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Note

Simple and rapid method for high-performance liquid chromatographic separation and quantification of soybean phospholipids

PAUL VAN DER MEEREN*, JAN VANDERDEELEN, MICHEL HUYS and LEON BAERT
State University of Ghent, Faculty of Agriculture, Department of Physical and Radiobiological Chemistry, Coupure Links 653, B-9000 Ghent (Belgium)
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The past ten years, numerous methods have been described for the separation of phospholipids. With few exceptions[1,2], silicic acid was used as the stationary phase, the mobile phase being either *n*-hexane–2-propanol–water[3–10], or acetonitrile–methanol–water[11–16]. However, detection of the phospholipids has been a major problem. Although lipids lack specific absorption peaks, UV detection has nevertheless mostly been used. The strong absorption in the 200–214 nm region is caused by the presence of unsaturated centres and functional groups such as carbonyl, carboxyl, phosphate, amino and quaternary ammonium. As the extinction coefficient depends on the degree of unsaturation of the phospholipids, UV detection does not allow a quantitative estimation. Furthermore, the mobile phase must be UV-transparent whereas gradients cause a baseline drift. Refractive index detectors are relatively insensitive and incompatible with gradient elution. IR detection on the other hand permits quantification, but necessitates the use of deuterated solvents. Another quantitative detection method is moving-wire flame ionization, but this detector is no longer manufactured commercially.

Recently, the evaporative light-scattering mass detector has been introduced[17,18]. The non-volatile lipids, remaining after evaporation of the nebulised mobile phase by a heated gas stream, form small droplets. Light scattered by this particle cloud is detected by a photomultiplier at an angle of 120° to the incident light beam. Using calibration curves, the absolute amount of lipids can be estimated from the peak areas of the chromatogram. Besides its capability of direct quantification, this detector is also characterized by its insensitivity to solvent changes and gradients. Moreover, the mass detector furnishes a very stable baseline. As all described methods involving this detector for the quantification of phospholipids necessitate gradient elution, it was our aim to develop a simple method for quantification of soybean phospholipids. Moreover, the method was elaborated so that it could easily be automated and scaled up for preparative work.

EXPERIMENTAL

Materials

HPLC-grade *n*-hexane and 2-propanol were supplied by Alltech. The water

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was deionized, distilled and used freshly. Commercial soybean lecithin and phosphatidylcholine (PC) were obtained from Lucas Meyer. Triolein, phosphatidylinositol (PI) and phosphatidic acid (PA) were suplied by Serdary Research Laboratories, and phosphatidylethanolamine (PE), phosphatidylserine (PS) and cerebroside (CER) were delivered by Sigma.

All lipids were dissolved in either chloroform or *n*-hexane–2-propanol (57.8:39) and filtered through a 0.2-μm Dynagard filter.

Instrumental set-up

A Waters Model 590 isocratic HPLC pump equipped with a solvent switcher (Waters) was used. The Waters Intelligent Sample Processor (WISP) could be programmed to inject up to 48 samples. The column consisted of 3 μm Spherisorb, packed in a 125 × 4.9 mm I.D. stainless-steel column (Hichrom Ltd.). To avoid particulate contamination of the column, a 0.2-μm Uptight precolumn filter (Upchurch Scientific) was inserted before the column. The phospholipids were detected by a mass detector (ACS). The peak areas were calculated by a Chromatopac C-R1A integrator (Intersmat). As an alternative, a Quartel data logger (Gulton) could be connected to the mass detector, storing each 5 s the value of the generated voltage. Using the Pronto software these data can be handled by a personal computer and plotted. Finally, a Frac-100 fraction collector (Pharmacia) was used for preparative fractionation. The Model 590 solvent-delivery module is connected to an event in/-event out box, and can be programmed in 40 steps, enabling the pump to be controlled by an external device or vice versa. The configuration is shown schematically in Fig. 1; both the tubings and the electrical connections are drawn.

Chromatographic conditions

The initial part of the separation was carried out with a mobile phase of *n*-hexane–2-propanol–water (57.8:39:3.2, v/v/v). After 9 min another mobile phase, *n*-hexane–2-propanol–water (52.6:42:5.4, v/v/v), was selected by the solvent switcher. After 18 min the first mobile phase was run again, so that a new sample could be

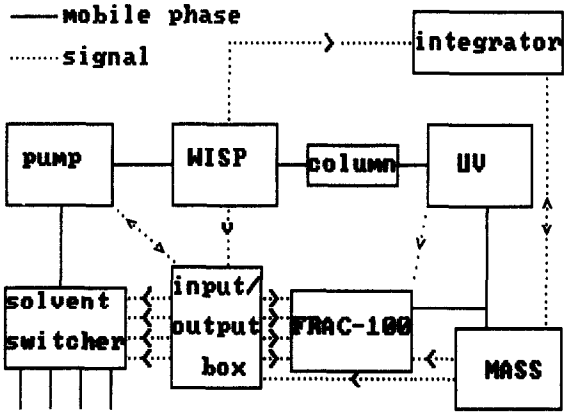

Fig. 1. Schematic representation of the HPLC equipment used. The tubings are shown as solid lines and the electrical connections as dotted lines.

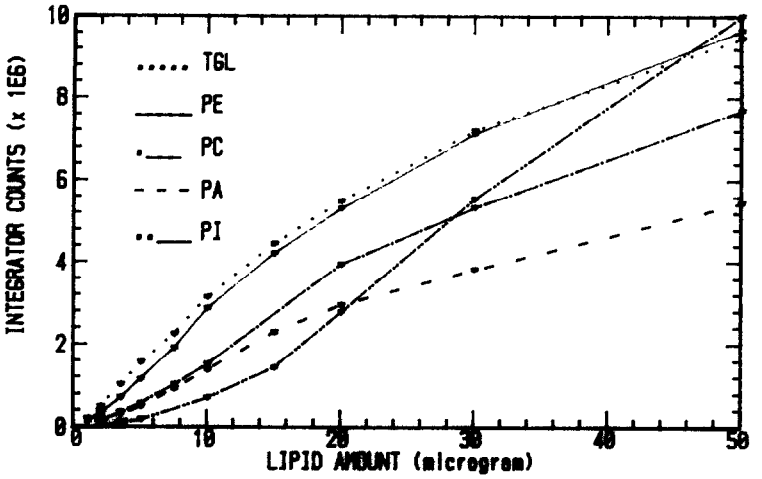

Fig. 2. Calibration curves of triolein (TGL), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidic acid (PA) and phosphatidylinositol (PI).

injected after *ca.* 25 min. From the pressure change it was concluded that newly selected solvent entered the column after *ca.* 3.5 min.

At the end of each day, the column was flushed with *ca* 50 ml of *n*-hexane or 2,2,4-trimethylpentane. Every month the column was rinsed with 100 ml of *n*-hexane–2-propanol–water (42:50:8, v/v/v) to remove polar contaminants.

Degassing of the solvents was achieved by flushing with helium.

RESULTS

To enable direct quantification of phospholipids, calibration curves were elaborated showing the relationship between the peak area as calculated by the integrator and the amount of phospholipids. Oppenheimer and Mourey[19] mentioned that the concentration response curves are sigmoidal when plotted on linear axes, whereas a double logarithmic plot reveals an exponential response. Depending on

TABLE I

COEFFICIENTS FOR THE CALIBRATION EQUATIONS

The equations $Y = aX^b$ and $Y = c(X-d)^e$ were fitted to the calibration curves of neutral lipids (NL), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidic acid (PA) and phosphatidylinositol (PI). Y represents the peak area, whereas X is the amount of phospholipids (in micrograms) and X_1 gives the limit for both equations. The (phospho)lipids are listed according to decreasing height-to-width proportion.

Lipid	*ln a*	*b*	*ln c*	*d*	*e*	X_1
NL	12.2	1.31	14.1	4.4	0.52	5
PE	11.7	1.35	14.1	5.6	0.53	10
PC	10.9	1.43	13.8	7.8	0.55	10
PA	10.7	1.47	13.5	6.7	0.53	10
PI	9.2	1.86	–	–	–	30

TABLE II

ANALYSIS OF CRUDE SOYBEAN LECITHIN AND COMMERCIAL POWDER LECITHIN

The last two columns represent the phospholipid composition of soybeans at 67 and 97 days after flowering, respectively, according to Privett and coworkers[20,21]. Values are in % wt.

	Crude lecithin	*Powder lecithin*	*Soybeans day 67*	*Soybeans day 97*
Neutral lipids	43.1	6.3	–	–
Cerebrosides	2.0	2.7	–	–
Glycolipids	1.0	3.0	–	–
Phosphatidylethanolamine	10.2	14.5	3.5	26.3
Phosphatidylglycerol	1.3	2.6	6.7	3.3
Phosphatidylinositol	10.6	17.1	12.5	14.1
Phosphatidic acid + phosphatidylserine	10.1	14.6	39.2	5.0
Phosphatidylcholine	18.6	33.9	10.2	45.0
Lysophosphatidylcholine	0.7	1.5	–	–
Others	2.4	3.8	27.9	6.3

the concentration range, the flow-rate of the nebulizer gas, and the composition and flow-rate of the mobile phase, the exponent ranged from 0.6 to 2. The calibration curves for neutral lipids (NL), PE, PI, PA and PC are given in Fig. 2. Plotting the response versus the amount of lipids on a double logarithmic scale, the linear region at low lipid concentrations revealed an exponential relation. For larger amounts, the equation $Y = c(X-d)^e$ yielded the best fit. The coefficients of these equations are summarized in Table I.

These results were used to analyse soybean phospholipids: for NL, PE, PI, PA and PC the corresponding equations were applied, whereas for the other components

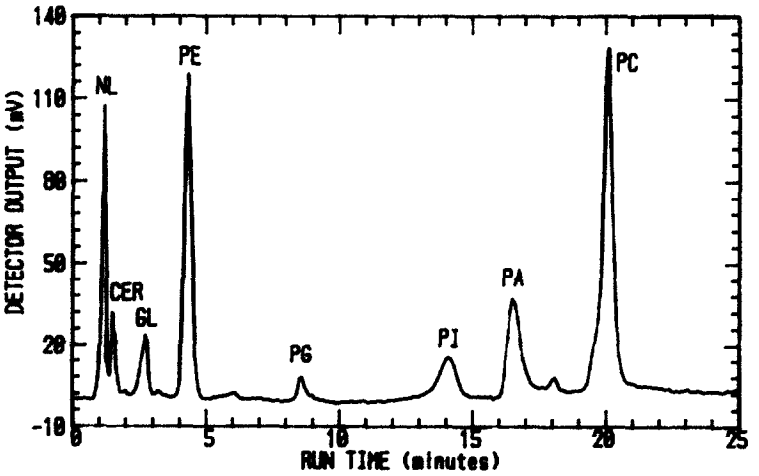

Fig. 3. HPLC separation of commercial powder lecithin; 115 μg were injected, dissolved in 5 μl of chloroform. The experimental conditions are described in the text.

the equation was used of the lipid to which the unknown substance showed most similarity in retention time and peak shape. The compositions of both a crude soybean lecithin and a commercial powder lecithin are shown in Table II, and the separation of the phospholipids of the commercial powder lecithin is represented in Fig. 3. The last two columns of Table II represent the phospholipid composition of soybeans at different stages of maturation, according to Privett and coworkers[20,21]. It can be concluded that our results agree very well with these previously reported values, supposing that the analysed lecithin originated from soybeans harvested about 90 days after flowering.

By repeated injections of the same sample, the standard deviation (S.D.) of this quantification method was shown to be *ca.* 3%. On the other hand, the S.D. of the injection volume was *ca.* 2% for all volumes exceeding 3 μl. Thus, the precision is mainly determined by the automatic injector, whereas the separation process has only minor influence. The detection limit was estimated as the lipid amount that resulted in a peak area of three times the largest background peak. Except for PI, the detection limit of all (phospho)lipids was less than 0.9 μg.

DISCUSSION

Optimization of mobile phase

The method proposed is based upon the procedure described by Nasner and Kraus[6], who used *n*-hexane–2-propanol–water (8:8:1) to separate soybean phospholipids. This solvent mixture results in a poor resolution between PE and neutral lipids, and PA and PI co-elute. The resolution of the latter compounds could be improved by decreasing the water content. However, this resulted in the permanent adsorption of PC on the stationary phase. Using a solvent switcher, two different mobile phases could be used: the first, which had a lower water content, was able to separate neutral lipids, glycolipids (GL) and PE, whereas the second contained more water in order to elute the acid phospholipids. The composition of the latter solvent was mainly of interest for the retention of PC; as already mentioned by Yandrasitz *et al.*[5], increasing both the water and the hexane content resulted in a shorter retention time for PC. The resolution between PA and PI couldn't be improved by altering the composition of the second solvent, but depended to a large extent on the first solvent; if less than 3% water was present, both PI and PA co-eluted with the front of the second solvent. A water content between 3 and 3.5%, however, resulted in a slow migration of PI, with PA remaining fixed to the column. At still higher water contents, PE was no longer separated from the neutral and glycolipids, whereas PI appeared as a broad peak. Besides their composition, the moment of changing the solvents was also very important: the resolution was improved by delaying the switch. Phosphatidylserine always eluted together with phosphatidic acid.

As stated by Tsimidou and Macrae[22], both the sample solvent and the injection volume influence the resolution to a large degree, especially when using chloroform or hexane. Injection volumes smaller than 5 μl did not affect the separation, but larger volumes mainly influenced the PE peak. The retention time was reduced from 3.76 min at 5 μl to 3.47 min at 25 μl and to 3.26 min at 200 μl, and the peak also became increasingly broad. On the other hand, the retention times of the neutral lipids and the glycolipids increased, resulting in a poor resolution of NL, GL and

PE when the injection volume was more than 10 μl. The remaining phospholipids were not influenced. This volume effect was eliminated by dissolving the samples in the first mobile phase used, omitting the water (*i.e.* *n*-hexane–2-propanol 57.8:39). A similar effect resulted if the column was not equilibrated with at least 10 column volumes after storage in *n*-hexane.

Optimization of detector output

The most decisive factors to optimise the detector output were the flow-rate of compressed air and the evaporator temperature. The latter was adjusted to maximize the signal-to-noise ratio. If the temperature was too low, the mobile phase was not fully evaporated, resulting in a baseline drift when the solvents were changed. On the other hand, at higher temperatures some low boiling components, such as some free fatty acids, began to evaporate. An evaporator setting of 70 was selected.

An inverse relationship between the air flow-rate and the peak area was observed. Therefore, the internal air pressure was fixed at 1 bar, whereas the external air pressure was set to 1.6 bar. It was noted that when the internal pressure was increased to 1.5 bar, the peak area decreased by *ca.* 33%, and at double the internal pressure (2 bar) the peak area was reduced by 61%.

From Table I, it may be concluded that an exponential response was obtained for all phospholipids if less than 10 μg was injected, whereas this limit for the neutral lipids amounts to *ca.* 5 μg. The coefficients in the equation $Y = aX^b$ reveal a distinct relationship between the peak area and the peak shape; it follows from the values of ln *a* that the peak area increased as the peaks became sharper, whereas the values of *b* deviated to a larger extent from the theoretical value of 2 (according to the Rayleigh theory) as the peak sharpness increased. For lipid amounts exceeding these limits, a very similar value of the exponent was found for the different (phospho)lipids when the equation $Y = c(X-d)^e$ was fitted to the data using the non-linear Marquardt regression procedure, indicating that the mass detector response is independent of the structure of the compound. An important feature of fitting equations to the experimental data is the greater reliability of inter- or extrapolations. Moreover, the analyses become faster and the results can be printed out in a standard report if these equations are inserted in a computer program.

Automation

One of the major advantages of our procedure is the high degree of automation. Using the automatic injector, up to 48 samples can be injected consecutively. Moreover, upon each injection a pulse is generated to restart the solvent program, and at the same time the integrator is initialized. The solvent program controls not only the flow-rate, but also the solvent switcher and the fraction collector. Safety settings are provided to avoid solvent accumulation in the mass detector when the compressed air flow is interrupted occasionally.

CONCLUSION

A method is described for the direct quantification of the most important soybean phospholipids. A main advantage is that the analyses can be performed with an isocratic HPLC pump using a solvent switcher. The method can be easily scaled

up for preparative fractionation. The mobile phase is also compatible with UV detection.

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