MAGNETIC RESONANCE IN CHEMISTRY
Magn. Reson. Chem. **36**, 907–912 (1998)

^{31}P NMR analysis of phospholipids in crude extracts from different sources: improved efficiency of the solvent system

Nicola Culeddu,[1] Marco Bosco,[2]* Renato Toffanin[2] and Piero Pollesello[3]

[1] CNR IATCAPA, via Vienna 2, I-07100 Sassari, Italy
[2] POLY-tech Scrl, Area Science Park, Padriciano 99, I-34012 Trieste, Italy
[3] Orion Corporation, Orion Pharma, R&D, Drug Design Unit, NMR Laboratory, P.O. Box 65, FIN-02101 Espoo, Finland

Received 27 April 1998; revised 15 July 1998; accepted 17 July 1998

ABSTRACT: Crude lipid extracts from different tissues were analysed by ^{31}P NMR spectroscopy in a mixture of triethylamine, dimethylformamide and guanidinium chloride. Higher dispersion of the ^{31}P NMR signals and better reproducibility of the chemical shifts were achieved with respect to the spectra acquired in the conventional chloroform–methanol–water solvent. In particular, the reproducibility of the chemical shifts allowed the unambiguous identification of all the components in the ^{31}P NMR spectra of complex phospholipid mixtures, which was not feasible using the chloroform–methanol–water system. The efficiency of the new solvent was tested on crude lipid extracts from different biological sources such as lecithin, mouse mammary carcinoma, porcine stomach, porcine spleen, porcine brain, human red blood cells, yeast, fungi and tuna fish muscle. The reproducibility of the chemical shift of the phospholipid signals was demonstrated in the presence of various amounts of neutral lipids (triglycerides) and sterols, which are commonly present in the Folch extracts. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{31}P NMR; chemical shifts; phospholipids; crude lipid extracts

INTRODUCTION

The phospholipids (PLs) are the main constituents of biological membranes, where they play many roles. The study of the PL composition of tissues, cell lines, organelles, plasma lipoproteins, etc., has become more important with time, as the phospholipids and their metabolism have been shown to be of crucial importance for basic cell functions such as cell trafficking and signal transduction.[1–3] Moreover, it has been demonstrated that the PLs play a role in pathologies such as various cardiovascular diseases and cancer.[4,5] In fact, since PLs stabilize the hydrophobic core in the blood plasma lipoproteins,[6] variations in their composition and concentration are associated with thrombosis. Furthermore, the PL composition is related to the evolution and growth of tumoral cells[7,8] and can be used as a marker to follow the effect of pharmacological treatments. As an example, the clinical analysis of serum PLs is a sensitive indicator in the monitoring of treatment in leukaemia.[9]

The analysis of phospholipids from any kind of biological source requires a lipidic extraction[10] followed by chemical analysis using chromatographic and/or spectroscopic techniques.[11–14] The separation of the PL components from a mixture by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) followed by chemical analysis of the total phosphorus content or by spectrophotometric (UV–visible) or spectrofluorimetric detection is required. These complicated procedures need chemical derivatizations and calibration using suitable internal standards. In contrast, NMR spectroscopic methods are non-degrading, easy, rapid and do not need any standardization. In particular, ^{31}P NMR is preferred to ^{1}H NMR techniques since the signals are better resolved and do not overlap with signals from neutral lipids usually present in the extracts.[15–17]

The solvent system commonly used in the NMR analysis of phospholipids is a chloroform–methanol–water mixture, which is a two-phase system. This may affect the reproducibility of the ^{31}P NMR signals primarily because their chemical shifts depend on the relative volume ratio of the three solvents.[18,19] However, reproducible results can be obtained provided that proper care is taken in preparing the samples. In previous work, to overcome these disadvantages, we tested a broad choice of different solvent systems;[19] we ultimately chose a combination of triethylamine, dimethylformamide and guanidinium chloride (Et_3N–DMF–GH^+), which is stable, as none of the components is volatile at room temperature and it is very efficient in the resolution of the PL signals in the NMR spectrum.

In this work, to demonstrate the efficiency of this new solvent system, we tested the reproducibility of the chemical shifts in the ^{31}P NMR analysis of phospholipids extracted from a wide range of different biological sources.

* *Correspondence to*: M. Bosco, POLY-tech Scrl, Area Science Park, Padriciano 99, I-34012 Trieste, Italy.
E-mail: bosco@poly01.tbs.trieste.it

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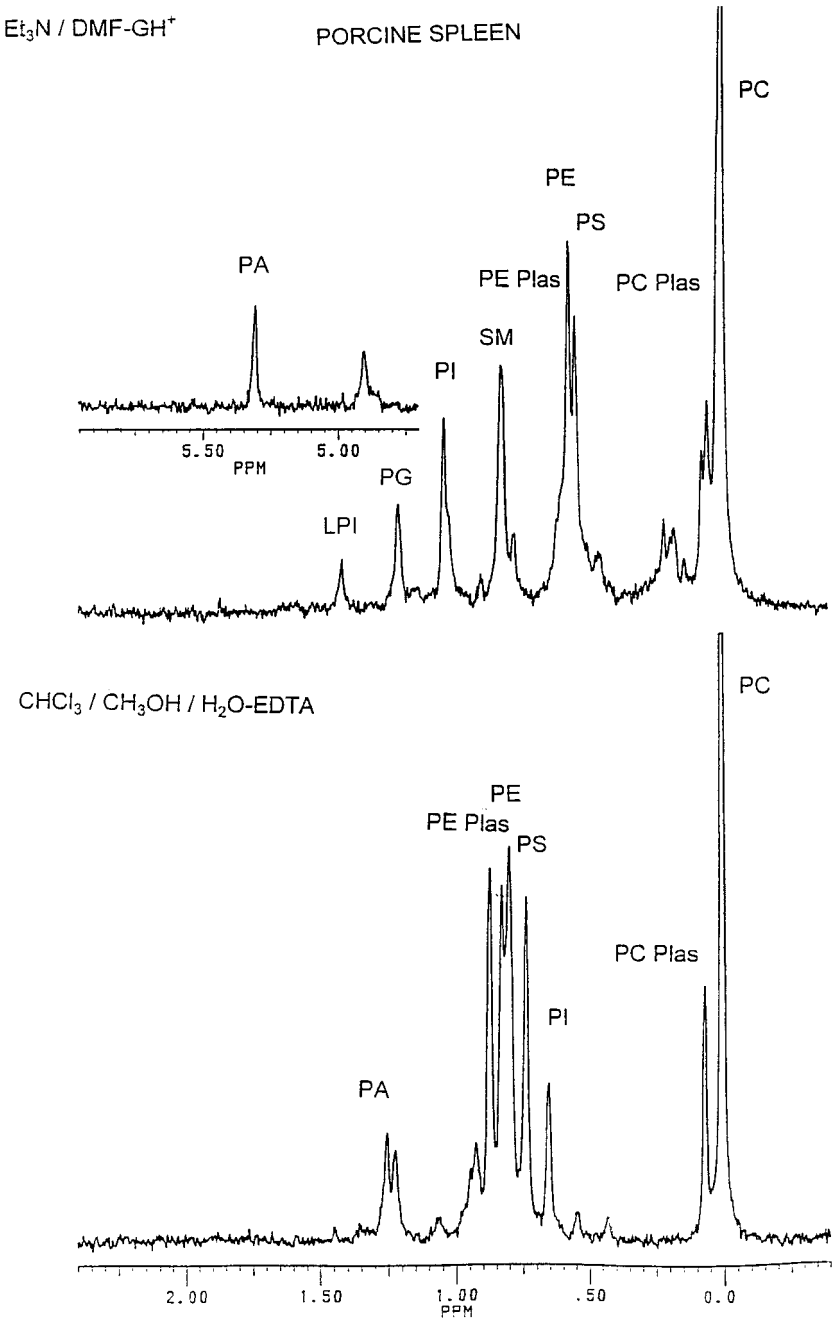

Figure 1. ^{31}P NMR spectra of a porcine spleen extract measured in $CHCl_3$–CH_3OH–H_2O–EDTA and Et_3N–DMF–GH^+.

RESULTS AND DISCUSSION

In previous work,[19] we demonstrated that the linewidth of the ^{31}P NMR signals is very sensitive to the composition of the solvent system. This probably means that the solvent can modulate the stability of PL micelles of different dimensions. It is well known that paramagnetic metal ions are a potential source of line broadening; however, the problem was solved by prewashing the samples with aqueous EDTA. Since the linewidths of the ^{31}P signals in our analysis are similar to those obtained in a water–detergent system, it can reasonably be deduced that the PLs are packed in small micelles and not, for instance, in double layers. This hypothesis is also supported by the T_1 values measured for lecithin in the same solvent system.[19] It should be considered that, in all organic (hydrophobic) solvents, inverse micelles are predicted. In this case, the phosphate groups do feel any variation of the environment inside the micelles and, as a consequence, their chemical shifts may vary significantly. In addition, the composition of the PL mixture or the amount of other neutral lipids can also affect the interactions among the phosphate groups and, as a consequence, their chemical shifts. For these reasons, the efficiency of the solvent system was checked on PLs extracted from a wide range of biological sources such as commercial lecithin, mouse mammary carcinoma (MCA), porcine stomach, porcine spleen, porcine liver, porcine brain, human red blood cells, yeast, fungi and tuna fish muscle.

Figures 1–3 show the ^{31}P NMR spectra of crude lipid extracts from porcine spleen, porcine stomach and mouse mammary carcinoma in Et_3N–DMF–GH^+ and $CHCl_3$–CH_3OH–H_2O–EDTA. For both solvent systems, the ^{31}P NMR signals of the most abundant PLs, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM) and phosphatidylinositol (PI), are well resolved. However, the spectra obtained in Et_3N–DMF–GH^+ show better resolution of the ^{31}P NMR signals of less abundant PLs such as lysophosphatidylcholine (LPC), phosphatidylglycerol (PG), *N*-monomethylphosphatidylethanolamine (MPE), *N*,*N*-dimethylphosphatidylethanolamine (DPE), phosphatidic acid (PA), their lyso derivatives and plasmalogens. This made the quantification of these components possible. Moreover, the better resolution revealed some further components, still unknown.

In comparison with the ^{31}P NMR spectra obtained in the chloroform–methanol–water system, those obtained in Et_3N–DMF–GH^+ show a better dispersion of the signals and generally a reduced linewidth.[16] For example, phosphatidylethanolamines (mono- and dimethyl) show separate signals (0.57, 0.47 and 0.27 ppm), as confirmed with commercial standards. Moreover, the signal due to phosphatidic acid and its lyso derivative, as a phosphomonoester, is approximately 5 ppm downfield from all the other PL signals. The ^{31}P chemical shifts of the PLs, obtained for the different crude lipid extracts in $CHCl_3$–CH_3OH–H_2O–EDTA

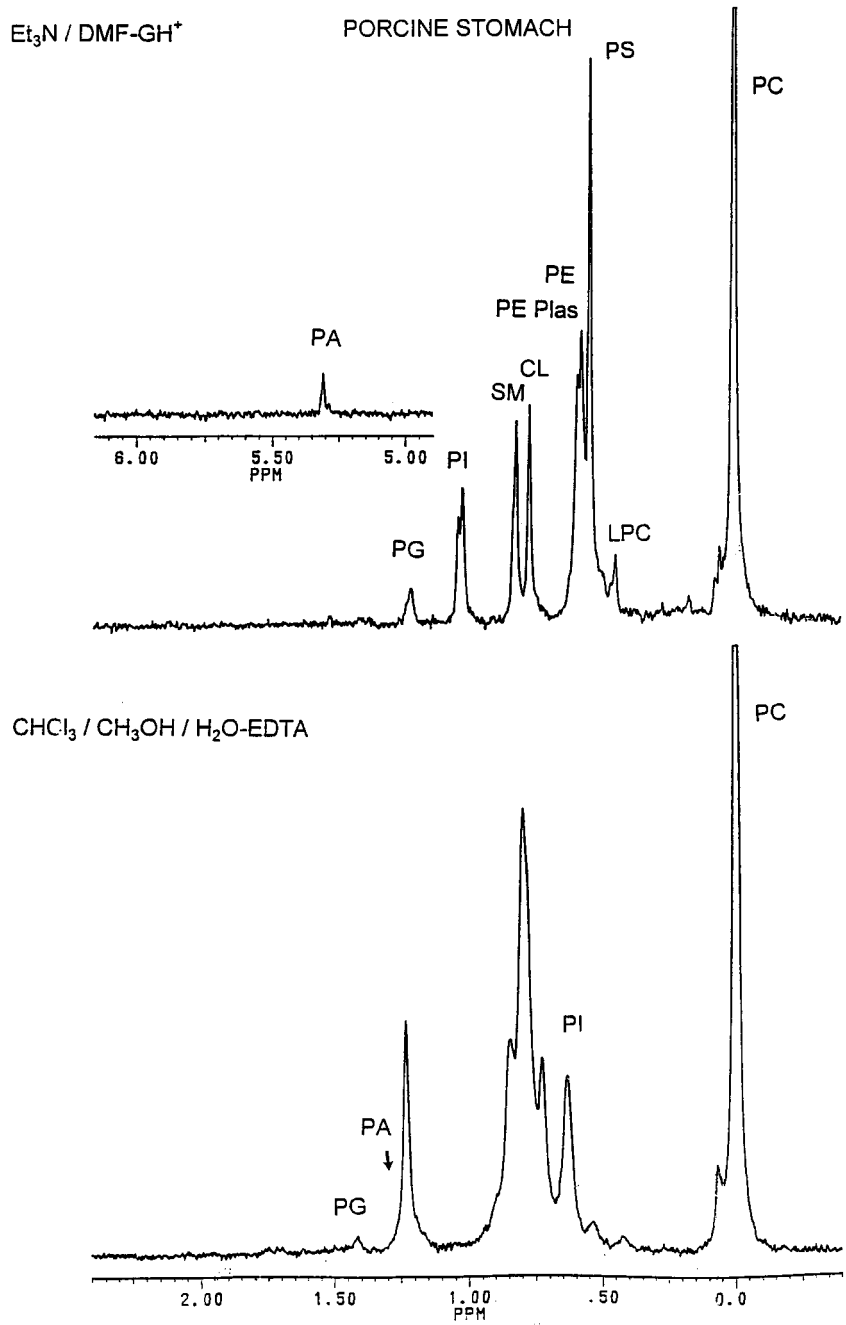

Figure 2. ^{31}P NMR spectra of a porcine stomach extract measured in $CHCl_3$–CH_3OH–H_2O–EDTA and Et_3N–DMF–GH^+.

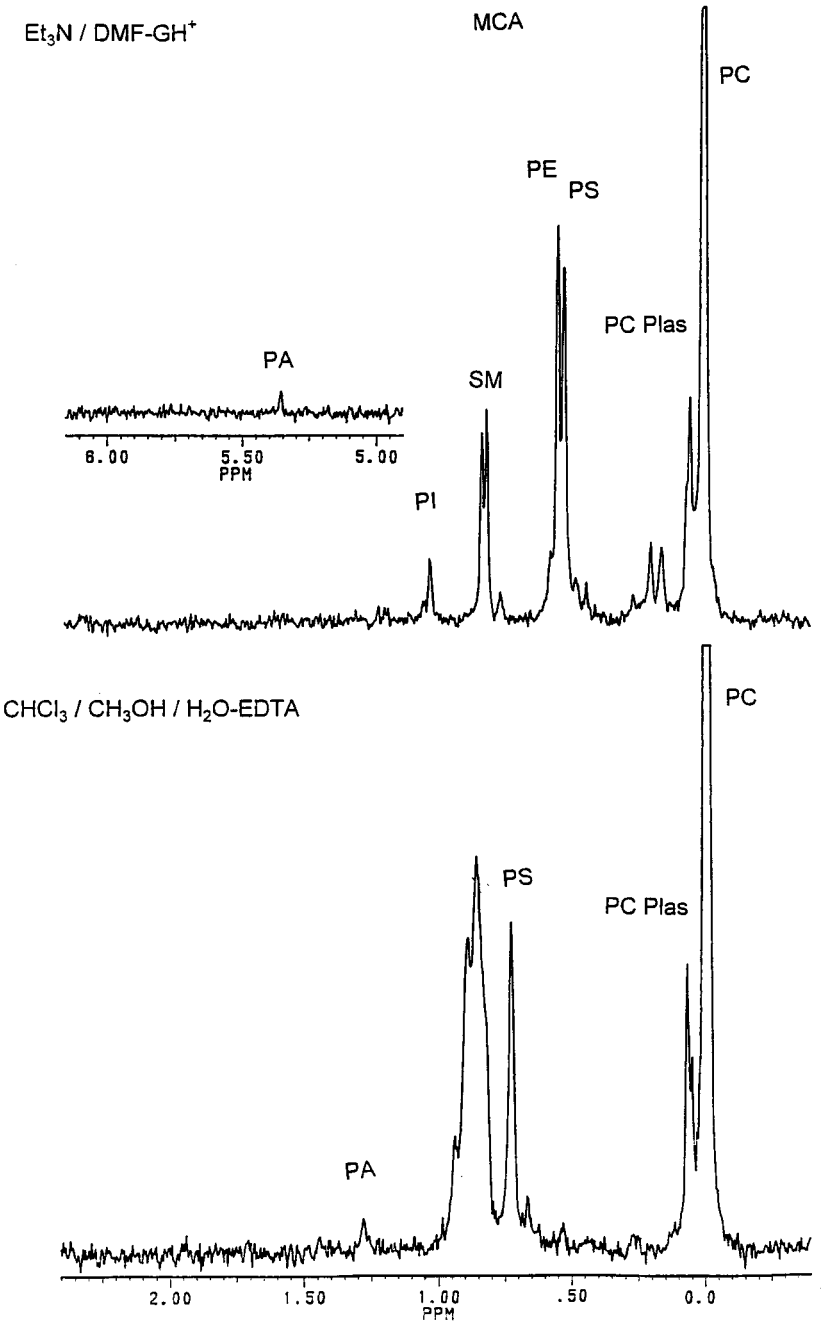

Figure 3. ^{31}P NMR spectra of a mouse mammary carcinoma (MCA) extract measured in $CHCl_3$–CH_3OH–H_2O–EDTA and Et_3N–DMF–GH^+.

and Et_3N–DMF–GH^+, are reported in Tables 1 and 2, respectively. For some of the samples in $CHCl_3$–CH_3OH–H_2O–EDTA, the signal overlap prevented unambiguous assignment; therefore, the relative chemical shifts are omitted in Table 1. The row denoted 'Literature' refers to data obtained by Glonek and coworkers in chloroform–methanol–water.[16,20,21]

In both tables, the average chemical shift values of the various PLs are also reported together with their relative standard deviations (σ). The separation, in ppm, of each signal from the neighbouring peaks ($\Delta\delta$) is calculated as $(\delta_{i+1} - \delta_{i-1})/2$, where δ_{i+1} and δ_{i-1} are the frequencies of the downfield and the upfield signals, respectively, with respect to the observed signal (δ_i). The comparison between σ and $\Delta\delta$ shows the probability that the signals overlap: if they are similar, the assignment of the relative PLs is uncertain. In the spectra obtained in $CHCl_3$–CH_3OH–H_2O–EDTA the separation of the signals in ppm ($\Delta\delta$) is as large as the standard deviation of their chemical shifts (σ). In contrast the analysis performed in Et_3N–DMF–GH^+ gives ^{31}P NMR spectra with a greater signal separation and smaller standard deviation; in this case $\Delta\delta$ is often ten times larger than σ. This means that the new solvent guarantees a highly reproducible ^{31}P NMR spectrum of crude extracts for a wide range of biological samples.

The average chemical shift values of the less abundant PLs are reported as follows: phosphatidylglycerol (PG), 1.25 ppm; lysophosphatidylinositol (LPI), 1.50 ppm; cardiolipin (CL), 0.77 ppm; *N*-

Table 1. ^{31}P chemical shift values of phospholipids in $CHCl_3$–CH_3OH–H_2O–EDTA, obtained for extracts from different biological tissues[a].

	LPC	PS	PE	SM	PI	PA
Literature	0.56	0.63/0.78	0.84	0.75	0.56	1.14/1.63
Lecithin	0.55		0.81		0.62	1.27
MCA	0.53	0.73	0.85	0.89	0.66	1.27
Porcine stomach	0.54	0.80	0.85	0.73	0.62	1.24
Porcine spleen	0.54	0.74	0.80	0.87	0.65	1.26
Porcine liver	0.56		0.80	0.72	0.42	1.19
Porcine brain						
Red blood cells		0.83	0.89	0.76	0.56	
Fungi	0.54				0.54	1.30
Yeast		0.79			0.51	1.33
Tuna fish muscle	0.56			0.86	0.68	1.28
$\delta_{average}$	0.55	0.74	0.82	0.80	0.60	1.28
σ	$\pm$0.01	$\pm$0.06	$\pm$0.02	$\pm$0.07	$\pm$0.08	$\pm$0.14
$\Delta\delta$	$\pm$0.05	$\pm$0.10	$\pm$0.02	$\pm$0.04	$\pm$0.09	0.46
			0.27			—

[a] All the chemical shifts (δ) are reported in ppm and referenced to PC. The standard deviation of the chemical shifts is reported as σ. $\Delta\delta$ is the distance between the signals as described in the text.

monomethylphosphatidylethanolamine (MPE), 0.47 ppm; *N*,*N*-dimethylphosphatidylethanolamine (DPE), 0.27 ppm; and lysophosphatidic acid (LPA), 6.02 ppm. Other minor PL components were present in only a few samples and hence their statistical parameters are not significant.

It is interesting that, in the extracts of human red blood cells and porcine brain (Table 2), a new signal at 5.46 ppm was found. Since in these samples there is a relevant amount of SM, it is likely that partially hydrolysed sphingomyelin molecules give a phosphomonoester similar to PA. Until now, this hypothesis could not be confirmed since a proper standard was not available.

The data clearly demonstrate the improved efficiency of the Et_3N–DMF–GH^+ solvent system in the ^{31}P NMR analysis of crude lipid extracts. The fact that the ^{31}P signals in NMR spectra obtained in this solvent have such a reproducible chemical shift, high spectral dispersion and reduced linewidth may be related to the packing of PLs in small homogeneous micelles which do not contain other kinds of lipids, especially neutral lipids. Since the use of the Et_3N–DMF–GH^+ system in the NMR analysis of phospholipids makes the identification and quantification of each component easier, it can certainly lead to a better understanding of the metabolic pathways in which the phospholipids are involved.

EXPERIMENTAL

Sample preparation and extraction

The crude lipid extracts were obtained with a modified version of the standard Folch method[10] from different

Table 2. ^{31}P chemical shift values of phospholipids in Et_3N–DMF–GH^+, obtained for extracts from different biological sources[a]

	LPC	PS	PE	SM	PI	PA
Lecithin	0.44		0.56		1.03	5.33
MCA	0.44	0.53	0.55	0.83	1.04	5.35
Porcine stomach	0.45	0.55	0.58	0.83	1.03	5.31
Porcine spleen	0.46	0.55	0.58	0.83	1.04	5.31
Porcine liver	0.44		0.56	0.83	1.05	5.33
Porcine brain	0.46		0.58	0.84	1.05	5.36
Red blood cells		0.53	0.58	0.84	1.07	
Yeast	0.44	0.54		0.83	1.03	5.32
Fungi	0.46	0.53			1.02	5.35
Tuna fish muscle	0.44		0.56	0.83	1.03	5.28
$\delta_{average}$	0.448	0.543	0.57	0.832	1.037	5.32
σ	$\pm$0.009	$\pm$0.009	$\pm$0.010	$\pm$0.007	$\pm$0.007	$\pm$0.020
$\Delta\delta$	$\pm$0.099	$\pm$0.059	$\pm$0.140	$\pm$0.237	$\pm$0.205	4.28
			0.59			—

[a] All the chemical shifts (δ) are reported in ppm and referenced to PC. The standard deviation of the chemical shifts is reported as σ. $\Delta\delta$ is the distance between the signals as described in the text.

biological sources: (a) lecithin, (b) mouse mammary carcinoma, (c) porcine stomach, (d) porcine spleen, (e) porcine liver, (f) porcine brain, (g) human red blood cells, (h) yeast, (i) fungi and (j) tuna fish muscle. In order to avoid the presence of divalent cations, the chloroform–methanol extracts were washed twice with the same volume of a 0.01 M Na–EDTA–0.1 M NaCl solution. In order to test if the water–methanol phase after the Folch extraction contained a significant amount of phospholipids, this phase was separated and lyophilized. The pellets were then resuspended in chloroform and their ^{31}P NMR spectra did not show any significant signal.

The organic phase was dried with anhydrous Na_2SO_4, filtered and evaporated under vacuum. The chloroform solution was dried to avoid a residual water content which causes relevant shifts of ^{31}P NMR signals from their typical values.

The extracts were divided into two aliquots, which were dissolved in Et_3N–DMF–GH^+ (150 ml + 500 ml + 50 mg) and $CHCl_3$–CH_3OH–H_2O–EDTA. The typical concentration of PLs was 2 mM. Some of these extracts were very rich in neutral lipids (triglycerides and cholesterol), as shown by their ^{1}H NMR spectrum in $CHCl_3$.[22] In this case, the addition of the Et_3N–DMF–GH^+ solvent did not lead to a clear solution and, after few minutes, a thin, insoluble layer separated above the solution. In a test experiment, this layer was collected and dissolved in $CDCl_3$–CD_3OD; the ^{1}H NMR spectrum showed a high concentration of neutral lipids whereas the ^{31}P NMR spectrum did not show any signal.

All chemicals were of HPLC grade from Aldrich. The use of lower quality solvents for the Folch extraction, especially chloroform, led to partial or extensive hydrolysis of the phosphate esters, probably due to HCl contamination of the solvent.

NMR measurements

^{31}P NMR spectra were recorded at 121.0 MHz on a Varian VXR 300-s instrument and at 81.015 MHz on a Bruker AC 200 spectrometer. Typically, ^{31}P NMR spectra were acquired unlocked at 297 K with a 90° pulse of 10 μs, 16K data points, a 1.5 s pulse repetition time and composite ^{1}H decoupling. In a test experiment the measurement was repeated also with longer repetition times (up to 6 s); the relative integrals of the different signals were constant for all the experiments. The data were processed with a 0.3 Hz line broadening factor and zero filling before Fourier transformation. The chemical shifts and linewidths were measured digitally and referenced to phosphatidylcholine (PC). The chemical shift of PC was also measured with respect to external 85% orthophosphoric acid ($\delta = +0.38 \pm 0.01$ ppm).[19]

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