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Quantitation of individual phospholipid molecular species by UV absorption measurements

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Abstract To validate the utility of on-line measurements of UV absorption for the direct quantitation of individual phospholipid molecular species isolated by reverse-phase HPLC, we synthesized 37 different individual molecular species of diradyl choline glycerophospholipids. UV absorbance response factors (integrated UV absorbance/nmole phospholipid) were calculated for all diacyl, alkenylacyl, and alkylacyl species by injecting varying amounts of the purified molecular species in the 2–100 nmole range and integrating the UV absorbance at 203 nm. There was excellent agreement between the results of the quantitation of individual molecular species determined by measurements of phospholipid mass in HPLC fractions with that based on measurements of total integrated UV absorption and the use of absorbance response factors. The on-line quantitation of individual phospholipid molecular species by UV absorption measurement should prove useful as an adjunct to other techniques of phospholipid quantitation, as a means to assist in the identification of individual molecular species in complex biologic mixtures, and as a stand-alone approach to phospholipid quantitation to facilitate studies of the metabolism of individual species particularly when coupled with radiolabeling techniques.—**McHowat, J., J. H. Jones, and M. H. Creer.** Quantitation of individual phospholipid molecular species by UV absorption measurements. *J. Lipid Res.* 1996. **37:** 2450–2460.

Supplementary key words phospholipid molecular species • UV absorption lipids • plasmalogens • plasmenylcholine • phosphatidylcholine • HPLC

The comprehensive characterization of phospholipid composition in samples derived from biologic sources requires the isolation and quantitation of individual phospholipid molecular species. This is particularly important for the characterization of the diradyl choline and ethanolamine phospholipid classes that are generally comprised of complex mixtures of alkylacyl, alkenylacyl (plasmalogen), and diacyl phospholipid species which are metabolized by distinct pathways that are separately regulated (1–3). The individual phospholipid molecular species are generally isolated by a multistep approach that is based on the recovery of phospholipids by organic solvent extraction, separation of the phospholipids into classes based on differences in polar headgroup composition, and finally, resolution of the individual phospholipid molecular species that differ in the composition of the aliphatic groups at the *sn*-1 and/or *sn*-2 positions of the glycerophospholipid molecule (4–6). While TLC techniques can be utilized to separate the phospholipid classes, HPLC approaches are preferred as a result of improved resolution and recoveries and less oxidation of unsaturated species with HPLC as opposed to TLC methods (5, 7). Only HPLC techniques permit isolation of the majority of individual molecular species present in the phospholipid classes derived from a complex biologic mixture. To isolate the individual species intact (i.e., without derivatization), isocratic reverse-phase HPLC is utilized using fully "end-capped" octadecylsilyl (C18)-derivatized silica microparticles (3–5 μm) as a stationary phase and using a mobile phase comprised of acetonitrile–methanol–water containing a cationic amine salt (e.g., choline chloride) with detection of individual species by measurements of UV absorption (8). The principal difficulties encountered with this approach are the incomplete resolution of all molecular species and the inability to perform "on-line" quantitation because of the previously uncharacterized relationship between UV absorption and phospholipid molecular species composition. Accordingly, the quantitation of individual species is generally accomplished by the "off-line" assay of lipid phosphorus in corresponding column fractions. This is

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Abbreviations: HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; UV, ultraviolet (refers to 190–350 nm wavelength range); PC, phosphatidylcholine (this term is also used to refer collectively to all diradyl choline glycerophospholipid species); PtdCho, diacyl phosphatidylcholine species; AlkCho, alkylacyl choline glycerophospholipid species; PlasCho, plasmenylcholine species (alkenylacyl choline glycerophospholipids); ARF, absorbance response factor; RRF, relative response factor; GC, gas chromatography; GC/MS, gas chromatography–mass spectrometry.

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particularly laborious because the presence of choline chloride in the HPLC mobile phase interferes with the standard phosphate determination, thus, removal of the choline chloride is required prior to assay (4). The present study was undertaken to determine whether direct "on-line" quantitation of individual phospholipid molecular species could be accomplished based on measurements of UV absorbance. To achieve this analytical objective, we synthesized 37 different individual homogeneous diradyl choline glycerophospholipid molecular species corresponding to those most likely to be encountered in tissue samples from mammalian species. The UV absorption characteristics of each compound after reverse-phase HPLC chromatography were determined and the relationship between molecular structure and the integrated UV detector response was established.

MATERIALS AND METHODS

Characterization of UV absorption spectra

The absorption spectra of isolated synthetic homogeneous phospholipid molecular species were characterized using a Beckmann DU-70 spectrophotometer and quartz cuvettes. Phospholipid samples were dissolved in the mobile phase used for reverse-phase HPLC separation (see below) and the absorbance was determined in the 190–340 nm wavelength range against a reference blank comprised of mobile phase only.

Isolation of individual phospholipid molecular species by gradient-elution reverse-phase HPLC

Individual phospholipid molecular species were isolated by a gradient elution modification of the technique originally developed by Patton, Fasulo, and Robins (8). Briefly, sample phospholipids were eluted for 30 min with a mobile phase comprised of methanol–water–acetonitrile 87:6:7 (v/v/v) containing 20 mM choline chloride (solvent A) followed by a linear increase over 60 min to a mobile phase comprised of methanol–water–acetonitrile 76:4:20 (v/v/v) containing 20 mM choline chloride (solvent B). Solvent composition was held constant at 100% solvent B for an additional 30 min after which the solvent composition was returned to 100% solvent A in a linear fashion over 10 min. With this approach, all phospholipid molecular species eluted within 110 min after injection. For those samples containing mixtures of synthetic phospholipid species, all individual compounds were completely resolved (i.e., baseline resolution was achieved for all synthetic mixtures). The stationary phase consisted of a 4.6 mm × 250 mm 5 μm Ultrasphere-ODS (C18) column with a C18 cartridge precolumn (Alltech). The HPLC system was comprised of two Waters 501 pumps, Rheodyne injector, Waters 486 Tunable UV Absorbance Detector, Waters System Interface Module, NEC PowerMate 386SX computer, and Waters Baseline Chromatography Software.

Assay of lipid phosphorus in reverse-phase HPLC column effluents

Quantification of individual phospholipid molecular species was achieved by measurement of lipid phosphorus in HPLC column effluents using a modification of the phosphomolybdate/malachite green method of Itaya and Ui (4, 9). Column effluents were first dried completely in a Savant centrifugal evaporator prior to digestion which was accomplished by adding 0.4 ml of analytical reagent grade (<0.0005% phosphate) perchloric acid to the dried residue and electrically heating the sample at 150°C for 2 h. The samples were allowed to cool to room temperature and excess perchloric acid was neutralized by addition of 1.0 ml of 4.5 N KOH. The neutralization effectively removes the excess perchlorate as a $KClO_4$ precipitate which is sedimented at 2000 *g* for 10 min; 600 μl of the clear supernate was removed for assay of lipid phosphorus as previously described (4).

Synthesis of individual phospholipid molecular species

For preparation of alkenylacyl choline glycerophospholipid species, lysoplasmenylcholine (1-O-alk-1′-enyl-*sn*-glycero-3-phosphocholine) was prepared by alkaline hydrolysis of bovine heart choline glycerophospholipid as described previously (10, 11). The product was isolated from the reaction mixture by silicic acid column chromatography using stepwise gradient elution (10). Characterization of the individual molecular species in the lysoplasmenylcholine product was achieved by gas chromatography and fast atom bombardment–mass spectroscopy (11). Homogeneous 1-O-hexadec-1′-enyl-*sn*-glycero-3-phosphocholine (16:0 lysoplasmenylcholine), 1-O-octadec-1′-enyl-*sn*-glycero-3-phosphocholine (18:0 lysoplasmenylcholine) and 1-O-octadec-1′,9′-dienyl-*sn*-glycero-3-phosphocholine (18:1 lysoplasmenylcholine) were isolated after the individual lysoplasmenylcholine molecular species were resolved by reverse phase HPLC (11). Individual plasmenylcholine (1-O-alk-1′-enyl-2-O-acyl-*sn*-glycero-3-phosphocholine, PlasCho) molecular species were prepared by incubating the appropriate lysoplasmenylcholine species with the corresponding fatty anhydride which was prepared by the dicyclohexylcarbodiimide-mediated condensation of the fatty acid (12). Lysoplasmenylcholine was acylated

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at the *sn*-2 hydroxyl group with fatty anhydride utilizing N,N-dimethyl-4-aminopyridine as a catalyst and the plasmenylcholine product was purified by passing the reaction mixture through an amine solid phase extraction column followed by normal phase HPLC using a Partisil SCX column (13, 14). Synthesis and purification of homogeneous phosphatidylcholine (1-O-acyl-2-O-acyl-*sn*-glycero-3-phosphocholine, PtdCho) and alkylacyl choline glycerophospholipid (1-O-alkyl-2-O-acyl-*sn*-glycero-3-phosphocholine, AlkCho) molecular species were performed similarly utilizing the appropriate fatty acid and palmitoyl-, stearoyl-, oleoyl-, or linoleoyl-lysophosphatidylcholine (16:0, 18:0, 18:1, or 18:2 lysophosphatidylcholine, respectively, from Avanti Polar Lipids or Sigma Chemical Co.) or 1-O-hexadecyl-*sn*-glycero-3-phosphocholine (16:0 lyso-platelet activating factor from Sigma Chemical Co.). The final purity of all synthetic products was confirmed by demonstration of comigration of synthetic product and commercially available standards (Avanti Polar Lipids) using two different TLC systems, normal phase HPLC and following reverse-phase HPLC separation of the individual phospholipid molecular species (4, 8, 13, 14). The aliphatic chain composition of each plasmenylcholine (PlasCho) species was confirmed by the demonstration of stoichiometric quantities of the dimethylacetal (DMA, corresponding to the *sn*-1 aliphatic group) and fatty acid methyl ester (FAME, corresponding to the *sn*-2 aliphatic group) derivatives produced after acid-catalyzed methanolysis and capillary GC analysis of the PlasCho species (4). After acid-catalyzed methanolysis and GC analysis, the composition of diacyl phosphatidylcholine (PtdCho) species was confirmed by the demonstration of stoichiometric production of FAME derivatives corresponding to the *sn*-1 and *sn*-2 aliphatic groups and for alkylacyl choline glycerophospholipids (AlkCho) species, the production of a single FAME derivative corresponding to the *sn*-2 aliphatic group. The concentration of each synthetic phospholipid molecular species was determined by assay of lipid phosphorus and the synthetic products were stored in the dark at −20°C in chloroform–methanol 1:1 (v/v) sealed under N_2 in a tapered glass vial with a Teflon "stop-go" cap (Supelco).

RESULTS

UV absorption spectra

The UV absorption spectra of diacyl, alkylacyl, and alkenylacyl choline glycerophospholipid molecular species containing 16 carbon atoms at the *sn*-1 position and

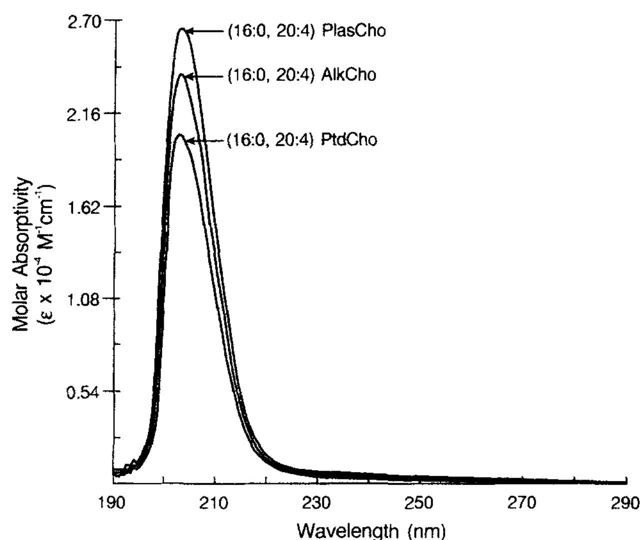

Fig. 1. Ultraviolet (UV) absorption spectra in the 190–290 nm wavelength range for equimolar concentrations of individual plasmenylcholine, alkylacyl choline glycerophospholipid, and phosphatidylcholine molecular species. All molecular species contained a 16 carbon atom aliphatic group at the *sn*-1 position and arachidonate esterified at the *sn*-2 position. The shorthand notation used to describe the composition of individual phospholipid species is described in the text and in the legend to Table 1. Phospholipids were dissolved in HPLC mobile phase and UV absorption spectra were recorded against a reference blank containing HPLC mobile phase alone. Note that the relative heights of each curve at the absorption maximum at 203 nm correspond to the ratios of absorbance response factors for the corresponding molecular species given in Table 1.

20 carbon atoms with 4 carbon–carbon double bonds at the *sn*-2 position (i.e., (16:0, 20:4)PtdCho, (16:0, 20:4)AlkCho, and (16:0, 20:4)PlasCho, respectively) are shown in **Fig. 1.** All three spectra demonstrate an absorbance maximum at 203 nm with parallel variations in absorptivity for each phospholipid molecular species in the 190–290 nm wavelength range. (There was no significant absorption at wavelengths >290 nm.) The absorption of UV energy in the 190–210 nm wavelength range is primarily the result of the Π–Π* electronic transition arising in molecules that contain nonconjugated C=C (carbon–carbon double bonds) (15). Note that all of the UV absorbance spectra in Fig. 1 were obtained from phospholipid molecular species with 4 nonconjugated C=C in the aliphatic group at the *sn*-2 position. The higher molar absorptivity of the (16:0, 20:4)PlasCho species results from the additional absorbance contribution of the vinyl ether (i.e.,–O–CH=CH–) group at the *sn*-1 position of plasmenylcholine molecular species. The 1-O-alkyl and 1-O-acyl functional groups at the *sn*-1 position of AlkCho and PtdCho species, respectively, would not be expected to make a significant contribution to the UV molar absorptivity of these species, thus, the UV absorbance of these species

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arises virtually exclusively from the Π–Π* transitions occurring in the nonconjugated C=C at the *sn*-2 position of these molecules. Accordingly, the greater molar absorptivity of the (16:0, 20:4)AlkCho species relative to the (16:0, 20:4)PtdCho species may reflect some differences in molecular conformation that influence molar absorptivity, specifically, some interaction between the UV absorbing C=C groups at the *sn*-2 position and the 1-O-alkyl and 1-O-acyl functional groups at the *sn*-1 position of these molecules, respectively (discussed in more detail below). For quantitation of phospholipid molecular species after reverse-phase HPLC separation, we selected to monitor UV absorbance at the peak absorption wavelength of 203 nm.

UV absorbance response of individual phospholipid molecular species

To characterize the relationship between UV absorbance, phospholipid mass, and molecular structure, we injected varying amounts of each individual synthetic phospholipid molecular species onto the HPLC column and monitored UV absorbance at 203 nm. The eluate from the column corresponding to each individual species was collected and subjected to assay of lipid phosphorus and the integrated UV detector response was determined by integration of the UV absorbance versus time chromatogram. The results for species containing 16 carbon aliphatic groups at the *sn*-1 position are summarized in **Fig. 2.** Note that the relationship between the integrated detector response (area (203 nm)) and phospholipid mass is linear for each phospholipid species. Accordingly, plotting the integrated detector response versus mass for a series of alkylacyl, diacyl, or alkenylacyl (plasmalogen) species with identical composition at the *sn*-1 position and aliphatic groups pos-

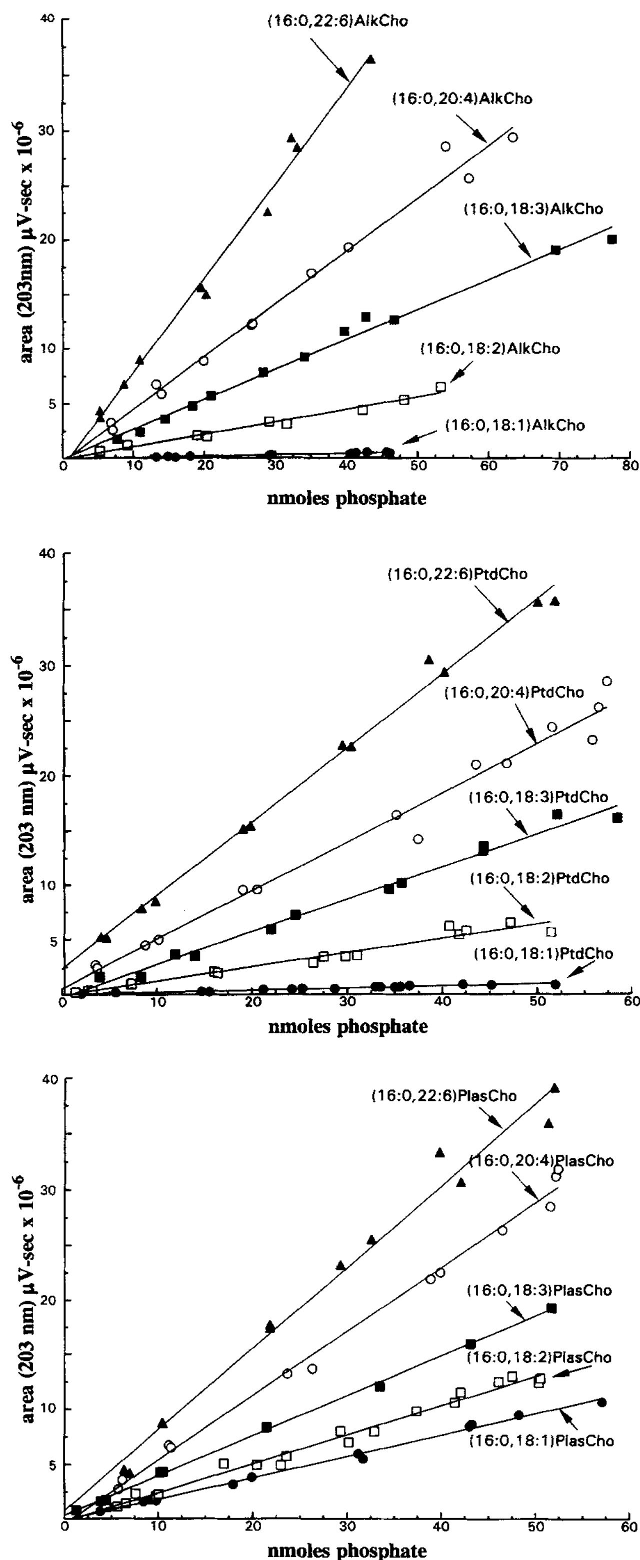

Fig. 2. Quantitative relationship between integrated UV detector response at 203 nm (area (203 nm) in units of µV-sec) and phospholipid mass determined by assay of lipid phosphorus in corresponding reverse-phase HPLC column effluents after injection of different amounts of each phospholipid molecular species. The slope of the "best-fit" linear regression line for each individual phospholipid molecular species (in units of µV-sec/nmol) defines the absorbance response factor for that species as described in the text. All phospholipid species shown in this figure contain 16 carbon atoms at the *sn*-1 position and 18, 20, or 22 carbon atoms with 1–6 double bonds at the *sn*-2 position. The shorthand notation used to describe the composition of individual phospholipid species is described in the text and in the legend to Table 1. Top panel, alkylacyl choline glycerophospholipid species ((16:0, R_2)AlkCho); middle panel, diacyl choline glycerophospholipid species ((16:0, R_2)PtdCho); and lower panel, alkenylacyl (plasmalogen) choline glycerophospholipid species ((16:0, R_2)PlasCo). A similar series of curves were constructed for the (18:0, R_2)PtdCho, (18:1, R_2)PtdCho, (18:2, R_2)PtdCho, (18:0, R_2)PlasCho, and (18:1, R_2)PlasCho species to define the absorbance response factors for these compounds.

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sessing increasing numbers of nonconjugated C=C at the *sn*-2 position gives rise to a series of straight lines that intersect near the origin and have different slopes as shown in Fig. 2. Notice that alkylacyl (top panel) and diacyl (middle panel) species containing a saturated 16 carbon group at the *sn*-1 position and an 18:1 group at the *sn*-2 position have very low detector response. Also note that the detector response for plasmalogen molecular species (bottom panel) is generally significantly higher than that for the corresponding alkylacyl and diacyl species. This difference is most pronounced for species containing fewer than 3 double bonds at the *sn*-2 position and becomes less apparent for highly unsaturated species (i.e., those species containing 20:4 or 22:6 *sn*-2 groups). The greater detector response for plasmalogen species reflects the highly significant additional absorbance contribution of the vinyl ether (-O-CH=CH-) linkage present at the *sn*-1 position of plasmalogens. The integrated detector response of the (16:0, 18:1)PlasCho species is significantly greater than that of the (16:0, 18:2)PtdCho species and approximates that for the (16:0, 18:3)PtdCho species, demonstrating that for some phospholipid molecular species, the vinyl ether linkage (-O-CH=CH-) at the *sn*-1 position of plasmalogens makes an absorbance contribution that is approximately 1.7 times the absorbance contribution of a carbon-carbon double bond (-CH=CH-) in the aliphatic group at the *sn*-2 position. (Note that when referring to plasmalogen molecular species, all molecular species contain a vinyl ether (-O-CH=CH-) group even though the shorthand notation given for the *sn*-1 group does not indicate the presence of this UV chromophore. Thus, a plasmalogen species designated (16:0, 18:1)PlasCho possesses two UV absorbing groups, one at the *sn*-2 position and the vinyl ether at the *sn*-1 position.) Because of the UV absorbance contribution of the vinyl ether linkage in plasmalogens, the series of curves shown in Fig. 2 (bottom panel) are not as "dispersed" as the series of curves shown in top and middle panels of Fig. 2.

The results summarized in Fig. 2 were all derived from phospholipid molecular species containing 16 carbon atoms at the *sn*-1 position. A qualitatively identical series of curves were also derived for diacyl phospholipid molecular species containing 18 carbon atoms and zero, one or two double bonds at the *sn*-1 position (i.e., species containing 18:0, 18:1 and 18:2 groups at the *sn*-1 position) and plasmalogen phospholipid molecular species containing 18:0 or 18:1 groups at the *sn*-1 position and aliphatic groups of varying unsaturation and chain length at the *sn*-2 position.

In Fig. 2, the value of the slope of the linear relationship between mass and detector response has the units of integrated area (in μV-sec)/nmole phosphate and represents the change in the integrated UV detector response expected for a given change in phospholipid mass. We define this quantity as the absorbance response factor (ARF). In **Fig. 3,** the ARF for alkylacyl, diacyl, and plasmalogen molecular species containing 16 carbon atoms at the *sn*-1 position is plotted against the number of double bonds present in the aliphatic group at the *sn*-2 position. For alkenylacyl (plasmalogen) phospholipid molecular species, the significant absorbance contribution of the vinyl ether linkage at the *sn*-1 position is evident by the larger value for the y-intercept. In Fig. 3, note that the curves for the diacyl and plasmalogen phospholipids are widely separated for species containing 1-2 double bonds at the *sn*-2 position but tend to converge for species containing 4-6 double bonds at the *sn*-2 position. Thus, the incremental increase in the ARF with an increase in the number of double bonds at the *sn*-2 position is greater for diacyl phospholipids than for plasmalogens. The most surprising finding was the steep slope for the alkylacyl phospholipids. For alkylacyl species containing 1-3 double bonds at the *sn*-2 position, the ARF is virtually identical to that for the corresponding diacyl phospholipid species; however, the ARF for alkylacyl species containing 4-6 double bonds at the *sn*-2 position is significantly greater. In fact, for species containing 22:6 aliphatic groups at the *sn*-2 position, the ARF is greatest for the alkylacyl species. Thus, the nature of the covalent linkage between the polar headgroup and the *sn*-1 aliphatic chain appears to exhibit a profound effect on the absorbance contribution of C=C groups at the *sn*-2 position. This may reflect the fact that in the nonaqueous solvent used for reverse-phase HPLC, the phospholipid molecules adopt a highly folded molecular conformation that places the covalent linkage at the *sn*-1 position in close spatial proximity to the C=C groups at the *sn*-2 position. This would allow the *sn*-1 polar headgroup composition to influence the absorbance contribution of the Π-Π* transition arising in C=C groups at the *sn*-2 position.

The absolute value of the ARF is expected to be dependent upon the functional characteristics of the UV detector, detector interface, and computerized integration system. For example, changing the detector lamp or lamp alignment could alter the absolute voltage change generated by the detector photodiode after the absorption of a given amount of UV light energy by a fixed amount of phospholipid. Accordingly, the application of absolute ARFs for the quantitation of phospholipid molecular species would require determination of response factors for each species for each different detector system. To overcome the problem of

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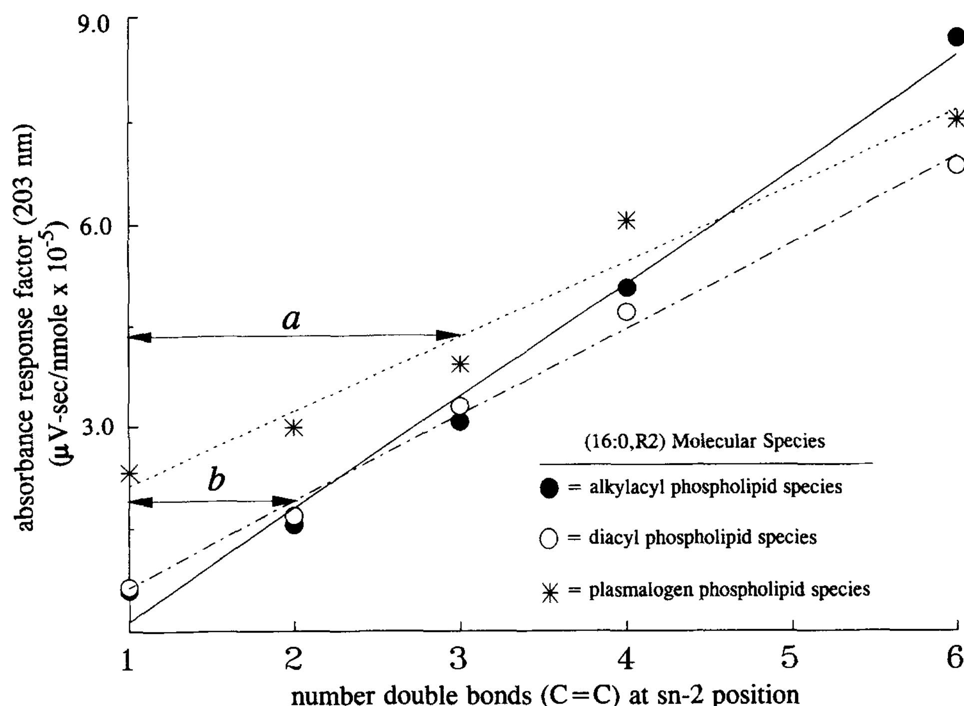

Fig. 3. Relationship between absorbance response factor (ARF, in units of µV-sec/nmol) and number of carbon–carbon double bonds (C=C) present in the aliphatic group esterified at the *sn*-2 position for alkylacyl, diacyl and plasmalogen diradyl choline glycerophospholipids containing 16 carbon atoms at the *sn*-1 position. Note that there is a direct linear relationship between the ARF value and the number of *sn*-2 C=C groups but that the slope of the line differs for alkylacyl, diacyl, and plasmalogen species suggesting that the nature of the covalent linkage of the aliphatic group at the *sn*-1 position influences the absorption contribution of C=C at the *sn*-2 position as discussed in the text. The large value for the y-intercept of the regression line for plasmalogen molecular species reflects the additional UV absorption contribution of the vinyl ether (–O–CH=CH–) group present at the *sn*-1 position of these species. The ratio of the y-intercepts of the horizontal lines labeled as "a" and "b" defines the calculated relative response factor for the (16:0, 18:3)PlasCho species as described in the text.

system-specific absolute UV detector responses, we define a new parameter called the relative response factor (RRF). The RRF for molecular species X is defined as follows:

$$\text{relative response factor (RRF)} = \frac{\text{ARF for species X}}{\text{ARF for (16:0, 18:2)PtdCho}}$$

By defining this quantity as a ratio of the absolute detector responses for two different molecular species, we would expect to minimize the effects of the system-specific variables that could influence detector responses. Accordingly, the RRF value should not be as dependent on the detector system. In order to define the RRF value, we selected the (16:0, 18:2) diacyl choline glycerophospholipid molecular species as the reference species because this compound is commercially available (Avanti Polar Lipids), relatively inexpensive, produces an easily measured absorbance response, and is relatively stable as it is not as prone to oxidation as other more unsaturated phospholipid species. The method used to calculate the RRF value is graphically illustrated for the (16:0, 18:3)PlasCho molecular species in Fig. 3. The y-intercept of the double-arrowed line indicated by the letter "a" in Fig. 3 approximates the expected value for the ARF for the (16:0, 18:3)PlasCho species. Similarly, the y-intercept of the double-arrowed line indicated by the letter "b" in Fig. 3 approximates the expected ARF for the reference species (16:0, 18:2)PtdCho. The ratio of these two y-intercepts approximates the RRF value for (16:0, 18:3)PlasCho. **Table 1** presents the calculated RRF values for all individual diacyl, alkylacyl, and alkenylacyl phospholipid molecular species. (The calculated RRF values shown in Table 1 were not determined graphically but were derived from the least-square linear regression analysis of the data for ARF versus number of double bonds at the *sn*-2 position.)

Examination of Fig. 3 and the calculated RRF values

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TABLE 1. Relative response factor (RRF) for diradyl choline glycerophospholipid molecular species

Composition[a]	RRF Observed[b]	RRF Calculated	Composition	RRF Observed	RRF Calculated
Diacyl phospholipid (PtdCho) molecular species					
(16:0, 18:1)	0.17 ± 0.06	0.12	(18:0, 18:1)	0.14 ± 0.04	0.11
(16:0, 18:2)	0.87 ± 0.13	1.00	(18:0, 18:2)	0.96 ± 0.03	1.00
(16:0, 18:3)	1.92 ± 0.11	1.88	(18:0, 18:3)	2.00 ± 0.08	1.98
(16:0, 20:4)	2.72 ± 0.23	2.77	(18:0, 20:4)	3.23 ± 0.15	2.97
(16:0, 22:6)	3.96 ± 0.31	4.53	(18:0, 22:6)	4.60 ± 0.23	4.94
(18:1, 18:1)	0.27 ± 0.06	0.23	(18:2, 18:1)	1.23 ± 0.11	1.03
(18:1, 18:2)	1.11 ± 0.08	1.02	(18:2, 18:2)	1.99 ± 0.15	1.81
(18:1, 18:3)	2.12 ± 0.18	1.85	(18:2, 18:3)	2.71 ± 0.11	2.65
(18:1, 20:4)	3.03 ± 0.21	2.70	(18:2, 20:4)	4.09 ± 0.39	3.49
(18:1, 22:6)	4.58 ± 0.29	4.39	(18:2, 22:6)	5.80 ± 0.60	5.17
Plasmalogen phospholipid (PlasCho) molecular species					
(16:0, 18:1)	0.82 ± 0.14	1.15	(18:0, 18:1)	1.17 ± 0.16	0.41
(16:0, 18:2)	2.09 ± 0.18	1.92	(18:0, 18:2)	1.87 ± 0.38	2.09
(16:0, 18:3)	2.28 ± 0.18	2.69	(18:0, 18:3)	2.27 ± 0.22	2.61
(16:0, 20:4)	3.52 ± 0.18	3.47	(18:0, 20:4)	3.80 ± 0.25	3.73
(16:0, 22:6)	4.73 ± 0.25	5.00	(18:0, 22:6)	5.79 ± 0.87	5.39
(18:1, 18:2)	1.94 ± 0.83	2.36			
(18:1, 20:4)	4.11 ± 0.58	4.35			
Alkylacyl phospholipid (AlkCho) molecular species					
(16:0, 18:1)	0.13 ± 0.04	0.09			
(16:0, 18:2)	0.92 ± 0.11	0.93			
(16:0, 18:3)	2.02 ± 0.16	2.08			
(16:0, 20:4)	3.18 ± 0.16	3.24			
(16:0, 22:6)	4.82 ± 0.43	5.55			

[a]The composition of individual phospholipid molecular species is described by the shorthand notation (a:b, c:d) where a and c represent chain length and b and d represent the number of carbon–carbon (C═C) double bonds for the aliphatic groups at the *sn*-1 and *sn*-2 position, respectively. For plasmalogen molecular species, the double bond present in the vinyl ether (–O–CH═CH–) linkage at the *sn*-1 position is not included in the shorthand notation for these species.

[b]The values shown for RRF observed represent the mean ± SD for 3–6 separate measurements determined by integration of UV detector response and assay of phospholipid phosphorus in column effluents corresponding to the indicated molecular species and the (16:0, 18:2)PtdCho reference species as described in the text.

shown in Table 1 reveals the following relationships between phospholipid molecular structure and UV detector response.

For species containing 1–4 C═C groups at the *sn*-2 position, the plasmalogen molecular species have significantly greater UV detector response than their corresponding diacyl or alkylacyl counterparts due to the high molar absorptivity of the vinyl ether (–O–CH═CH–) UV chromophore at the *sn*-1 position of plasmalogens. As the number of double bonds at the *sn*-2 position increases from 1 to 4, the proportional contribution of the UV absorbance of the *sn*-1 vinyl ether linkage of plasmalogens to the total absorbance response diminishes. The molar absorptivity and RRF of alkylacyl and diacyl species of comparable aliphatic chain composition are very similar for species containing 1–4 double bonds, however, for species containing 6 double bonds, the molar absorptivity and RRF of the alkylacyl species is greater than that of the corresponding diacyl or plasmalogen species.

Increasing chain length from 16:0 to 18:0 at the *sn*-1 position has minimal effect on the UV detector response.

The UV detector response is not determined solely by the degree of unsaturation. For example, the RRF value for (16:0, 18:3)PtdCho or (18:0, 18:3)PtdCho is significantly different from that of (18:1, 18:2)PtdCho or (18:2, 18:1)PtdCho despite the fact that all of these species contain 3 nonconjugated C═C bonds. Similarly, the RRF for (16:0, 20:4)PtdCho or (18:0, 20:4)PtdCho differs from that of (18:2, 18:2)PtdCho or (18:1, 18:3)PtdCho. A more rigorous confirmation of these observations would require additional studies with other, less abundant molecular species such as those containing 16:1 aliphatic groups at the *sn*-1 position or 20:5 or 22:5 groups at the *sn*-2 position.

For diacyl phospholipid species, the absorbance contribution of a given aliphatic group is unaffected by the location of the group at the *sn*-1 or *sn*-2 position (note that the RRF value for (18:1, 18:2)PtdCho is virtually identical to that for (18:2, 18:1)PtdCho).

Phospholipid molecular species with aliphatic groups

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containing only one C=C exhibit an unexpectedly low detector response. Thus, for example, the diacyl and alkylacyl species containing saturated groups at the *sn*-1 position (16:0 or 18:0) and 18:1 groups at the *sn*-2 position have anomalously low detector responses. Similarly, the RRF for (18:1, 18:1)PtdCho is considerably lower than that for the (16:0, 18:2)PtdCho or (18:0, 18:2)PtdCho species. Also note that all diacyl phospholipid species containing an 18:1 aliphatic group at the *sn*-1 position have nearly the same RRF values as compared to the corresponding species containing saturated (16:0 or 18:0) *sn*-1 groups.

To verify the potential utility of the RRF values for the quantitation of phospholipid molecular species, we prepared several mixtures of different synthetic, homogeneous diacyl, alkylacyl, and alkenylacyl molecular species. The mixture of species was selected to provide a sequence of peaks which were all "baseline resolved." To each mixture, we added (16:0, 18:2)PtdCho. Column effluents corresponding to each phospholipid species were collected and subjected to phosphate assay. Integrated UV detector responses were then determined by electronic integration of each peak. These results were used to define the ARF for each species (ratio of integrated area/nmol phosphate). The RRF value was then determined by calculating the ratio of the ARF for each species to that of the (16:0, 18:2)PtdCho reference species measured during the same HPLC run. The results of these experiments are shown in Table 1 where the values for RRF observed represent the RRF values determined after injection of the various phospholipid synthetic mixtures and phosphate analysis. As seen in Table 1, there is in general excellent agreement between the calculated and observed RRF values, in fact, the calculated RRF value was within the 95% confidence interval for the observed RRF value for all species.

Figure 4 and **Table 2** illustrate the use of RRF values for the quantitation of individual phospholipid molecular species in a mixture based on UV absorbance measurements at 203 nm. For this experiment, (16:0, 18:2)PtdCho was added as an internal standard to a mixture of plasmalogen phospholipids containing an 18:0 aliphatic group at the *sn*-1 position and *sn*-2 groups with differing levels of unsaturation. To quantify the different phospholipid molecular species using RRF values, it is first necessary to define the ARF for the (16:0, 18:2)PtdCho reference species for the chromatographic system. This is most easily accomplished by determining the integrated UV detector response and quantifying lipid phosphorus in the column effluent corresponding to this species. After establishing the ARF for (16:0, 18:2)PtdCho (i.e., the ratio of integrated area/phospholipid mass), the following equation can be used to calculate the mass of all other phospholipid species in the mixture:

$$\text{calculated mass of species X} = \frac{\text{integrated UV detector response for species X}}{\text{(ARF (16:0, 18:2)PtdCho)} \times \text{(RRF of species X)}}$$

Table 2 compares the results of this calculation with the results obtained by direct measurements of lipid phosphorus in the corresponding column effluents. As indicated in Table 2, there is excellent agreement between the results of the determination of phospholipid mass by UV absorbance measurement and direct quantitation of lipid phosphorus. From Table 2, note that there is less variability in the determination of phospholipid mass based on integrated detector response and RRF values than for direct mass measurements based on phosphate assay. For the analysis of complex biologic mixtures or other phospholipid samples that contain additional molecular species that coelute with (16:0, 18:2)PtdCho, the ARF for the reference species could be determined by separate injection of synthetic, homogeneous (16:0, 18:2)PtdCho and quantifying lipid phosphorus in the corresponding column effluent.

In addition to the use of RRF values for quantitation, the RRF can also be used to provide information that may help to identify an unknown peak in a complex mixture. For example, the integrated UV detector response could be determined for the unknown peak and the corresponding column effluent could be collected and subjected to phosphate analysis and the ARF could be calculated. The RRF value could then be determined and by comparing the observed RRF and retention time of the unknown peak with the retention times of known species and the RRF values shown in Table 1, a tentative identification could be made. Confirmation of peak identity, however, would require additional characterization of the molecular species in the unknown peak using GC or GC/MS techniques.

DISCUSSION

The present study demonstrates the utility of UV absorbance measurements for the quantitation of diradyl choline glycerophospholipid molecular species after their isolation by chromatographic techniques. With currently available chromatographic systems that include computer control of the chromatographic process and sophisticated data storage and analysis software, it should be possible to incorporate ARF and RRF values into the post-run data analysis program to permit direct reporting of phospholipid mass after separation of a mixture of molecular species. In order to apply this approach to the quantitation of the complex mixture of diradylglycerophospholipid molecular species present in phospholipid classes derived from biologic

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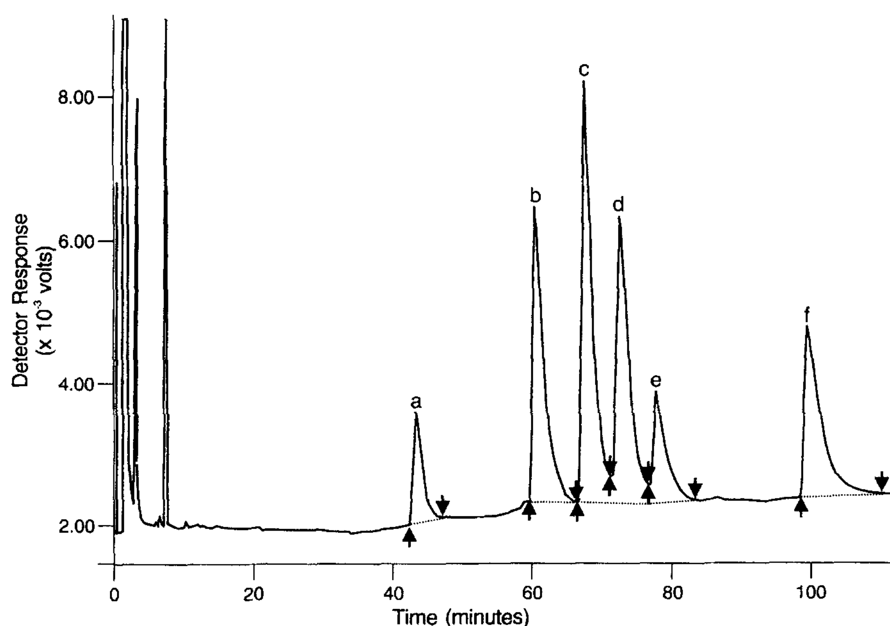

Fig. 4. A representative reverse-phase HPLC chromatogram (UV detector response at 203 nm versus elution time) for a mixture of plasmalogen phospholipid molecular species containing 18 carbon atom aliphatic groups at the *sn*-1 position and *sn*-2 groups of varying chain length and degree of unsaturation. Peak "a" represents (16:0, 18:2)PtdCho which was added as an internal reference species. The other peaks in the chromatogram represent (18:0, 18:3)PlasCho (peak "b"), (18:0, 22:6)PlasCho (peak "c"), (18:0, 20:4)PlasCho (peak "d"), (18:0, 18:2)PlasCho (peak "e"), and (18:0, 18:1)PlasCho (peak "f") (the shorthand notation used to describe the composition of individual phospholipid species is described in the text and in the legend to Table 1). The up and down arrows indicate integration start and stop points, respectively, and the dotted line at the base of each peak represents the integration baseline. The results of integration of UV detector responses and lipid phosphorus quantitation in column effluents corresponding to each peak are given in Table 2.

TABLE 2. Comparison of results for phospholipid mass determined by phosphate assay with calculated mass based on integrated detector response and relative response factors

Phospholipid Molecular Species	Integrated UV Detector Response (μV-sec $\times 10^{-6}$)	Relative Response Factor	Phospholipid Mass (nmol)	
			Calculated	Measured
(18:0, 18:1)PlasCho	7.83 ± 0.24	1.41	28 ± 0.9	34 ± 3.7
(18:0, 18:2)PlasCho	3.85 ± 0.20	2.09	9 ± 0.5	11 ± 1.8
(18:0, 18:3)PlasCho	9.52 ± 0.37	2.61	19 ± 0.7	21 ± 0.9
(18:0, 20:4)PlasCho	9.30 ± 0.07	3.73	13 ± 0.1	12 ± 0.8
(18:0, 22:6)PlasCho	12.47 ± 0.31	5.39	12 ± 0.3	11 ± 1.6

Values shown represent the mean ± SD of three separate injections of a sample mixture containing the indicated plasmalogen molecular species and 15 nmol of (16:0, 18:2)PtdCho added as an internal reference. From the integrated detector response and known mass of the internal reference species, the absolute response factor (ARF) was determined (ARF = integrated response/mass = 199136 μV-sec/nmol for the reference species in this sample mixture). Based on the ARF for the reference species, the mass of each individual plasmalogen molecular species was calculated using the corresponding relative response factor (RRF, from Table 1) and the equation given in the text. The results for the calculated mass are compared with the phosphate assay results in the corresponding reverse-phase HPLC column effluents. There were no significant differences between the calculated and measured phospholipid mass at the 5% level of confidence using a paired *t*-test. The shorthand notation used to describe the composition of individual phospholipid molecular species is described in the text and in the legend to Table 1. A representative chromatogram of the phospholipid molecular species in this sample mixture is shown in Fig. 4.

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sources, three prerequisites must be met: *1*) the individual species must contain at least one C=C group at the *sn*-1 or *sn*-2 position in order to generate a detectable UV absorbance response; *2*) the aliphatic composition of the *sn*-1 and *sn*-2 groups of the individual species in each peak must be known; and *3*) the individual species must not coelute (i.e., each species must produce a separate peak; if a peak contains more than one species, the composition and relative amount of each species must be known). In biologic samples, the overwhelming majority of phospholipid molecular species contain at least one C=C and the *sn*-1 and *sn*-2 aliphatic composition of most species can be established by capillary GC analysis of the volatile fatty acid methyl ester and dimethylacetal derivatives produced after acid-catalyzed methanolysis of column effluents corresponding to each peak. Therefore, the greatest difficulty arises from the need to achieve adequate separation of all species in the mixture. This is often difficult to accomplish with current reverse-phase HPLC techniques; however, with partial resolution of species and post-run data analysis using commercially available systems with sophisticated deconvolution algorithms, reasonably accurate estimates of the areas of overlapping peaks should be obtainable. Accordingly, we are presently engaged in studies comparing the results of phospholipid quantitation "on-line" using integrated peak areas and RRF values with the results of conventional "off-line" measurements of phospholipid mass to characterize the phospholipid composition of complex biologic mixtures in an effort to further validate our proposed method for quantitation of individual phospholipid molecular species. In preliminary studies, we have found that the RRF values presented in Table 1 can be used to provide accurate estimates not only of the mass of individual choline diradylglycerophospholipid molecular species, but also of the corresponding ethanolamine diradylglycerophospholipids for those peaks corresponding to the 5–10 most abundant species in the mixture. In other words, early preliminary studies suggest that UV detector response is not significantly affected by the composition of the phosphobase group esterified at the *sn*-3 position of the phospholipid molecule, thus, application of integrated UV detector responses for the quantitation of all major phospholipid classes may be possible. Of course, further studies are needed to confirm these preliminary observations.

There is an alternative approach to the quantitation of phospholipid molecular species utilizing HPLC and UV detection that deserves special mention. This approach is based on the measurement of UV absorbance at 230 nm of the diradylglycerobenzoate derivatives of individual species formed after phospholipase C (PLC)-catalyzed hydrolysis and reaction of the resulting diradylglycerol products with benzoyl chloride (16–18). With this approach, the molar absorptivity of each molecular species is presumed to be the same as the detector is responding to electronic transitions arising in the aromatic ring and this UV absorption at 230 nm is apparently not affected by the composition of the aliphatic groups at the *sn*-1 and *sn*-2 positions. This approach has the advantage of improved sensitivity for detection and quantitation of highly saturated species and offers improved resolution of individual molecular species, however, there are several potential problems and limitations: *1*) additional time and effort must be expended for the hydrolysis, derivatization, and isolation of the diradylglycerobenzoate product (this also provides additional opportunity for oxidation and other undesirable covalent modification of sample molecules); *2*) conditions for PLC hydrolysis and derivatization must be selected that result in quantitative conversion of all diacyl, alkenylacyl (plasmalogen), and alkylacyl species with no selectivity for hydrolysis of a specific subclass or selective loss during derivatization; and *3*) the 1,2-diradylglycerol product produced by PLC hydrolysis undergoes further intramolecular rearrangement to form 1,3-diradylglycerol species. The 1,2- and 1,3-diradylglycerobenzoate derivatives have different chromatographic retention characteristics, thus, biological samples containing complex mixtures of many different phospholipid molecular species may give rise to even more complex mixtures of 1,2- and 1,3-isomers that may be difficult or impossible to accurately quantify. For diacyl phospholipids, the 1,2-diacylglycerols undergo near quantitative rearrangement to 1,3-diacylglycerols, however, the ether-linked phospholipids (alkylacyl and plasmalogen subclasses) do not undergo complete rearrangement and the result is a mixture of 1,2- and 1,3-diradylglycerol species (17) that obviously complicates any efforts to quantify these species in mixtures containing all three phospholipid subclasses. *4*) The source of PLC must provide enzyme that is not contaminated by other phospholipases that could alter sample composition or by phospholipids that could accompany the enzyme during purification; *5*) in radiolabelling experiments using [^{3}H]arachidonate, the radiolabeled diradylglycerobenzoate may eluate as a separate peak before the corresponding unlabeled species (18); *6*) with phospholipids radiolabeled at the *sn*-3 phosphobase group, the diradylglycerobenzoate approach cannot be used if measurements of both mass and radioactivity in isolated individual molecular species are desired; and *7*) the diradylglycerobenzoates are only partially soluble in the HPLC mobile phase (17, 18), thus, great care must be exercised in dissolving and transferring samples in mobile phase prior to injection. The present approach to UV absorption-based phos-

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pholipid quantitation avoids most of these problems by providing a method that permits analysis of the intact molecular species. Our approach will be even more attractive when improvements in the resolution of intact molecular species by reverse-phase HPLC techniques are achieved.

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