methods

Quantitative determination of phospholipid compositions by ESI-MS: effects of acyl chain length, unsaturation, and lipid concentration on instrument response

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Abstract **Electrospray ionization-mass spectrometry (ESI-MS) is a very promising tool for the analysis of phospholipid compositions, but is hampered by the fact that not all molecular species are detected with equal efficiency. We studied this and other issues that need to be taken into account to obtain truly quantitative compositional data. The key findings were as follows: First, the instrument response for both saturated and unsaturated phospholipid species decreased with increasing acyl chain length. This effect became increasingly prominent with increasing overall lipid concentration. Second, the degree of acyl chain unsaturation also had a significant effect on instrument response. At the highest concentration studied (10 pmol/μl), polyunsaturated species gave 40% higher intensity than the fully saturated ones. The effect of unsaturation diminished and nearly disappeared with progressive dilution. Third, the instrument response for the different head group classes varied markedly depending on the infusion solvent used. Notably, inclusion of ammonia in the infusion solvent eliminated sodium adduct formation in the positive ion mode, thus greatly simplifying the interpretation of the spectra. The fact that instrument response is dependent on many structural features, overall lipid concentration, solvent composition, and instrument settings makes it necessary to include several internal standards for each phospholipid class to obtain accurate data. Preferably, both unsaturated and saturated standards should be used. Finally, we quantified the major phospholipid classes of BHK cells using ESI-MS. The data agreed closely with those obtained with thin-layer chromatography and phosphorus analysis. This study indicates that quantitative compositional data can be obtained with ESI-MS, provided that proper attention is paid to experimental details, particularly the choice of internal standards.—** Koivusalo M., P. Haimi, L. Heikinheimo, R. Kostiainen, and P. Somerharju. **Quantitative determination of phospholipid compositions by ESI-MS: effects of acyl chain length, unsaturation, and lipid concentration on instrument response. *J. Lipid Res.* 2001.** 42: **663–672.**

Supplementary key words lipid metabolism and trafficking • electrospray mass spectrometry • membrane lipid composition

Compositional analysis of phospholipids is of considerable interest because they are involved in many crucial cellular functions such as signal transduction, apoptosis, and protein sorting (1, 2). At present, the analysis is complicated by the low sensitivity and complexity of the commonly available methods. Regarding the former, hundreds of nanomoles of total phospholipid are needed to establish the molecular species profiles of the different classes (3). This precludes detailed analyses when subcellular fractions obtained from cultured cells, for instance, are studied. The complexity, on the other hand, derives from the fact that several chromatographic steps and/or enzymatic reactions are required to establish the molecular species patterns. In view of these complications, it is hardly surprising that, so far, a complete molecular species profile (i.e., including all major classes) has been quantitatively determined for very few, if any, mammalian cells or membranes.

Mass spectrometry (MS) offers an attractive alternative for the analysis of phospholipid compositions because of its high sensitivity, specificity, and (apparent) simplicity. However, MS has rarely been used for this purpose until very recently. The main reason for this is that the ionization methods previously available (fast-atom bombardment, etc.) cause extensive fragmentation of the lipid molecules; this, along with the extreme complexity (hundreds of different molecular species) of most biological samples, has precluded compositional analysis. The recent introduction of "soft" ionization methods has opened completely new vistas in this field. In particular,

Abbreviations: ESI-MS, electrospray ionization-mass spectrometry; TLC, thin-layer chromatography; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; HPTLC, high performance TLC; SM, sphingomyelin; HPLC, high performance liquid chromatography.

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electrospray ionization-mass spectrometry (ESI-MS) has been shown to be a very promising technique (4–12). However, there are still many issues to be resolved before ESI-MS can become a routine tool for quantitative determination of lipid compositions. A key problem is that different molecular species are not detected with equal efficiency (6, 9, 13). For instance, it has been shown that the detection sensitivity (instrument response) can depend markedly on the acyl chain length (9). However, other important factors remain to be studied. First of all, it is not clear whether and how the instrument response depends on the degree of unsaturation of phospholipid acyl chains. Second, it is unknown whether or not the relative response factors are affected by the overall lipid concentration. The data on the effect of the phospholipid head group structure on instrument response are somewhat contradictory. In this study, we have investigated these and other issues that need to be considered when using ESI-MS for quantitative determination of (phospho)lipid compositions.

MATERIALS AND METHODS

Lipids and other materials

Synthetic PC, PE, and PS standards were obtained from Avanti Polar Lipids (Birmingham, AL), fatty acids from Larodan AB (Malmö, Sweden), and sphingosylphosphorylcholine from Matreya, Inc. (Pleasant Gap, PA). Phospholipase D from *Streptomyces species* (type VII) was obtained from Sigma (St. Louis, MO), and silica gel 60 high performance thin-layer chromatography (HPTLC) plates from Merck (Darmstadt, Germany). Sphingomyelins (SMs) with a 14:0, 17:0, 23:0, or 25:0 fatty acid residue were synthesized from sphingosylphosphorylcholine and the respective fatty acid as described previously (14, 15). The SM product was purified using a NH_2-bonded silica cartridge (16), followed by reverse-phase HPLC on an Ultrasphere ODS column (Beckmann, 5-μm particle size, 250 × 4.6 mm) eluted with 5% chloroform in methanol at 1 ml/min. Di-16:0 and di-18:0-PI species were isolated from hydrogenated yeast PI by reverse-phase HPLC. Alternatively, Di-16:0-PI was obtained from Larodan AB. Di-21:0-PE and -PS were synthesized from the corresponding PC by using phospholipase D-mediated transesterification (17). The products were purified on a Lichrosphere 100 DIOL column (5-μm particle size, 250 × 4.6 mm; Alltech, Deerfield, IL) as described previously (18). All solvents were of high performance liquid chromatography (HPLC) or analytical grade and were purchased from Merck or Rathburn Chemicals Ltd. (Walkerburn, Scotland).

Phospholipid standard mixtures

All standard mixtures were prepared in silane-treated autosampler vials (Alltech) in chloroform/methanol (C/M) 1:2 to avoid loss due to adsorption to glass. Several different equimolar PC mixtures were prepared. The compositions of these mixtures are indicated in the respective figure legends. PE, PS, and PA standard mixtures were prepared from these PC mixtures by phospholipase D-mediated transesterification or hydrolysis (see above). For the quantification of the cellular phospholipid species, a mixture consisting of the following internal standards was used: 14:0/14:0-PC, 19:0/19:0-PC, 21:0/21:0-PC, 24:0/24:0-PC, 14:0-SM, 17:0-SM, 23:0-SM, 25:0-SM, 14:0/14:0-PE, 16:0/16:0-PE, 21:0/21:0-PE, 14:0/14:0-PS, 16:0/16:0-PS, 21:0/21:0-PS, 16:0/16:0-PI, and 18:0/18:0-PI.

Cell culture and lipid extraction

BHK-21 cells were cultured on plastic dishes (Nunc, Roskilde, Denmark) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (200 U/ml), and streptomycin (200 μg/ml) under 5% CO_2 at 37°C. Cellular lipids were extracted (19) in silane-treated screw-cap tubes, and the extract was divided into three aliquots. One aliquot was used to determine the total phospholipid content of the extract (20), the second aliquot was used for lipid class distribution analysis by TLC (21) and phosphorus analysis (20), and the third aliquot was spiked with PC, SM, PE, PS, and PI internal standards and used for ESI-MS analysis.

ESI-MS and data analysis

Most experiments were carried out both with an ion trap instrument (Esquire-LC, Bruker-Franzen Analytik, Bremen, Germany) and a triple quadrupole instrument (Perkin Elmer Sciex API 300). The lipids were dissolved in C/M 1:2 with or without NH_4OH (0.25–1%) or 0.1 mM NaCl, and were infused to the electrospray source via a 50-μm id fused silica capillary using a syringe pump at the flow rate of 5 μl/min. With the ion trap, nitrogen was used as the nebulizing (at 5–6 psi) and the drying gas (5–7 l/min at 200°C). The potentials of the spray needle, capillary exit, and skimmer 1 were set to ± 4,000, 90–150, and 25–50 V, respectively. For each spectrum 100–500 scans were averaged. With the triple quadrupole instrument, the spray capillary voltage was 4,000 V and orifice voltage 25 V in positive and negative ion ESI. Synthetic air was used as the nebulizing gas, and nitrogen was used as the curtain and collision gas. The m/z range scanned in the MS measurements was from 500 to 1,000 (5 s/scan), and in MS/MS from m/z 50 to 10 mass units above the m/z value of precursor ion (15–300 s/scan). Collision-activated dissociation was applied in the second quadrupole at a collision energy of 25–40 eV. The phospholipid species were identified on the basis of *i*) their characteristic m/z value, *ii*) fragmentation analysis, and *iii*) precursor ion or neutral loss scans. In the positive ion mode, PE and PS give the characteristic neutral losses of 141 or 185, respectively, whereas PC and sphingomyelin can be identified by scanning for precursors of m/z 184 (9, 22, 23), and PI species can be selectively detected by scanning for precursors of m/z 241 in the negative ion mode (9).

The instrument response can vary depending on the phospholipid acyl chain length (9). To account for this and for the isotope effect, several internal standards at equimolar concentrations were included. A correction function $f_{std}(m)$ can then be defined by plotting the measured intensity of the internal standards versus m/z. The concentration of an analyte species can be obtained from the equation: $C(I,m) = I * C_{std}/f_{std}(m)$, where C_{std} is the concentration of the internal standards and m and I are the m/z value and peak intensity of the analyte species, respectively. At low total lipid concentrations, the instrument response is a linear function of m/z, thus correction function is of the form $f_{std}(m) = k * m + c$. At high total lipid concentrations the exponential function $f_{std}(m) = a * e^{bm}$ better approximates the chain length dependency. The degree of lipid acyl chain unsaturation can also affect the instrument response. In principle, it is possible to correct for this by including unsaturated internal standards as well. However, because such standards were not available for all lipid classes (e.g., PI), the samples were measured at low total lipid concentrations, at which the effect of unsaturation is eliminated or at least greatly diminished (see Results).

Other methods

Phospholipid classes were separated on HPTLC silica gel 60 plates using chloroform–methanol–acetic acid–formic acid–water 70:30:12:4:2, (v/v/v) as the solvent (21). The lipid bands

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were scraped off the plates and their phosphorus content was determined (20).

RESULTS

Effect of acyl chain length and on instrument response

A previous study indicated that the acyl chain length of PC can have a significant effect on the instrument response (9). To study this issue in more detail, an equimolar mixture of PC standards containing 11 saturated and 5 diunsaturated species (each at 5 pmol/μl) was prepared and analyzed with the ion trap instrument. As apparent from **Fig. 1**, the response decreased markedly and in a nearly linear manner with increasing acyl chain length for both the saturated and diunsaturated species. Notably, however, the instrument response for the unsaturated species was somewhat higher than for the corresponding saturated species. This effect of acyl chain unsaturation will be studied further below. Parallel results were obtained with the triple quadrupole instrument, and also for PE, PS, and PA standard mixtures (data not shown).

To study whether the total phospholipid concentration influences the chain length dependency of instrument response, an equimolar mixture of saturated PC species was diluted to 10, 5, 1, 0.5, 0.2, or 0.1 pmol/μl per species (120–1.2 pmol total lipid/μl). At the highest concentration (10 pmol/μl), the relative response decreased strongly and in a seemingly exponential manner with the acyl chain length (**Fig. 2**), which is in agreement with the previous study (9). However, when the concentration was decreased to 5 pmol/μl (60 pmol/μl total lipid) and below, the response was an essentially linear function of the chain length. At the same time, the differences between the short and long chain species diminished. For example, the response for 24:0-PC was only 2.5-fold higher than for 48:0-PC at 0.1 pmol/μl, whereas it was 5-fold higher at 10 pmol/μl (Fig. 2).

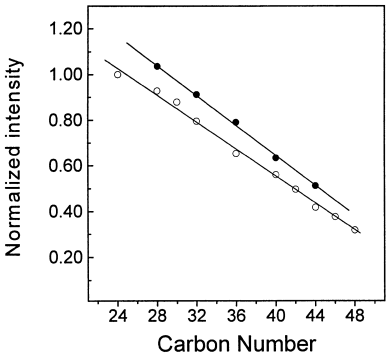

Fig. 1. Effect of acyl chain length on instrument response. An equimolar mixture of 10 saturated (open circles) and 5 diunsaturated (closed circles) PC species (5 pmol/μl each) was prepared and analyzed by the ion trap instrument. Each data point is an average of five replicate samples with standard deviation of less than 5%. The infusion solvent was C/M 1:2 + 1% NH_4OH. Data have not been corrected for the isotope effect, as such correction has only a modest effect on curves. Parallel data were obtained for the corresponding PE, PS, and PA standard mixtures analyzed in the negative ion mode, either with the ion trap or the triple quadrupole instrument (data not shown).

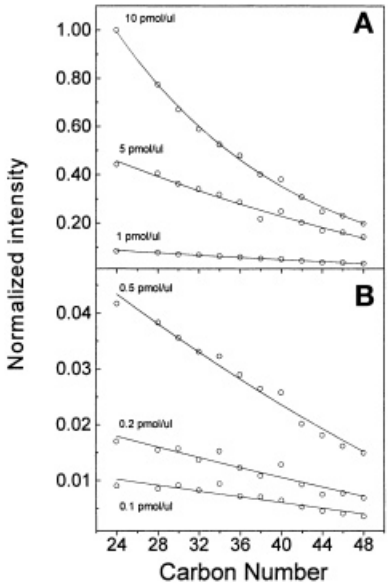

Fig. 2. Effect of concentration of saturated phosphatidylcholines on instrument response. An equimolar mixture of 12 saturated PC species of the indicated chain length was prepared, diluted to obtain the concentration of 10, 5, 1, 0.5, 0.2, or 0.1 pmol/μl per species, and then analyzed with the ion trap instrument. A: Data for the three highest concentrations. B: Data for the three lowest concentrations. Other details as given in the legend for Fig. 1.

We also studied the chain length dependency of instrument response with (di)unsaturated PC species (**Fig. 3**). Analogous to the saturated species, *i*) the response decreased with acyl chain length, and *ii*) the decrease became more prominent with increasing lipid concentration. However, the response remained a linear function of the acyl chain length, even at the highest concentration studied (10 pmol/μl). Parallel data were obtained with PE, PS, and PA standards, both with the ion trap and the triple quadrupole instrument (data not shown).

Effect of acyl chain unsaturation on instrument response

To study the effect of lipid unsaturation and concentration on the instrument response in more detail, an equimolar mixture of PC standards, including five 36-carbon PC species with 0, 1, 2, 4, or 6 double bonds, was analyzed at various concentrations. As can be seen in **Fig. 4**, the response was markedly dependent on lipid unsaturation, particularly at the higher concentrations. For instance, at 10 pmol/μl per species (140 pmol/μl total lipid), the response for the polyunsaturated species was 40% higher than that for the fully saturated ones. However, when the mixture was progressively diluted, the effect of unsaturation gradually diminished, and virtually disappeared at 0.1 pmol/μl per species (1.4 pmol/μl of total lipid).

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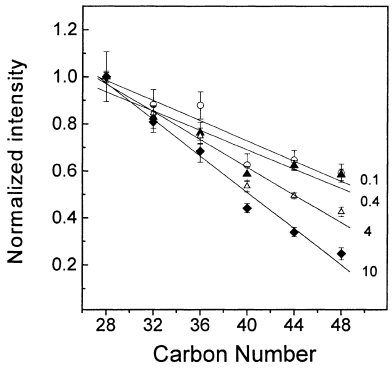

Fig. 3. Effect of concentration of diunsaturated phosphatidylcholines on instrument response. An equimolar mixture of 14 PC species, including 6 diunsaturated PC species, was diluted to obtain the concentration of 0.1 (open circles), 0.5 (closed triangles), 4 (open triangles), or 10 (diamonds) pmol/μl per species (1.4–140 pmol/μl total lipid) and then analyzed with the ion-trap instrument. The data were normalized by setting the peak intensity of the 28:2 species equal to 1.0 at each concentration. The error bars give the standard deviation ($n = 4$). PC species in the mixture were 26:0, 28:0, 28:2, 36:0, 36:1, 36:2, 36:4, 36:6, 40:0, 40:2, 44:0, 44:2, 48:0, and 48:2. The lines represent linear fits to the data. For other details, see legend for Fig. 1.

Effect of polar head group structure on instrument response

The influence of the phospholipid head group structure on instrument response in ESI-MS has been studied previously, but the results are somewhat contradictory (see Discussion). In addition, the response for PI relative to other phospholipids has not been studied using acyl chain matched species. Therefore, an equimolar mixture of six dipalmitoyl species, i.e., 16:0/16:0-PC, -PE, -PS, -PG, -PA, and -PI was prepared, diluted to a varying degree in C/M 1:2 with or without 1% NH_4OH, and spectra were then obtained with the ion trap instrument operated in the negative or positive ion mode. In the negative ion mode with C/M 1:2 as the solvent, there were major differences in instrument response between classes, as shown in **Fig. 5A**. The highest response was observed for PG, whereas PI, PA, and PS gave intermediate responses, and PE and the chloride adduct of PC gave low responses. Inclusion of ammonia (1%) in the infusion solvent markedly increased the response for PE and PA relative to other species (Fig. 5C). Notably, the relative responses did not vary significantly with lipid concentration, independent of whether ammonia was included or not (Fig. 5B and C).

In the positive ion mode, with C/M 1:2 as the solvent, the highest peak was observed for protonated PC, followed by the Na^+-adducts of PC, PE, and other lipids (Fig. 5D). It is notable that in the case of PE and most other lipids, the Na^+-adduct was much more prominent than the protonated form, whereas the opposite was true for PC (Fig. 5E). When ammonia was included, the relative in-

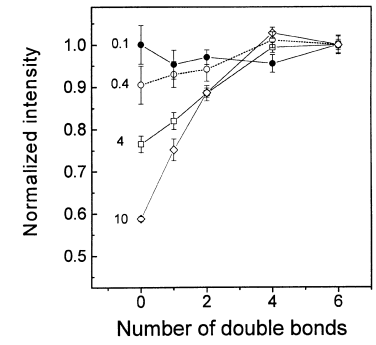

Fig. 4. Effect of degree of unsaturation on instrument response at different concentrations. An equimolar mixture of 14 different PC species, including 5 species with 36 acyl carbons and 0, 1, 2, 4, or 6 double bonds, was diluted to 0.1 (closed circles), 0.4 (open circles), 4 (squares), or 10 (diamonds) pmol/μl per species (1.4–140 pmol/μl total lipid) and then analyzed with the ion trap instrument. The response values have been normalized relative to that for the 36:6 species at each concentration. The error bars indicate the standard deviation ($n = 5$). For other details, see legend for Fig. 2.

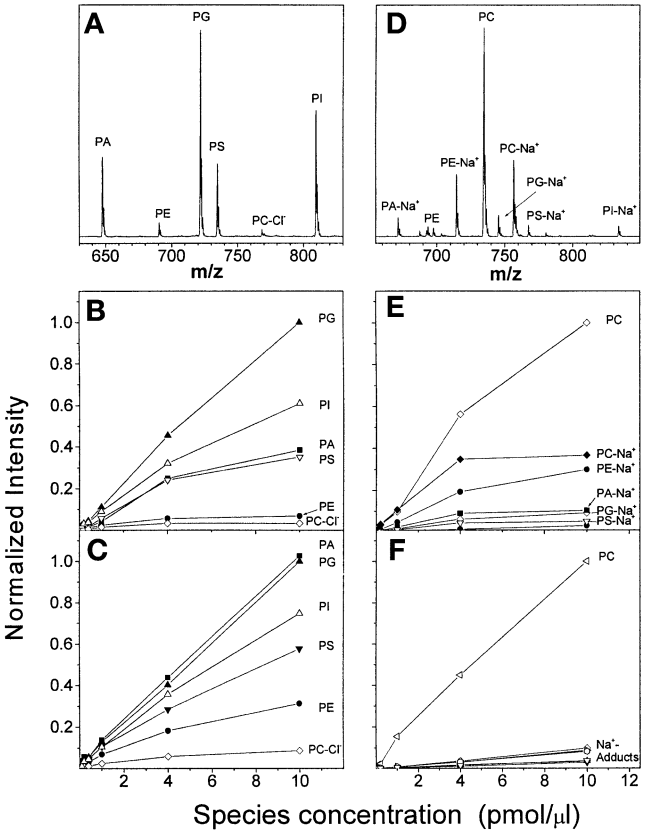

Fig. 5. Effect of the polar head group structure and solvent composition on instrument response. An equimolar mixture of dipalmitoyl-PC, -PE, -PS, -PG, -PI, and -PA was prepared and analyzed with the ion trap instrument at various dilutions. A: Negative-ion spectrum at concentration of 10 pmol/μl per species with C/M 1:2 as the solvent. B: Concentration dependency of instrument response in the negative ion mode with C/M 1:2 as the solvent. C: As in B, but C/M 1:2 + 1% NH_4OH as the solvent. D: Positive-ion spectrum at concentration of 10 pmol/μl per species with C/M 1:2 as the solvent. E: Concentration dependency of instrument response in the positive ion mode with C/M 1:2 as the solvent. F: As in E, but C/M 1:2 + 1% NH_4OH as the solvent.

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tensities changed dramatically (Fig. 5F). At this point the intensity of protonated PC far exceeds that of the others, probably because under these basic conditions PC is a zwitterion, and the other lipids exist as anions. The relative responses varied only slightly with the total lipid concentration (Fig. 5E and F). Note that, in agreement with previous studies (24), the adjustment of certain instrument parameters can markedly change the relative (and absolute) responses from those shown in Fig. 5 (data not shown).

Linearity of response

The linearity of instrument response is obviously an important concern in quantitative analysis. Previous studies have shown that a linear response can be obtained with a quadrupole instrument over at least two orders of magnitude (6, 22). To determine whether this is the case with an ion trap, an equimolar mixture of seven PC species varying in chain length and unsaturation was prepared and analyzed at various dilutions (0.1–10 pmol/μl per species), using the ion trap instrument. As shown in **Fig. 6A**, the intensities were linearly dependent on the lipid concentration up to about 1 pmol/μl, but tended to level off at higher concentrations. Such signal saturation is a typical phenomenon in ESI-MS and results from the saturation of the surface of the spray droplets by the analyte molecules (see Discussion). Importantly, however, the relative responses were linearly dependent on the concentration up to at least 5 pmol/μl, as shown for some species in Fig 6B. Parallel results were obtained for the PE, PS, and PA standard mixtures (data not shown).

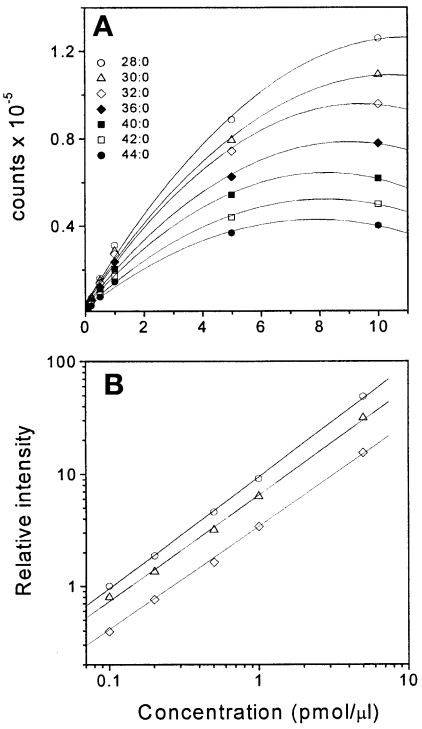

Fig. 6. Concentration dependency of instrument response. An equimolar mixture of seven saturated PC species was prepared, diluted to 10, 5, 1, 0.5, 0.2, or 0.1 pmol/μl (0.7–70 pmol/μl total lipid), and then analyzed with the ion trap instrument. A: Peak intensity versus concentration. B: Response for selected species relative to that for the 26:0 species. The data for the other species were omitted for clarity. Note the logarithmic scales. Symbols are as in A.

To investigate whether such linear behavior is also observed under more biologically relevant circumstances, an equimolar mixture of five PC species (30:0, 32:2, 34:4, 38:0, and 44:2) was prepared and diluted to concentrations varying from 0.1 to 10 pmol/μl per species. Each diluted mixture was then combined with BHK cell total lipid extract containing 140 pmol/μl of total phospholipid and analyzed with the ion trap instrument. The relative response was a linear function over the whole 100-fold concentration range studied for each PC species (data not shown). Thus a linear response over a considerable range of concentrations can be obtained for phospholipids, even in the presence of other cell-derived lipids and other compounds present in the crude lipid extract.

Molecular species composition of BHK-21 cells

After having determined the effect of structural factors and lipid concentration on instrument response, we used this information to quantitatively determine the molecular species profiles of the major phospholipids of BHK-21 cells. To this end, a total lipid extract was prepared and spiked with a mixture of internal standards for each major phospholipid class, and then analyzed with the triple quadrupole instrument using class-specific detection (9). Representative spectra are shown in **Fig. 7**. Because unsaturated standards were not available for all phospholipid classes (see Materials and Methods), saturated standards were used throughout. These allowed us to correct for acyl chain length dependency of instrument response, but obviously not for the unsaturation dependency. To overcome this problem, the samples were progressively diluted until the relative peak sizes did not change. This should, as indicated by Fig. 4, eliminate the need to correct for unsaturation (see Discussion).

The molecular species compositions of the major glycerophospholipid classes are shown in **Table 1**. The major PC species were 34:1 (24.9%), 36:2 (23.0%), 34:2 (10.5%), and 36:1 (8.0%), whereas the major PE species were 36:2 (25.0%), 36:1 (9.6%), 34:1 (7.4%), 38:4 (6.8%), and 34:2 (5.7%). The most abundant PI species were 38:4 (23.3%), 38:3 (19.2%), 36:2 (14.0%), and 36:1 (8.6%), whereas the most prominent PS species were 36:1 (31.3%), 36:2 (14.3%), 40:6 (10.8%), and 34:1 (8.6%). Only three significant SM peaks were observed, i.e., those with *m/z* of 703, 813, and 815. These probably correspond to lipids with a sphingosine backbone and a 16:0 (65.0%), 24:1 (20.0%), or 24:0 (15.0%) fatty acid residue, respectively. However, some of the peaks should also contain small amounts of isobaric dihydrosphingosine derivatives, as these have been reported to be present in BHK cells (25). Note that the MS method used (i.e., head group-specific neutral-loss or precursor ion scanning) does not allow the identification of the molecular species at the level of individual fatty acid residues.

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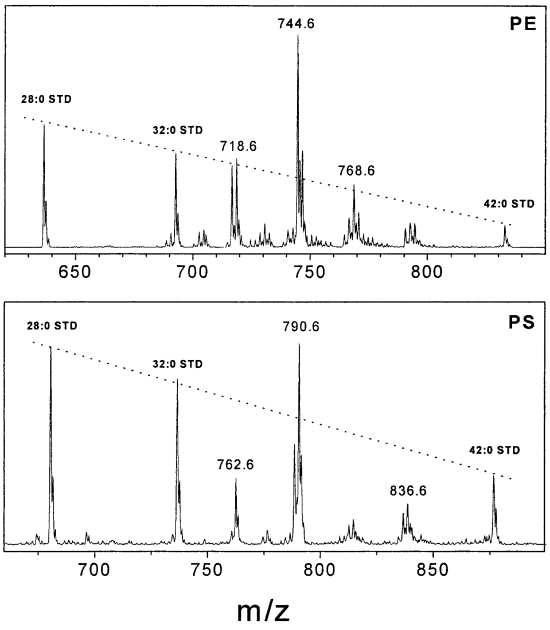

Fig. 7. Quantification of phosphatidylethanolamine and phosphatidylserine molecular species in crude BHK cell extract by using internal standards and head group-specific scans. A total lipid extract of BHK cells was spiked with a mixture of saturated internal standards (see Materials and Methods) and then analyzed with the triple quadrupole instrument in the positive ion mode by scanning for the neutral loss of 141 or 185 specific for PE (upper panel) and PS (lower panel), respectively. Internal standards and some major species are indicated. The dashed lines indicate the dependency of instrument response on *m/z*. The indicated PE species are *m/z* 718.6 = 34:1; *m/z* 744.6 = 36:2; and *m/z* 768.6 = 38:4. The indicated PS species are *m/z* 762.6 = 34:1; *m/z* 790.6 = 36:1; and *m/z* 836.6 = 40:6. PC and sphingomyelin were quantified analogously by scanning for precursors of *m/z* 184 in the positive mode; PI was quantified by scanning for precursors of *m/z* 241 in the negative ion mode (not shown).

TABLE 1. Molecular species composition of major glycerophospholipid classes in BHK-21 cells

Molecular Species	% of a Class[a]			
	PE	PC	PI	PS
32:2	0.5[c]			
32:1	1.1	7.0		1.1
32:0		3.9		
34:3 ether[b]	0.3			
34:2 ether		2.2		
33:2 acyl	0.7			
33:1 acyl/34:1 ether	0.9	6.1		
34:2	**5.7**	**10.5**	3.0	2.1
34:1	**7.4**	**24.9**	4.2	**8.6**
34:0			3.1	1.0
35:1				4.5[c]
36:5 ether	0.4[c]			
36:4 ether	0.4			
36:3 ether	0.8			
36:2 ether	2.1			
36:1 ether	1.4			
35:2 acyl		3.0		
35:1 acyl		2.0		
36:5	0.5			
36:4	1.7	2.4	1.7	
36:3	2.8	2.6	3.7	
36:2	**24.9**	**23.0**	**14.1**	**14.3**
36:1	**9.6**	**8.0**	**8.6**	**31.3**
36:0	1.3[c]			
38:6 ether	1.1[c]			
38:5 ether	1.2			
38:4 ether	0.9			
38:3 ether	0.5[c]			
38:2 ether	0.5[c]			
38:6	1.5[c]			
38:5	4.5	2.5	**6.9**	
38:4	**6.8**	1.9	**23.3**	2.8
38:3	3.5		**19.2**	4.7
38:2	1.8		**6.0**	3.7
38:1	0.8			
38:0	0.8			
40:7	3.1[c]			7.9[c]
40:6	**5.2**		2.0	**10.8**
40:5	3.8		2.1	4.4
40:4	1.5[c]		1.4	2.4
40:3	0.6[c]		1.0	2.7[c]
40:2	0.5[c]			
Unidentified				0.4 (*m/z* 696 pos)

The molecular species were identified and quantified as detailed in Materials and Methods. The amounts of the major species are shown in bold.

[a] Percentage of a species of the total in a phospholipid class. Each value represents an average of 5–9 replicate samples. Standard deviation varied from 2% (major species) to 20% (minor species).

[b] Ether lipid refers to a species with an alkyl or an alkenyl ether-linked chain in the sn-1 position of the glycerol moiety.

[c] Identification based on *m/z* value only.

Comparison of phospholipid class distribution analyses performed by MS and TLC methods

To estimate the accuracy of the MS method, a lipid extract of BHK cells was prepared and divided into three aliquots. One aliquot was analyzed by the triple quadrupole instrument, using internal standards and class specific detection, i.e., precursor ion and neutral loss scans. The second aliquot was subjected to phospholipid class distribution analysis by TLC; the third aliquot was used for the determination of the total lipid phosphorus content. As shown in **Fig. 8**, the abundance of most phospholipid classes as determined by the MS method agree very well with those determined by the TLC method in this study here or previously (25). This is particularly true for the SM, PI, and PS classes. The somewhat lower PE content given by the MS method could result from the fact that the alkyl- and alkenyl-ether species, abundant in PE, may give lower responses than the diacyl species when determined using the specific neutral loss scan of 141 mass units in the ion positive mode (9, 12). We attempted to quantify the PE species by scanning for precursors of *m/z* 196 in the negative ion mode, because it has been reported that this mode is less discriminative toward the ether species (9). However, for unknown reasons, useful spectra could not be obtained. The lack of PE ether standards precluded further studies of this issue. In case of PC, the MS method produced a somewhat higher value than the TLC method. This may be due to the fact that despite extensive dilution of the sample, full suppression of the double-bond effect was not achieved (see Discussion). This would result in overestimation of the PC content of the cells, as the saturated internal standards would give lower responses than presumed.

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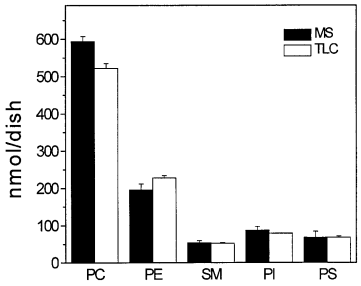

Fig. 8. Comparison of BHK cell phospholipid class compositions determined by mass spectrometry or thin-layer chromatography. BHK cells from one 145-cm^2 dish were extracted and analyzed by mass spectrometry (closed columns) or thin-layer chromatography (open columns) as detailed in Materials and Methods. Error bars indicate the standard deviation ($n = 3$).

On the basis of the phosphate assay of the lipid extract, BHK cells contained 974 ± 40 nmol of total phospholipid/dish. From the data shown in Fig. 8, it can be calculated that the MS method produced a value of 952 ± 63 nmol/dish for the sum of PC, PE, PS, PI, and SM. Because the minor classes, i.e., cardiolipin, lysobisphosphatidic acid, lyso-PC, and PA (which could not be included in the analysis owing to lack of appropriate standards) represent about 5 mol% of total BHK cell phospholipids (25), the MS method would give approximately 1,000 nmol/dish for the total phospholipid content. This is close to the value given by the phosphate determination and thus further supports the accuracy of the MS data.

DISCUSSION

Factors affecting instrument response

This study shows that in ESI-MS, the instrument response for phospholipids can depend on many factors. These include *i*) acyl chain length, *ii*) acyl chain unsaturation, *iii*) structure of the polar head group, *iv*) total lipid concentration, *v*) solvent composition, and *vi*) instrument settings. These factors, some of which have also been indicated by previous studies, will now be discussed briefly.

The acyl chain length was found to have a marked effect on instrument response, the short-chain lipids being detected with higher sensitivity than the long-chain ones (Fig. 1). Although this phenomenon has been described previously (9), the present study appears to be the first to show that the effect is markedly dependent on (total) lipid concentration, i.e., it became more pronounced with increasing lipid concentration. In addition, the shape of the instrument response versus acyl chain plot was not constant, but changed from linear to exponential when lipid concentration increased (Fig. 2).

The enhanced chain length dependency of the instrument response at higher lipid concentrations probably relates to the ionization process proper, rather than ion transport or other processes within the mass analyzer and/or the detector (which, on the other hand, are likely to be responsible for the residual, concentration-independent decrease of response with increasing acyl chain length; see Figs. 2 and 3). We speculate that *i*) at higher lipid concentrations, the surface of the sprayed droplets becomes crowded, and *ii*) under such conditions, the molecules with shorter acyl chains, being more surface active, become enriched in the surface layer and are therefore more efficiently ionized than the molecules with a long acyl chain. The alternative possibility, that the observed discrimination against long-chain species would simply relate to solubility of the species in the infusion solvent (C/M 1:2), seems very unlikely for the following reasons: first, all standard mixtures used were completely clear solutions, which is to be expected because phosphatidylcholines are commonly thought to be highly soluble in C/M mixtures; and second, the solubility of the species with the longest saturated chains, i.e., 48:0-PC in C/M 1:2, was tested and found to be more than 10 nmol/μl at RT, which exceeds the highest concentration of this standard used (100 pmol/μl) by two orders of magnitude.

The most important novel finding of the present study is that unsaturation of phospholipid acyl chains can have a major effect on instrument response (Fig. 5). Although this finding is not surprising, considering that a similar effect has been observed previously with triglycerides using either electrospray (23) or atmospheric pressure chemical ionization (26), it seems not to have been considered in previous MS studies focused on quantification of phospholipids. A probable reason for this is that the effect of acyl chain unsaturation is concentration dependent (Fig. 4) and has therefore gone unnoticed when tested with standards, typically used at low (total) concentrations. The importance of taking the unsaturation effect into account is emphasized by the present finding that the response can vary by as much as 40% depending on the total number of double bonds and the lipid concentration (Fig. 4). Although the double bond-effect is alleviated or even abolished upon progressive dilution of the sample, the use of highly diluted samples unavoidably compromises the quantification of the less abundant molecular species. Furthermore, as we discuss below, with biological samples the unsaturation effect may not be fully eliminated even by dilution.

It is not fully clear why the instrument response is affected by acyl chain unsaturation. One possibility is that the unsaturated species are, like the short-chain saturated ones, more surface active and thus become more efficiently ionized. Additionally, the double bonds could weaken the intermolecular interactions in the droplet surface layer, thus enhancing the evaporation of the molecule to gas phase. This would be analogous to the enhanced efflux of unsaturated lipid molecules from membranes to the aqueous phase (27).

The structure of the polar head group structure was also found to have a major effect on the instrument response (Fig. 5). In the negative ion mode, the acidic phospholipids PG, PI, PA, and PS gave a much higher response than PE or PC. This finding, which agrees with a previous study (13), is not unexpected, because the latter two lipids are zwitterionic at neutral pH. Inclusion of ammonia in

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the infusion solvent markedly increased the response for PE, obviously because the higher pH favors deprotonation of the NH_4^+ group of ethanolamine (6). Previously, sodium or lithium hydroxide has been used to achieve this effect (6, 10, 28, 29). In another study (6), PA, PG, and PS were found to give very similar responses and PE gave only a somewhat lower response. These results do not seem to be in accord with the present data, but this is probably only due to differences in experimental conditions, e.g., the presence of NaOH in the infusion solvent, different lipid concentration, or different instrument settings.

In the positive-ion mode and when using C/M 1:2 as the solvent, major peaks corresponding to the sodiated species were detected along with the protonated ones (Fig. 5). This phenomenon, also reported previously (4, 5, 30, 31), can greatly complicate the analysis of biological samples. Therefore, it is satisfying that inclusion of ammonia in the infusion solvent greatly reduced the formation of the sodium adducts. Alternatively, sodium or lithium salts could be included to suppress formation of protonated ions (28). However, we have not been able to obtain full suppression of the protonated ions by inclusion of sodium salts (unpublished observations). It is notable that although sodium adducts do not interfere when a precursor ion scan is used to selectively detect the choline lipids (9, 32), this was found not to be the case for PE- and PS-specific neutral loss scans, for which major sodium adduct peaks were observed. However, inclusion of ammonia virtually eliminated the sodiated species, thus greatly simplifying the interpretation of the spectra (data not shown).

Optimization of phospholipid quantification by ESI-MS

The fact that the instrument response is sensitive to such a variety of factors, including lipid structural parameters, solvent conditions, and instrument settings, makes it obligatory to use internal standards when truly quantitative data are expected. A minimum of three internal standards for each head group class needs to be included so that the acyl chain length dependency (linear vs. nonlinear) can be unambiguously determined. Saturated standards might be used if the samples can be diluted to very low total lipid concentrations. However, even then, full suppression of the unsaturation effect may not be obtained (particularly if detergent or other surface-active impurities are present). In addition, extensive dilution compromises quantification of the less abundant molecular species, e.g., those of PS, PA, PG, and SM, because these classes constitute only a relatively minor fraction of the total phospholipids in eukaryotes. Therefore, it seems safer to employ (di)unsaturated standards, as they give a response more similar to that of the (usually unsaturated) natural phospholipids. For the most accurate results, both unsaturated and saturated standards should be included, as only then can one fully correct for the chain length and unsaturation-dependent effects, independent of sample concentration.

In this study, most experiments were carried out with both an ion trap and a triple quadrupole instrument. Standards for both were analogous except for the somewhat higher sensitivity of the ion trap. However, with real samples, the triple quadrupole instrument has the significant advantage of allowing direct analysis of crude lipid extracts by using selective detection modes, i.e., class-specific precursor ion or neutral loss scans (9, 22, 32, 33). Such a selective detection is particularly useful when the negatively charged lipids are being analyzed, since the negative ion spectrum of lipid extracts is typically highly crowded due to extensive overlap of species belonging to different head group classes. The use of head group-specific scans also allows the analysis samples containing impurities from, e.g., gradient materials, which tend to obscure the direct MS spectrum (13). Finally, such specific scans, together with isotopically labeled precursors, provide a powerful and convenient tool for studies on phospholipid metabolism, as have been demonstrated recently (32). The major drawback of this approach is that the detailed chemical structures of the molecules, including the *sn*-positions of the individual acyl residues and double bond positions, cannot be determined.

Because the ion trap and other mass spectrometers are unable to perform precursor or neutral loss scans,[2] preseparation of the phospholipid classes is necessary with most samples to allow reliable quantification. Such preseparation is most conveniently done by HPLC, either on line (5, 31, 34) or off line (10, 12, 29). In the latter case, special care should be exercised when collecting the fractions, because, depending on the chromatographic system used, the retention times of the standards can be significantly different from each other as well as from those of the sample lipids (unpublished observations). A particular advantage of performing phospholipid class preseparation is that the individual molecular species can be determined by using fragmentation analysis (4, 6, 9, 10, 29). Also, the *sn*-positions of the individual acyl residues can be determined (24, 28, 30, 31, 35). However, a truly quantitative determination of the individual molecular species in isobaric peaks is very complicated, because the probabilities of cleavage of the *sn*-1 and *sn*-2 residues can strongly depend on the acyl chain length and unsaturation of the individual acyl residues (24, 28, 30). Preseparation of classes is also necessary when certain minor but functionally important phospholipids such as phosphatidic acid, lysobisphosphatidic acid, or cardiolipin (36, 37) are to be analyzed, as no specific scan mode seems to be available for these lipids. Yet, preseparation may be necessary when the sample contains high concentrations of detergents or other impurities interfere with the ionization process (13).

Finally, it should be emphasized that regardless of the method used, data analysis is likely to become the rate-limiting step in quantitative analysis of phospholipid compositions, particularly in any type of routine application

[2] Recent ion trap models are reported to produce neutral loss and precursor ion scans, albeit post-run (Finnigan Corporation Sales Information brochure).

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involving many samples. Developments in the MS software field will, one hopes, offer a remedy for this problem in the near future.

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