Rapid separation and identification of phosphatidylethanolamine molecular species

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Abstract A novel high performance liquid chromatography method is presented for the separation and identification of intact molecular species of phosphatidylethanolamine (PE). After isocratic separation, detection of species can be achieved by measurement of UV absorbance as well as by the quantitative method of light scattering detection. A mathematical relationship exists between *i*) the relative retention time of a PE molecular species and *ii*) the number of carbon atoms and double bonds in the aliphatic groups of the species. This relationship can aid in the identification of the species. Furthermore, the absence of non-volatile components in the solvent allows the use of electrospray mass spectrometry to identify the eluting components and to establish the position of the individual radyl groups at the glycerol backbone. Using this method, samples of bovine heart PE (rich in plasmalogens) and rat liver PE (rich in diacyl species) have been analyzed.—Brouwers, J. F. H. M., E. A. A. M. Vernooij, A. G. M. Tielens, and L. M. G. van Golde. **Rapid separation and identification of phosphatidylethanolamine molecular species. *J. Lipid Res.* 1999.** 40: **164–169.**

Supplementary key words ether lipids • phospholipids • plasmalogens • mass spectrometry • HPLC

Phosphatidylethanolamine (PE) is a major phospholipid in membranes from bacterial as well as from eukaryotic origin and has received special attention due to several interesting characteristics. Among the best studied are the asymmetric distribution in the plasma membrane of cells and the particular richness of ether-linked aliphatic groups often found in this phospholipid class. Furthermore, PE is rich in polyunsaturated fatty acyl groups. After hydrolysis by phospholipase A_2, released fatty acids play important roles in signal transduction by serving as precursors for biologically active molecules such as prostaglandins and leukotrienes. For a thorough characterization of membrane phospholipids it is therefore necessary to determine not only phospholipid headgroup composition, but also the individual molecular species of the phospholipids.

Analysis of intact molecular species of phospholipids by high performance liquid chromatography (HPLC) is nowadays the method of choice. Without the need of derivatization, the risk for the introduction of artefacts is reduced to a minimum, and the information about the combinations of fatty acids present in the lipid class is preserved. Whereas phospholipid classes can easily be separated and sensitively quantified by a variety of procedures (1–3), separation of the molecular species within a single phospholipid class without removal of the polar headgroup, is more problematic (reviewed in ref. 4). Patton, Fasulo, and Robins (5) published a method applicable for a variety of phospholipid classes, including PE. Quantification, however, is difficult using that method, as the response of the UV detector is dependent on the degree of unsaturation of the aliphatic groups, making careful calibration of each component necessary (6). Light scattering has proven to be an excellent choice for the detection of molecular species, as it is relatively inexpensive, very sensitive and, in contrast to a UV detector, the response is independent of the number of double bonds in the molecule (7). Recently, we published a method for the quantitative analysis of intact molecular species of PC using a light scattering detector (8). However, this method is not applicable as such for PE, because the presence of triethylamine in the solvent causes all PE molecular species to elute from the column without retention. We have now developed a method for the separation of intact molecular species of PE that enables the use of a light scattering detector and the isolation of individual species in a pure form.

Abbreviations: ECN, equivalent carbon number; ELSD, evaporative light scattering detection; ESI, electrospray ionization; FAB, fast atom bombardment; (RP-) HPLC, (reverse phase-) high performance liquid chromatography; MS, mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine (referring collectively to all diradyl ethanolamine glycerophospholipid species); PtdEth, diacyl phosphatidylethanolamine; PlasEth, plasmalogen PE species (1-alk-1′-enyl 2-acyl ethanolamine glycerophospholipids); R'_t, relative retention time.

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A PE sample from a biological origin usually contains a large number of fatty acyl combinations bonded via different types of radyl linkage, making identification of each and every peak a laborious task. Mass spectrometry (MS) has been used successfully in the past to aid in the identification of molecular species of PE and its derivatives (9–12). The electrospray ionization procedure used here poses the same restrictions on the mobile phase as an evaporative light scattering detection (ELSD). Both detectors display best performance when no salts are present and the solvent is volatile. In this paper we describe an HPLC method for the rapid analysis of intact PE molecular species, which is capable of good component separation and quantification. Identification of the individual molecular species and the assignment of the position of the acyl chains at the glycerol backbone was achieved with on-line electrospray mass spectrometry.

MATERIALS AND METHODS

Isolation of PE

Bovine heart PE and rat liver PE were purified by normal phase HPLC according to the method of Letter (3) with some minor modifications as published previously (8).

High performance liquid chromatography

All solvents were from Labscan, Dublin, Ireland and of HPLC grade. Molecular species of approximately 50 nmol of PE were separated on two 250 × 4 mm Lichrospher RP-18 endcapped columns in series (Merck, Darmstadt, Germany). Isocratic elution was performed with a solvent consisting of acetonitrile–methanol 3:7 (v/v) containing 5 μm ethanolamine, or a solvent consisting of acetonitrile–methanol 2:3 (v/v) containing 2 μm ethanolamine at a flow rate of 1.25 $ml \cdot min^{-1}$. The column effluent was monitored at 206 nm using an LKB 2251 Uvicord (Pharmacia, Upsala, Sweden) and subsequent ELSD was performed using a Varex MKIII obtained from Alltech, (Deerfield, IL) operating at a gas flow rate of 1.9 $l \cdot min^{-1}$ and a drift tube temperature of 100°C. The detector was calibrated by triplicate injections of 1, 2.5, 5, 7.5, 10, 15, 20, 25, and 30 μg (approx. 1–40 nmol) dioleoyl phosphatidylethanolamine. For on-line mass spectrometry, the flow rate of the HPLC was adjusted to 1 $ml \cdot min^{-1}$ and a flow splitter was inserted between column and mass spectrometer. The flow into the mass spectrometer was set to approximately 0.1 $ml \cdot min^{-1}$.

Mass spectrometry

A Fisons VG Platform II single quadrupole mass spectrometer (Micromass, Manchester, UK), fitted with an electrospray ion source operated at atmospheric pressure, was used for the identification of components eluting from the column. Nitrogen was used as nebulizer gas (0.2 $l \cdot min^{-1}$) and as curtain gas (7 $l \cdot min^{-1}$). The capillary voltage was set to 3.0 kV and the cone voltage to 100 V.

Data analysis

Data obtained from the ELSD were recorded and analyzed using EZChrom chromatography data system (Scientific Software, San Ramon, CA). MS data were recorded and analyzed using Masslynx software (Micromass, Manchester, UK).

RESULTS AND DISCUSSION

High performance liquid chromatography

Non-specific interaction is known to occur between the phospholipid headgroup and column material and to interfere with an optimal separation. In the past, this has been prevented by the addition of either choline chloride or triethylamine to the solvent system (5, 8). Here, we were able to reduce the interaction between the PE headgroup and the column material by the addition of low concentrations of ethanolamine (2–5 μm) and frequent regeneration of the column with acetone, hexane, and ethanol. Despite the fact that two columns in series are used for the separation of molecular species, the total time required for analysis is only 75 min at a flow rate of 1.25 $ml \cdot min^{-1}$, which compares favorably with the well-known method published by Patton et al. (5), requiring 150 min at a flow rate of 2 $ml \cdot min^{-1}$. **Figure 1** shows the separation of intact PE molecular species from bovine heart as detected by UV absorbance and subsequent ELSD. Although both detectors yield comparable chromatograms, the increased response of the UV detector to highly unsaturated species (e.g., (16:0–20:5)PlasEth, peak 4) relative to more saturated species (e.g., (18:0–18:2)PtdEth, peak 16) is evident. For the quantification of species based on the absorbance at 206 nm, response factors for the individual molecular species are required, thereby excluding the analysis of samples with an unknown composition or fully saturated species (6). Light scattering detection, on the other hand, permits quantification at the nanomole level and is unaffected by the degree of unsaturation of lipids, but careful calibration of the detector is necessary as the response of the detector is at low concentrations nonlinear with the amount of sample (1, 8).

After calibration of the light scattering detector in the range of 1 to 40 nmol with (18:1–18:1)PtdEth, the molecular species of PE and their abundance in rat liver and bovine heart were determined as listed in **Table 1** and **Table 2**. The species composition of PE isolated from rat liver has previously been described (5, 13, 14), and although the exact amounts of individual molecular species vary somewhat between the different studies, the main characteristics do not. Typical is the low amount of ether lipids in rat liver found in the literature (5, 13, 14) as well as in the present study; all common species are of the diacyl subclass. The molecular species (16:0–20:4) PtdEth, (16:0–22:6) PtdEth, (18:0–20:4) PtdEth, and (18:0–22:6) PtdEth are invariably reported as the most abundant species. These are also major components according to our analysis (Table 1). Earlier analysis of membranes from (canine) heart tissue by reverse phase (RP)-HPLC and fast atom bombardment mass–spectrometry (FAB-MS) has revealed that the PE fraction of these membranes is rich in plasmalogens and that arachidonic acid is the predominant fatty acyl at the *sn*-2 position (15–17). The (bovine) heart PE fraction analyzed here (Table 2), is also rich in plasmalogens (53% of total PE), and contains arachidonic acid as the most common fatty acyl at the *sn*-2 position (40%).

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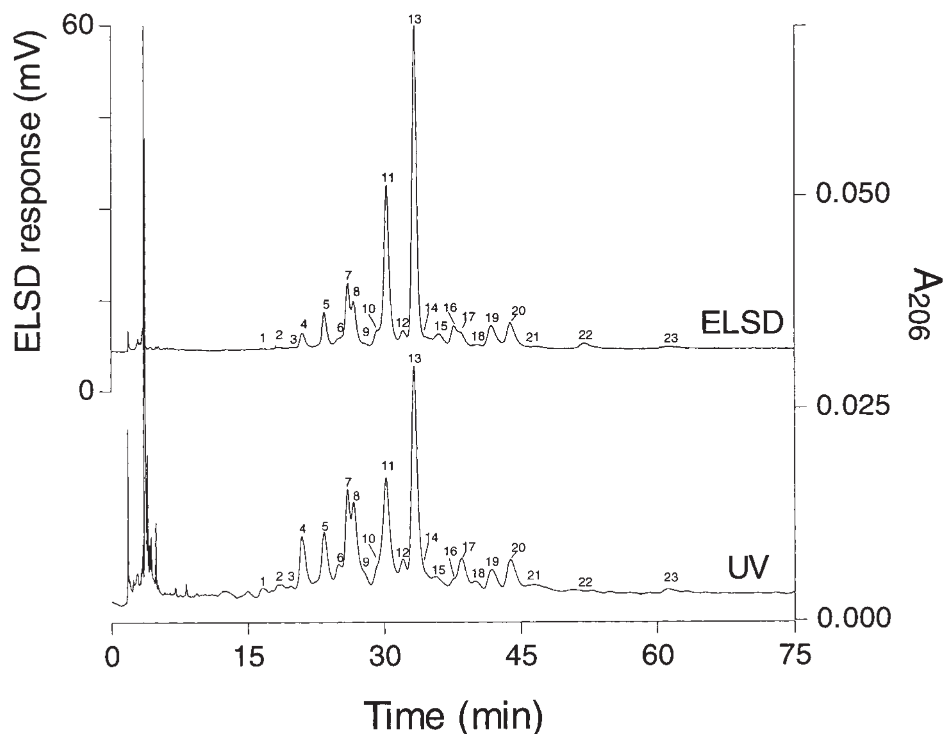

Fig. 1. The separation of intact PE molecular species from bovine heart. Molecular species were resolved on two Lichrosphere RP-18 columns in series using a mobile phase consisting of 5 μm ethanolamine in methanol–acetonitrile 7:3 (v/v). Detection was performed by measurement of the absorbance at 206 nm (**UV**) and subsequent light scattering detection (**ELSD**). Peak numbers correspond to species listed in Table 2.

Identification of species

In the past, the high sensitivity of electrospray ionization mass spectrometry (ESI-MS) has successfully been used for the analysis of phospholipids and their molecular species by direct injection of chloroform extracts (18–21). Although direct injection does allow detection of subpicomole amounts of phospholipids (18, 21), individual species cannot be isolated for further use or analysis. Furthermore, complex mixtures of lipids require tandem mass spectrometry (MS/MS), making their analysis time-con-

TABLE 1. Molecular species of phosphatidylethanolamine found in rat liver

	Component	Abundance
		mole %
1	(16:0–18:1)PtdEth	3.6[a]
2	(16:0–18:2)PtdEth	8.0
3	(16:0–20:3)PtdEth	1.8
4	(16:0–20:4)PtdEth	18.3
5	(16:0–22:6)PtdEth	21.4
6	(18:0–18:1)PtdEth	tr
7	(18:0–18:2)PtdEth	3.4
8	(18:0–20:3)PtdEth	1.6
9	(18:0–20:4)PtdEth	23.0
10	(18:0–22:6)PtdEth	4.6
11	(18:1–18:2)PtdEth	1.7
12	(18:1–20:4)PtdEth	2.5
13	(18:1–22:6)PtdEth	4.6
14	(18:2–18:2)PtdEth	tr
15	(18:2–20:4)PtdEth	tr

Mole percentages were calculated from the ELSD response using a calibration curve constructed with dioleoyl PE as described in Materials and Methods section; tr, trace amounts detected (<1%).
[a]Also contained (18:1–18:1)PtdEth.

TABLE 2. Molecular species of phosphatidylethanolamine found in bovine heart tissue

Peak Number	Component	Abundance
		mole %
1	(15:0–20:5)PlasEth	tr
2	(15:0–18:3)PlasEth	tr
3	(14:0–18:3)PlasEth	tr
4	(16:0–20:5)PlasEth	2.3
5	(16:0–18:3)PlasEth	4.5
6	(15:0–18:2)PlasEth	1.3
7	(18:0–20:5)PtdEth	6.9
8	(16:0–20:4)PlasEth	5.5
9	(18:0–20:5)PlasEth	tr
10	(16:0–16:1)PlasEth	2.1
11	(16:0–18:2)PlasEth	19.8
12	(16:0–20:3)PlasEth	2.4
13	(18:0–20:4)PtdEth	31.8
14	(16:0–17:1)PlasEth	tr
15	(16:0–18:1)PtdEth	2.8
16	(18:0–18:2)PtdEth	2.2
17	(18:0–20:4)PlasEth	2.8
18	(18:0–20:3)PtdEth	tr
19	(16:0–18:1)PlasEth	4.2
20	(18:0–18:2)PlasEth	4.8
21	(18:0–20:3)PlasEth	tr
22	(18:0–18:1)PtdEth	1.0
23	(18:0–18:1)PlasEth	tr

The abundance of species was calculated as in Table 1. Peak numbers correspond to the peaks in Fig. 1; tr, trace amounts detected (<1%).

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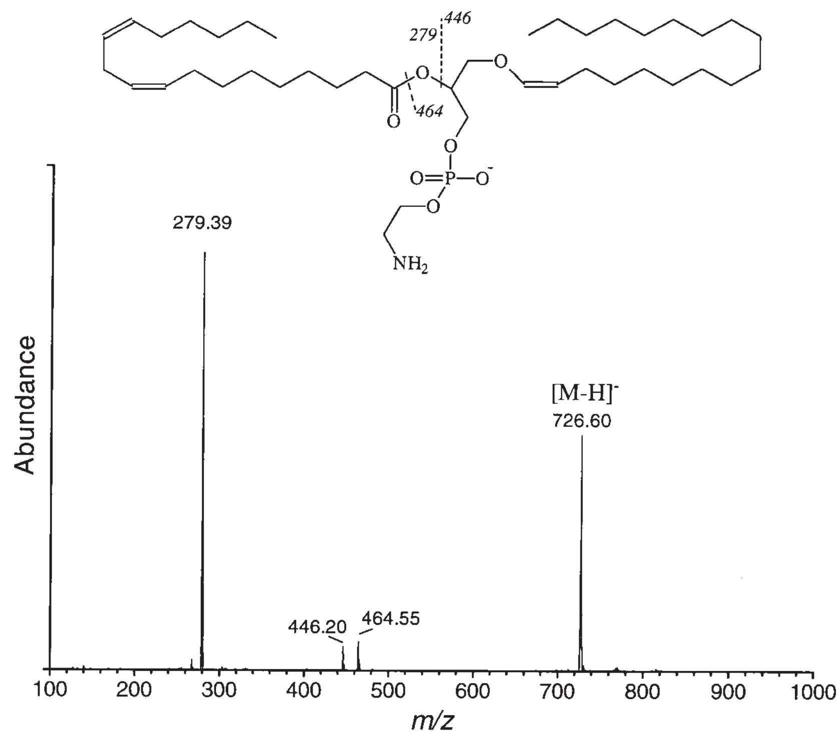

Fig. 2. Negative ion mass spectrum recorded during the elution of component 20 in Fig. 1 ((18:0–18:2)PlasEth). Observed ions correspond to [M–H]$^-$ (*m/z* 726.6) and ions resulting from fragmentation at the sites indicated in the structure formula of the [M–H]$^-$ anion.

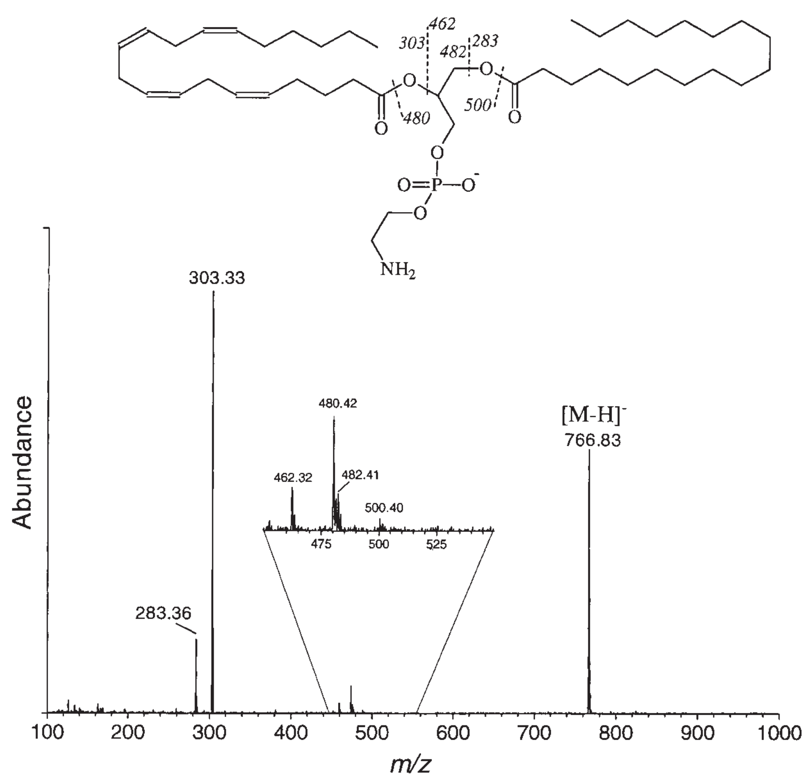

Fig. 3. Negative ion mass spectrum during the elution of (18:0–20:4) PtdEth in a sample of rat liver PE. Elution was performed as in Fig. 1. Observed ions correspond to [M–H]$^-$ (*m/z* 766.8) and ions resulting from fragmentation at the sites indicated in the structure formula of the [M–H]$^-$ anion.

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suming and difficult to automate. Here, we achieved on-line identification of individual species eluting from the column using a single quadrupole mass spectrometer fitted with an electrospray ion source operating in the negative ion mode. Spectra of eluting plasmalogen species displayed the fatty acid carboxylate anion derived from the *sn*-2 position, the molecular ion $[M-H]^-$, and two ions resulting from fragmentation at the ester bond (**Fig. 2**). Fragmentation of the vinyl-ether bond at the *sn*-1 position did not occur. These (single MS) spectra thereby showed large resemblance to tandem MS spectra obtained after collision-induced dissociation of PE molecular ions (21, 22).

Eluting diacyl species also had fragmentation patterns that were comparable to previously published data from more complex tandem mass spectrometry (**Fig. 3**, refs. 19, 21, 22). Characteristic is the presence of the molecular ion $[M-H]^-$ and both the fatty acid carboxylate anions from the *sn*-1 (*m/z* 283) and *sn*-2 (*m/z* 303) position, the *sn*-2 carboxylate anion being more abundant than the *sn*-1 carboxylate anion, thereby enabling determination of the position of the fatty acyl groups along the glycerol backbone. A recently published HPLC/ESI-MS method for the determination of acyl chain position in PC molecular species is based on the relative abundance of the *sn*-1 and *sn*-2 lyso-PC fragment ions (23). Similarly, further confirmation of acyl position in PE species could be obtained from the abundance of the *sn*-1 and *sn*-2 lyso-PE ions in the mass spectrum (see inset, Fig. 3). Loss of the acyl group from the *sn*-2 position as $R_2 = C = O$ (leading to the fragment ion at *m/z* 480) was considerable. From the *sn*-1 position, loss of $R_1 = C = O$ was hardly observed (*m/z* 500). From both the *sn*-1 and *sn*-2 position the radyl group was lost as intact fatty acid (leading to *m/z* 482 and *m/z* 462, respectively).

Relation between retention time and carbon number

The isocratic elution in this system had another advantage in that a mathematical correlation existed between the relative retention time (R'_t) and the equivalent carbon number (ECN) of a molecular species (**Fig. 4**). We found that the ECN of a given species can be calculated from the number of carbon atoms (CN) and double bonds in the aliphatic groups (n) of the species by the empirically obtained formulas $ECN = CN - 1.505n$ (diacyl species) and $ECN = CN - 1.538n$ (plasmalogen species). The formulas relating R'_t to ECN are illustrated in Fig. 4 for plasmalogen and diacyl species of the analyzed samples of bovine heart and rat liver PE, respectively, and can assist in the identification of unknown PE species when a mass spectrometer is not available.

Method performance

In conclusion, the HPLC method described here separates a wide variety of molecular species of PE in an intact form using solvents that enable UV detection as well as ELSD. The sensitivity of ELSD for mass rather than for degree of unsaturation enables the sensitive and quantitative analysis of PE samples of unknown composition. Furthermore, unequivocal species identification, including the assignment of acyl chain position, can be achieved by on-line electrospray MS. The observed relationship between ECN and retention time can aid in the identification of unknown species. The method can be applied for the isolation of individual PE species in order to detect their biological activities.

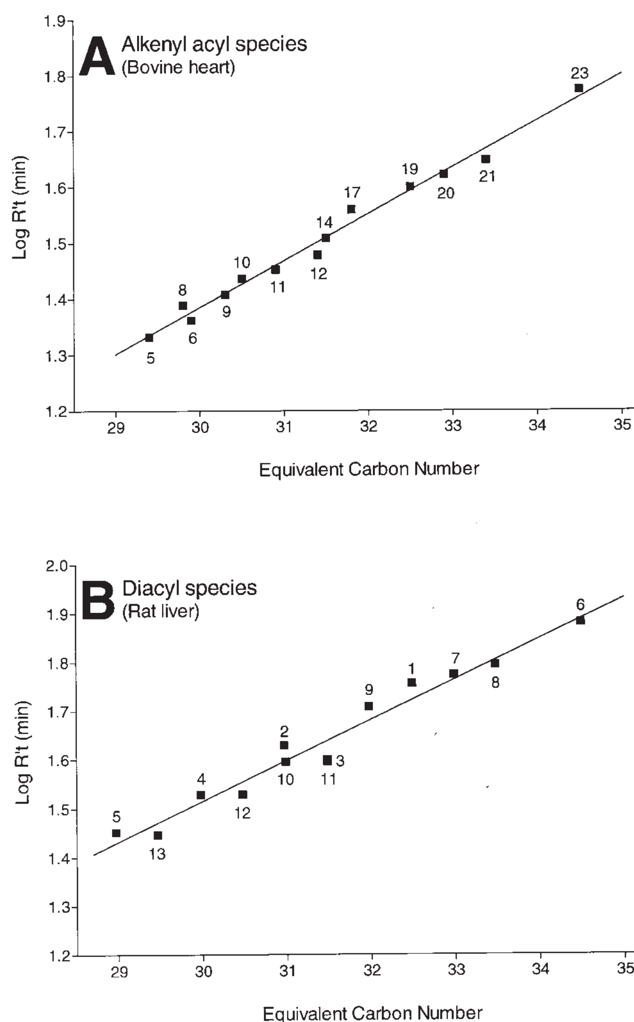

Fig. 4. Exponential relationship between the relative retention time of molecular species and their equivalent carbon numbers. **A:** Plasmalogen species of bovine heart PE eluted with methanol–acetonitrile 7:3 (v/v) from two endcapped Lichrosphere RP18 columns in series in the presence of 5 μm ethanolamine. Numbers refer to the species listed in Table 2. **B:** Diacyl species of rat liver PE eluted with methanol–acetonitrile 3:2 (v/v) in the presence of 2 μm ethanolamine. Numbers refer to the species listed in Table 1.

This work was supported by the Netherlands Foundation for Chemical Research (SON) and the Life Science Foundation (SLW) of The Netherlands Organization for Scientific Research (NWO).

Manuscript received 23 April 1998 and in revised form 1 September 1998.

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