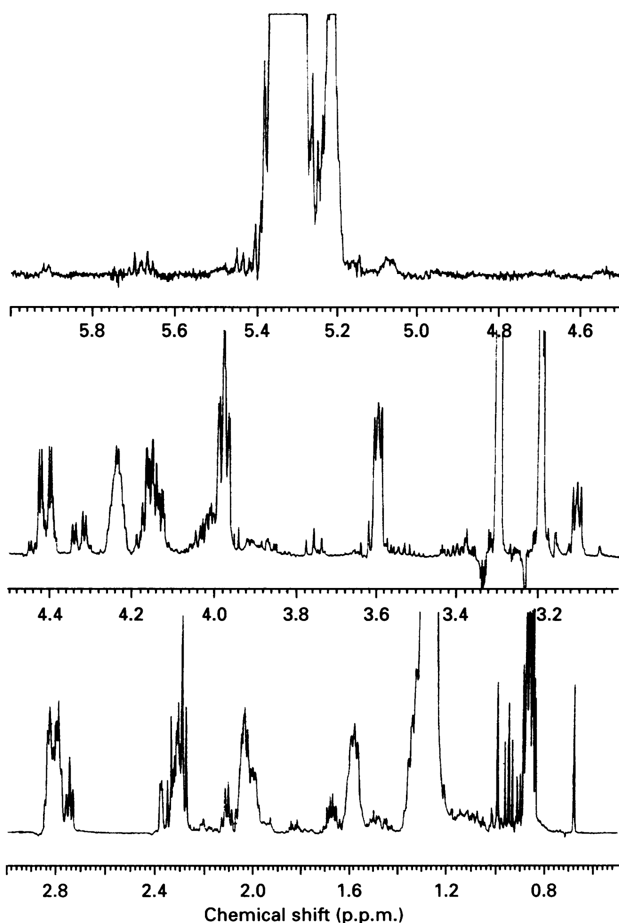

Figure 1 Expanded 1D proton n.m.r. spectrum of lipid extract from rat liver

The lipid extract contains 0.5 mg in 0.6 ml of $C^2HCl_3/C^2H_3O^2H$ (1:2, v/v).

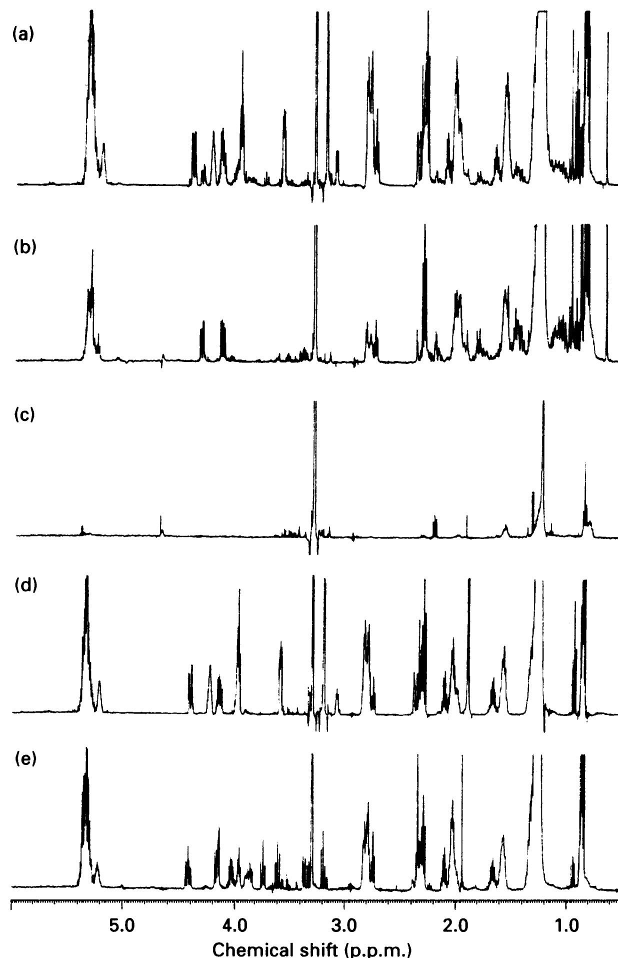

Figure 2 1D n.m.r. spectra of liver extract with and without Bond Elut separation

(**a**) Spectrum of lipid extract without separation, (**b**) spectrum of fraction I (eluted neutral lipids and cholesterol), (**c**) spectrum of fraction II (eluted non-esterified fatty acids), (**d**) spectrum of fraction III (eluted non-acidic phospholipids), (**e**) spectrum of fraction IV (eluted acidic phospholipids).

Bond Elut ion-exchange chromatography can efficiently separate lipids into four fractions: neutral lipids, especially glycerides and steroids (fraction I); non-esterified fatty acids (fraction II), non-acidic phospholipids (fraction III), and acidic phospholipids (fraction IV). The 1D proton n.m.r. spectrum of total liver lipids is shown expanded in Figure 1. Figure 2 shows the comparison of this spectrum with those of the four chromatographic fractions. The efficiency of this fractionation, also checked by t.l.c., was readily seen by the presence of marker cholesterol resonances and glyceride resonances in Figure 2(b) but not in Figures 2(c)–2(e). Significantly, the spectra in Figures 2(b) and 2(c) contained no detectable phospholipid or acidic phospholipid signals. Cholesterol and glyceride analysis was therefore made straightforward by the absence of overlapping fraction III and fraction IV lipid spectra. Tables 1 and 2 show that the loss of

Table 1 Quantification of lipids from rat liver determined from n.m.r. spectra of Bond Elut fractions

Sub-columns of a, b and c within each fraction represent repeated experiments. TG/DG, triacylglycerol/diacylglycerol.

Chemical species	Chemical shift (p.p.m.)	Area before separation	Fraction ...	Area after separation Ia	Ib	Ic	IIIa	IIIb	IIIc	IVa	IVb	IVc
Total phospholipids	4.40	0.91		–	–	–	0.68	0.73	0.71	0.18	0.17	0.17
PC	3.20	0.62		–	–	–	0.54	0.54	0.55	–	–	–
PE	3.08	0.25		–	–	–	0.19	0.22	0.19	–	–	–
PI	3.75	0.11		–	–	–	–	–	–	0.09	0.09	0.09
PS	3.75	–		–	–	–	–	–	–	–	–	–
Cholesterol	0.70	0.15		0.15	0.15	0.14	–	–	–	–	–	–
Sphingomyelin	5.70	0.04		–	–	–	0.3	0.04	0.03	–	–	–
Plasmalogen	5.90	0.03		–	–	–	0.01	0.03	0.03	–	–	–
TG/DG	4.30	0.28		0.27	0.25	0.24	–	–	–	–	–	–

Table 2 Recoveries of lipids after Bond Elut chromatography as determined by comparative 1D proton n.m.r. spectra

TG/DG, triacylglycerol/diacylglycerol.

Chemical species	Chemical shift (p.p.m.)	Area before separation	Fraction ...	Area after separation I	III	IV	Recovery (%)
Total phospholipids	4.40	0.91		–	0.70 ± 0.02	0.17 ± 0.01	95.9
PC	3.20	0.62		–	0.55 ± 0.01	–	87.5
PE	3.08	0.25		–	0.20 ± 0.01	–	79.4
PI	3.75	0.11		–	–	0.09 ± 0.001	86.9
PS	3.75	–		–	–	–	–
Cholesterol	0.70	0.15		0.15 ± 0.003	–	–	97.3
Sphingomyelin	5.70	0.04		–	0.03 ± 0.01	–	91.7
Plasmalogen	5.90	0.003		–	0.002 ± 0.001	–	66.7
TG/DG	4.30	0.28		0.25 ± 0.01	–	–	89.4

cholesterol and glycerides during Bond Elut fractionation was minimal (97% and 89% recoveries respectively).

The extractable non-esterified fatty acids (Figure 2c) were principally saturated fatty acids, as reflected in the ratio of the multiple resonances at approx. 0.9, 1.3, 1.5, 2, 2.8 and 5.4 p.p.m. As expected, most fatty acids in the liver were covalently or strongly bound to glycerides and phospholipids or carrier proteins.

Fraction III, the non-acidic phospholipids, was readily analysed by the absence of resonances from fraction I and IV lipids (Figure 2d). Again, Tables 1 and 2 show that the levels of these lipids were relatively unaffected by chromatographic fractionation. As previously shown [10] the unsaturated ether and sphingoid lipids, although present at low concentrations, were more accurately quantified by their CH=CH resonances below 5.6 p.p.m. (Figure 2d). A new feature, however, was that total ether lipids could now be measured from the approx. 4.3 p.p.m. glycerol moiety that was previously hidden by acyl glycerol resonances. Thus most of the ether lipids were of the plasmalogen or vinyl ether type. This was confirmed by the low-abundance resonances of the non-vinyl ether lipids at 3.44 and 1.59 p.p.m., and by the integrated areas of the CH2 and CH1 resonances of ether lipid glycerol moieties.

The ratio of total choline to total ethanolamine lipids was obtained from the areas of the 3.2–3.1 p.p.m. head group (CH2′) signals, namely 1.34–0.48. The existence of at least two different choline lipids in the ratio of 6.8:1 was obtained from their separate $N^+(CH_3)_3$ resonances at approx. 3.20 p.p.m. These corresponded to total phosphatidylcholine (PC) (3.20 p.p.m.) and ether lipids (3.19 p.p.m.) respectively. The latter value also corresponded to total ether lipids as determined also from the $CH1^d$ glycerol resonance in the fraction II spectrum. The absence of a third sphingoid $N^+(CH_3)_3$ resonance at 3.20 p.p.m. indicated that ceramide ethanolamine was the principal sphingoid. Total sphingolipids were quantified by their CH2 sphingosine resonance at 5.07 p.p.m. due to the adjacent NHCO group. Thus the two sphingoid resonances at 5.68 p.p.m. (CH=CH) and at 5.02 p.p.m. (CH2) yielded 1.05 as the ratio of sphingenine lipids to sphingenine plus sphinganine lipids. The *N*-methyl resonance of lysophophatidylcholine (LPC), glycerophosphocholine (GPC) and the methylated ethanolamines were too small to measure under these conditions.

Acidic phospholipids

Analysis of acidic lipids before chromatographic fractionation was quite difficult. Of the common acidic phospholipids, the inositols and serine lipids have characteristic head-group resonances that can distinguish them. Cardiolipin, PG and PA, on the other hand, do not have head groups that give resonances significantly removed from the glycerol moiety resonance between 4.5 and 3.9 p.p.m.

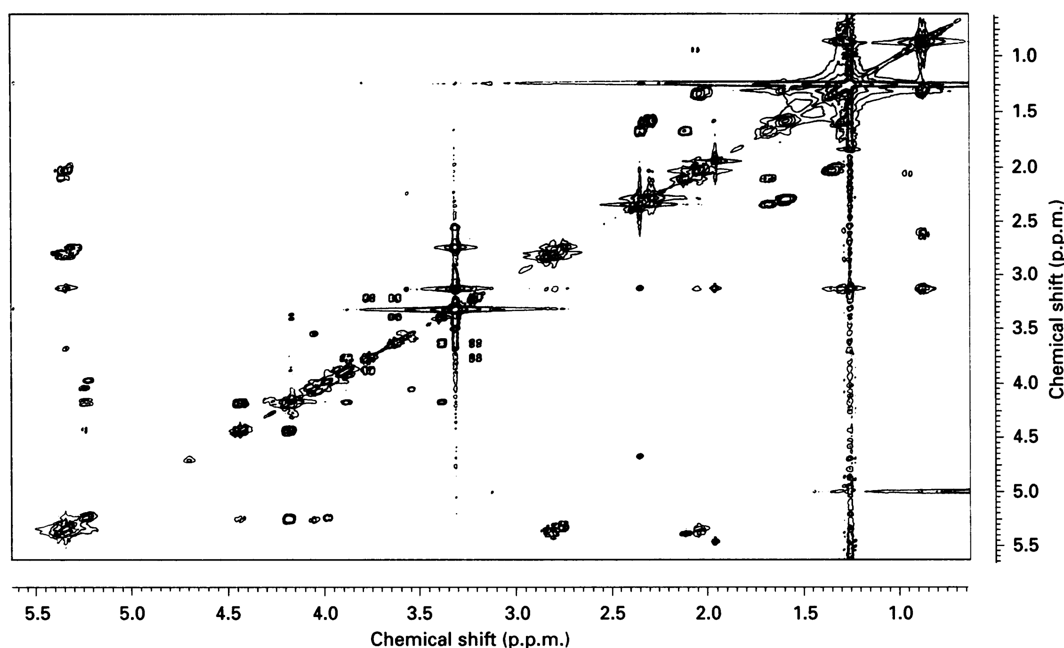

Figure 3 2D COSY proton n.m.r. spectrum of acidic phospholipids separated by Bond Elut chromatography

PI was shown as the principle component in this particular fraction. Detail assignments were published previously [1,2]

Table 3 Fatty acid analysis of n.m.r. spectra of Bond Elut fractions

Row 1 (5.61 p.p.m.) includes the vinyl –CH=CH– groups of every unsaturated fatty acid. Row 2 (2.80 p.p.m.) includes all alkylic CH_2 groups for PUFAs containing more than two unsaturated fatty acid chains [e.g. $-CH{=}CH-\mathit{CH_2}-CH{=}CH-$ (excluding linoleic acid)]. Row 3 (2.75 p.p.m.) represents the $-CH{=}CH-\mathit{CH_2}-CH{=}CH-$ alkylic resonances of linoleic acid and diene fatty acids. Row 4 (2.40 p.p.m.): the multiplet includes all alkylic groups in fatty acids containing the italicized structural features in $-CH{=}CH_2\mathit{CH_2}\mathit{CH_2}CO_2-$. Row 5 (2.00 p.p.m.): this multiplet arises from alkylic groups external to a mono- or poly-unsaturated fatty acid (e.g. structures such as $-\mathit{CH_2}CH{=}CH-(CH_2-CH{=}CH)_x\mathit{CH_2}-$, in which $x \geqslant 0$]. Row 6 (1.70 p.p.m.): This resonance arises from β alkyl groups of fatty acids containing the italicized structure features in $-CH{=}\mathit{CH_2}CH_2CH_2CO_2-$. The γ alkylic group resonance of this type of fatty acid occurs at 2.1 p.p.m. Arachidonic acid is a primary example of this class of fatty acid. Row 7 (0.95 p.p.m.): this methyl triplet arises from fatty acid chains containing a high degree of unsaturation (e.g. docosahexaenoic acid and linolenic acid) and arises from structural features such as $\mathit{CH_3}CH_2CH_2CH{=}CH-$. Information on highly unsaturated fatty acid can also be obtained from the resonances which arise from $-CH{=}CH-\mathit{CH_2}\mathit{CH_2}CO-$. Row 8 (0.85 p.p.m.) includes the CH_3 groups of fatty acid chains, both saturated and unsaturated. In fraction 1 this also includes cholesterol methyl groups. Row 9 = UFA, unsaturated fatty acids.

Rows	Group chemical shift (p.p.m.)	Area before separation	Area after separation Fraction... I	III	IV	Recovery (%)
1	5.61	4.74	0.85 ± 0.021	2.72 ± 0.030	0.82 ± 0.004	92.6
2	2.80	3.10	0.42 ± 0.011	1.92 ± 0.016	0.46 ± 0.009	90.3
3	2.75	0.52	0.13 ± 0.001	0.23 ± 0.001	0.13 ± 0.001	94.2
4	2.40	0.16	0.03 ± 0.002	0.10 ± 0.001	0.01 ± 0.000	87.5
5	2.00	1.28	0.37 ± 0.008	0.64 ± 0.006	0.07 ± 0.004	84.4
6	1.70	0.50	–	0.31 ± 0.007	0.05 ± 0.004	72.0
7	0.95	0.33	0.11 ± 0.005	0.15 ± 0.001	0.05 ± 0.004	93.9
8	0.85 (Total)	2.83	0.76 ± 0.028	1.52 ± 0.014	0.63 ± 0.011	102.6
9	Total UFA	1.69	0.39 ± 0.009	0.85 ± 0.041	0.28 ± 0.009	89.9

The resonances for the phosphatidylinositol (PI) inositol group permitted its easy quantification (Figures 2e and 3; Table 2); these occurred at 3.75, 3.85, 3.60 and 3.35 p.p.m.

The integral of the triplet at 3.85 p.p.m. arising from the CH1′ resonance of PI was significantly lower from that of the CH2, CH1 and CH3 glycerol moiety signals in this spectrum, indicating the presence of the other acidic lipids.

The 2D COSY had a single cross-peak in the head-group region at 4.05, 3.55 p.p.m. which corresponded closely to the β and α proton signals of the serine head-group of PS.

Analysis of the 1D and 2D spectral components of cardiolipin, PG and PA was not achieved in this analysis, but the relative integrals of the glycerol proton resonances indicate that one or more of these is present.

Fatty acid analysis of intact lipids

Previously it was shown that 1D and 2D n.m.r. gave a great deal of information about the overall fatty acid composition of liver lipids, but not about the composition of individual classes of

lipid [10,11]. By separating lipids into four fractions, the n.m.r. spectrum of each fraction could then be analysed for the fraction-specific fatty acid composition.

A complete fatty acid analysis of fraction III lipids by n.m.r. has not yet been achieved, but a great deal of information can be easily gained from Figure 3. For example, docosahexanoic and related acids have a distinctive ω-methyl triplet at 2.40 p.p.m., arachidonic and related acids have their characteristic β and γ methylene resonances at 2.2 p.p.m. and 1.70 p.p.m. respectively, and linoleic acids gave alkylic CH_2 resonances at 2.75 p.p.m. These structure-specific resonances yielded accurate quantification of these fatty acid components.

The ratio of total fatty acid chains to polyunsaturated fatty acid (PUFA) chains of approx. 2:1 was derived from the integrated ratios of the ω-CH_3 resonances (0.9 p.p.m.) to that of total vinyl CH=CH resonances (at 5.4 p.p.m.).

Table 3 summarizes the fatty acid analysis of the intact neutral lipids, non-acidic phospholipids and acidic phospholipids. Analytical information was obtained as follows.

(1) The spectral areas in rows 8 and 9 gave the ratio of total unsaturated fatty acid chains to fatty acid chains in control sample as 1.69:2.83, equal to 0.6. These values were reasonably constant from fractions I, III and IV and were 0.51, 0.56 and 0.45, compared with the control value of 0.57.

(2) The linoleic acid plus diene fatty acid composition was higher in non-acidic phospholipids compared with both neutral and acidic phospholipids (Table 3, row 3).

(3) PUFAs containing structural features analogous to arachidonic acid were not detectable in triacylglycerols, as evidenced by the absence of the structure-specific resonance at 1.7 p.p.m. compared with the control and fractions III and IV. The percentages of fatty acid chains containing this structural feature (principally arachidonate) were different in the control, non-acidic and acidic phospholipids. The values respectively were 30.0, 36.0 and 18.0 % obtained from the ratio of rows 6 and 9.

(4) PUFAs containing low-field-shifted methyl groups were analysed as depicted in Table 3, row 7. Docosahexaenoic acid is a prime example. The percentage of chains with this feature varied from the control to fractions I, III and IV respectively as 0.12, 0.14, 0.10 and 0.08 %.

(5) The 2.4 p.p.m. resonances (Table 3, row 4), also gave information on highly unsaturated fatty acid chains throughout the lipid fractions. Thus the composition varied as follows: control, 6 %; fraction I, 3 %; fraction III, 7 %; fraction VI, 15 %.

A more detailed fatty acid analysis of these fractions is possible from 2D heteronuclear correlation spectroscopy (HETCOR) and 1D ^{13}C spectra, and is currently being carried out.

Conclusions

The combination of Bond Elut chromatography plus 1D and 2D proton n.m.r. spectroscopy has been shown to give superior qualitative and quantitative analysis than either technique alone. The neutral lipid fraction was analysed for steroids and glycerides, whereas the non-acidic lipids were analysed for the various structural lipids containing choline and ethanolamine; these included ether, diacylglycerol and sphingoid lipids. Specifically, two major choline lipids (PC and vinyl ether lipids) were detected, and the major sphingoid lipid was ceramide ethanolamine. The ratio of sphinganine to sphingenine lipids was easily determined by these procedures.

Considerable information on the saturated, unsaturated and polyunsaturated fatty acid composition of each fraction of lipid was obtained rapidly and quantitatively by this technique, without the need for lipid hydrolysis.

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