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DETERMINATION OF THE PHOSPHOLIPID PROFILE OF LECITHINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Results of a collaborative study and the standardized method

(Technical Report)

Prepared for publication by

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Determination of the phospholipid profile of lecithins by high performance liquid chromatography: Results of a collaborative study and the standardized method (Technical Report)

Abstract - An HPLC method was tested for the direct determination of phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol (PI), and phosphatidylcholine (PC) in commercial soybean lecithin concentrates. The CV_r for PC ranged from 0.7 to 7.0 and the CV_R for PC was 7.3 in the two years of the study.

INTRODUCTION

A previous IUPAC collaborative study on the phospholipid composition of commercial lecithin involved fractionation by thin-layer chromatography and the determination of the phosphorus content of the separated fractions. The repeatability of results with this method in each laboratory was satisfactory but the reproducibility among laboratories was unsatisfactory. A direct HPLC method would reduce the number of analytical steps and the time for the determination of phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol (PI), and phosphatidylcholine (PC) in lecithin concentrates.

COLLABORATIVE STUDY AND RESULTS

1. The samples studied in 1988 were de-oiled soy lecithin, a mixture of it with a standard phosphatidylcholine and samples of crude lecithin, two of which (samples 4 and 5) were identical. Participants used their own HPLC equipment with a pretreated column as described in the method.

Twelve laboratorties representing 9 countries reported the results of duplicate analyses on each sample. Statistical analyses were done according to IUPAC guidelines for each phospholipid in each sample. Cochran's Test (α = 0.001 for outliers and α = 0.05 for stragglers) identified any large differences in duplicate results. Outliers were deleted and the remaining data were test using Grubb's Test (α = 0.01 for outliers) to identify extraordinarily high or low results. The outliers were deleted; the steps were repeated until no outliers were detected. A summary of the results is presented in Table 1.

2. In 1989, four lecithin samples were studied in twelve laboratories. The reference sample contained PC, PE, PI and PA and was provided by Lucas Meyer, Germany. The range of PC to be tested was 8.8 to 12.3% of the lecithin mixtures provided. Of the results reported, no outliers were detected in PC, but the highest CV_R was associated with PC in sample 3. The CV_r for the 4 phospholipids in the 4 samples rantged from 1.7 to 5.0, but the CV_R ranged from 5.7 to 13.6. During the two years of testing the average CV_R for PC was 7.3. A summary of the results is presented in Table 2.

CONCLUSION

HPLC is a relatively rapid method which allows reliable determination of the individual phospholipids in lecithin concentrates of plant origin. Although there was a tendency for the results obtained using the method to show a somewhat high level of variation between laboratories, this variation was considerably less than that experienced when determining the phospholipid profile by TLC and phosphorus analysis (method 5.301). Accordingly, on the basis of the results, the Commission decided to adopt the method.

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Table 1. Summary Statistics for IUPAC Phospholipid Collaborative Study in 1988

Phospholipid	sample	n[a]	mean	S_r[b]	CV_r[c]	S_R[d]	CV_R[e]
PE[f]	1	11	14.04	0.17	1.18	0.74	5.29
	2	10	15.80	0.15	0.98	1.10	6.98
	3	11	10.24	0.23	2.29	0.88	8.55
	4	10	9.45	0.09	0.98	0.77	8.10
	5	10	9.49	0.09	1.00	0.59	6.18
	6	11	11.18	0.17	1.54	1.19	10.65
PA	1	11	12.58	0.27	2.14	1.55	12.28
	2	11	11.68	0.17	1.45	1.12	9.56
	3	10	4.68	0.21	4.58	0.96	20.61
	4	10	12.18	0.28	2.34	2.51	20.63
	5	10	12.09	0.27	2.23	2.54	21.01
	6	11	7.18	0.17	2.37	0.61	8.46
PI	1	9	11.60	0.49	4.20	1.07	9.20
	2	10	12.43	0.52	4.22	1.47	11.80
	3	9	7.67	0.27	3.52	1.55	20.18
	4	9	7.84	0.32	4.08	0.85	10.88
	5	10	8.86	0.56	6.28	3.45	38.89
	6	8	9.16	0.19	2.11	0.63	6.89
PC	1	10	17.24	0.20	1.19	0.97	5.61
	2	10	19.01	0.49	2.60	1.12	5.89
	3	11	14.44	0.34	2.44	0.86	5.95
	4	8	9.64	0.13	1.40	0.22	2.25
	5	11	9.33	0.41	4.35	0.66	7.04
	6	11	14.74	0.40	2.68	0.83	5.66

Table 2. Summary Statistics for IUPAC Phospholipid Collaborative Study in 1989

Phospholipid	sample	n[a]	mean	S_r[b]	CV_r[c]	S_R[d]	CV_R[e]
PE[f]	1	12	10.12	0.29	2.83	0.99	9.75
	2	12	9.98	0.24	2.39	0.98	9.85
	3	12	11.12	0.26	2.30	0.77	6.93
	4	11	11.50	0.51	4.45	0.66	5.77
PA	1	11	13.89	0.37	2.67	1.89	13.61
	2	9	5.92	0.10	1.74	0.34	5.79
	3	11	5.76	0.28	4.84	0.33	5.73
	4	12	5.83	0.29	5.03	0.45	7.66
PI	1	11	7.22	0.42	5.81	0.80	11.03
	2	11	8.19	0.31	3.75	0.68	8.26
	3	10	7.98	0.33	4.17	0.58	7.26
	4	11	7.95	0.37	4.67	0.92	11.56
PC	1	12	8.50	0.54	6.11	0.70	8.00
	2	12	11.55	0.81	6.99	0.96	8.31
	3	12	12.23	0.72	5.92	1.65	13.51
	4	12	12.32	0.46	3.76	0.95	7.74

[a] number of labs remaining after elimination of outliers

[b] standard deviation of repeatability

[c] coefficient of variation for repeatability

[d] standard deviation of reproducibility

[e] coefficient of variation for reproducibility

[f] PE, phosphatidylethanolamine PI, phosphatidylinositol

PA, phosphatidic acid PC, phosphatidylcholine

5.302 Determination of the phospholipid profile of lecithins by high performance liquid chromatography

1. SCOPE

This Standard describes a method for the quantitative determination of individual phospholipids in commercial soybean lecithin preparations.

2. FIELD OF APPLICATION

The method allows the determination of phosphatidyl ethanolamine (PE), phosphatidic acid (PA), phosphatidyl inositol (PI), and phosphatidyl choline (PC) in commercial soybean lecithin preparations.

3. PRINCIPLE

Separation of the individual phospholipids by direct high performance liquid chromatography (HPLC) and their detection by ultraviolet light at 206 nm. Quantitation using external standards.

4. APPARATUS

4.1 High performance liquid chromatograph, consisting of an HPLC pump and an injection valve with 10 µl sample loop.

4.2 UV-detector system to measure absorbance at 206 nm.

4.3 Column: stainless steel tube, length 200 mm and internal diameter about 4.6 mm, packed with microparticulate spherical silica (5 µ) [Note 1].

4.4 Recorder and/or integrator.

4.5 Syringe, 25 µl graduated in 1 µl

4.6 Flasks, volumetric, 10, ml, 500 ml and 1000 ml

5. REAGENTS

5.1 n-Hexane, HPLC grade

5.2 2-Propanol, HPLC grade

5.3 Water, double distilled, filtered through a 0.45 µ pore size membrane filter.

5.4 Sodium acetate, 0.2M. Weigh 27.22 g sodium acetate [$3H_2O$], analytical grade, into a 1000 ml flask (4.6) and make up to volume with water. Mix.

5.5 Acetic acid, 0.2M. Weigh 6.006 g glacial acetic acid, analytical grade, into a 500 ml flask (4.6) and make up to volume with water. Mix.

5.6 Acetate buffer (Walpole), pH 4.2. Prepare by mixing 2.65 ml sodium acetate solution (5.4) and 7.35 ml acetic acid solution (5.5).

5.7 HPLC mobile phase. Prepare by mixing n-hexane (4.10), 2-propanol (4.2), and acetate buffer (5.6) in the proportion 8 : 8 : 1 (V/V).

5.8 Reference phospholipids: phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol (PI), and phosphatidylcholine (PC).

6. PROCEDURE

6.1 Stabilization of the column

To achieve a stable operating performance of the column it must be charged with a suitable quantity of buffer. This is carried out by passing mobile phase (5.7) through the column for at least 3 days at a flow rate of 0.2 ml/min until a stable baseline is obtained. A stable baseline will result in the retention times of peaks also being stable.

6.2 HPLC chromatography

Use the mobile phase (5.7) at a flow rate of 2 ml/min.

6.3 Calibration curve

Accurately weigh 10, 20, 30 and 40 mg quantities of each reference phospholipid (5.8) into separate 10 ml flasks (4.6). Dissolve the phospholipid in about 3 ml mobile phase (5.7), make up to volume with the same solvent and mix well [Note 2].

Construct a calibration curve for each phospholipid by injecting exactly 10 µl of each of the calibration solutions, recording the peak areas and plotting the latter against the corresponding quantity of phospholipid.

6.4 Sample analysis

Weigh accurately 50 to 250 mg of the lecithin sample into a small beaker and dissolve in about 3 ml mobile phase (5.7). Transfer quantitatively to a 10 ml flask (4.6), make up to volume with the same solvent, and mix well. In the case of some lecithins a sediment may form after some time. Inject 10 µl of the upper layer. The sediment has no influence on the analysis but can affect the column's performance [Note 3].

Record the individual peak areas of the eluted phospholipids, identifying them from the chromatogram (Fig. 1) or by comparison with the retention times observed during the chromatography (6.3) of the reference phospholipids (5.8).

7. CALCULATION AND EXPRESSION OF RESULTS

7.1 Read off from the calibration curves the amount of phospholipids in the injected volume of the solution of the lecithin sample (6.4).

7.2 The phospholipid content (P), expressed as per cent (m/m), is given by the formula:

$$P = \frac{A \times 100}{m}$$

where

A is the amount of phospholipid, in mg, read from the calibration curve (7.1)

m is the mass of sample weight, in mg (6.4)

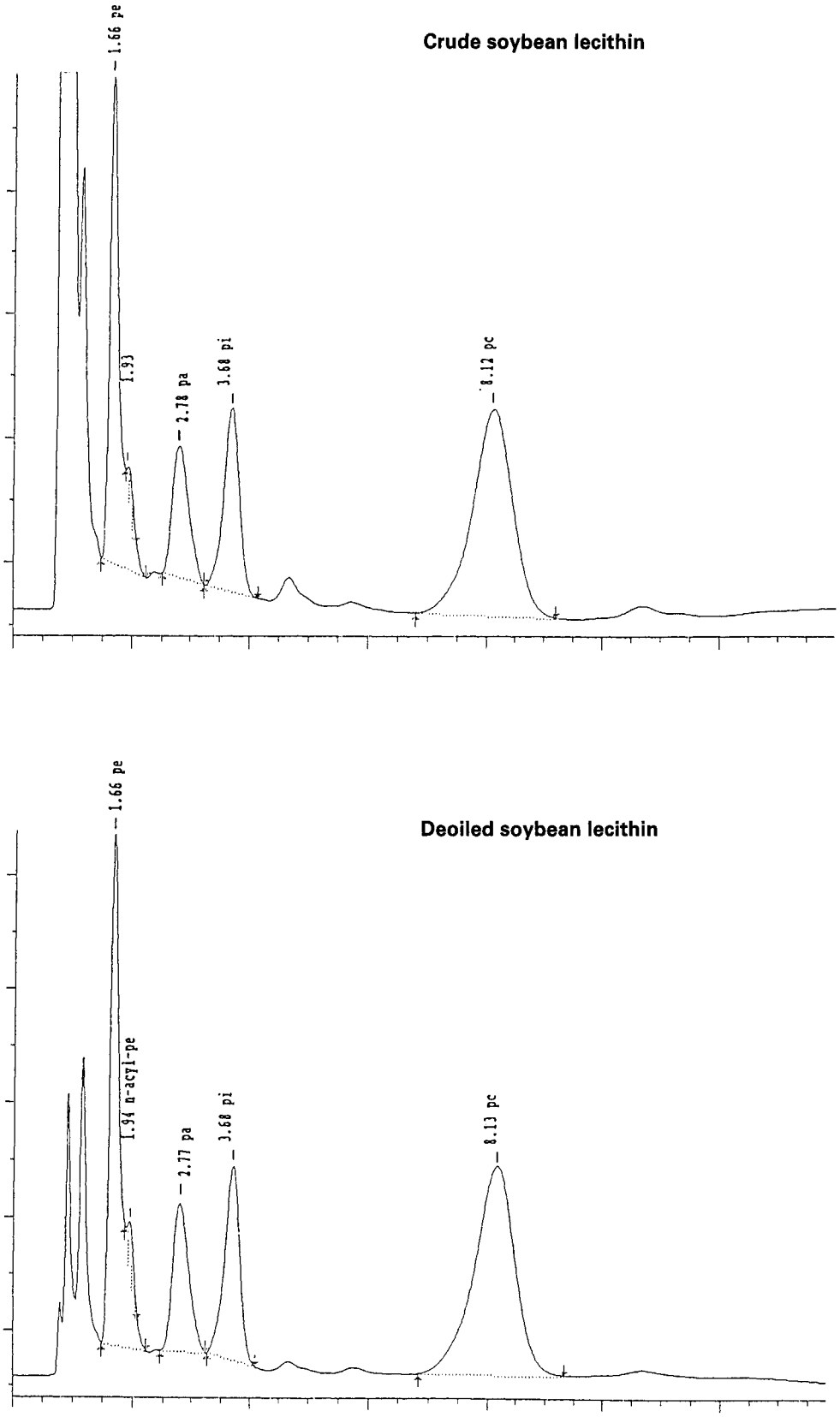

Fig. 1 Chromatograms of soybean phospholipids

8. QUALITY ASSURANCE

8.1 For *general principles* of analytical quality control see the section on *Quality Assurance* in the introductory part of the Compendium of Standard Methods.

8.2 For *specific applications* of analytical quality control see the *Annexe* to this standard method.

9. NOTES

1. Example of a suitable commercially available column:
Nucleosil 50-5 (Macherey-Nagel).

2. An exposure of a few minutes in an ultrasonic water bath of the standard and sample solutions may facilitate complete homogenisation of phospholipids and lecithins, respectively.

3. A deterioration in the performance of the column is indicated by a poor separation of the phosphatidylethanolamine and N-acyl-phosphatidyl ethanolamine peaks. The column should be refilled with fresh support to ensure good reproducibility of chromatograms. After completion of analyses the pump/head system should be rinsed, first with acetone, then with water, and again with water at a very low flow rate.

ANNEXE: ANALYTICAL QUALITY CONTROL

1. Repeatability

When the mean value [m] of two single test results obtained under **repeatability conditions***, lies within the range of the values shown in the tables below, the absolute difference between the two test results obtained should not be greater than the **repeatability limit** (r) deduced by linear interpolation from the data in the tables.

***repeatability conditions:** conditions where independent test results are obtiained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time.

2. Reproducibility

When the values of two single test results obtained under **reproducibility conditions****, lie within the range of the values shown in the tables below, the absolute difference between the two test results obtained should not be greater than the **reproducibility limit** (r) deduced by linear interpolation from the data in the tables.

****reproducibility conditions:** conditions where test results are obtained with the same method on identical test material in different laboratories with different operators using different equipment.

3. Results of the interlaboratory test

An interlaboratory test carried out at the international level in 1989 by the IUPAC Commission on Oils, Fats and Derivatives, in which 11 laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with ISO 5725-1986) summarised in the following tables:

Phosphatidic acid

Sample reference	1	2	3	4	5	6
Number of laboratories	11	11	11	11	11	11
Number of laboratories retained after elimination of outliers	11	11	10	10	10	11
Mean value (g/100 g sample)	12.58	11.68	4.68	12.18	12.09	7.18
Repeatability standard deviation (S_r)	0.27	0.17	0.21	0.28	0.27	0.17
Repeatability relative standard deviation	2.14	1.45	4.58	2.34	2.23	2.37
Repeatability limit (r) ($2.83 \times S_r$)	0.74	0.48	0.59	0.79	0.74	0.48
Reproducibility standard deviation (S_R)	1.55	1.12	0.96	2.51	2.54	0.61
Reproducibility relative standard deviation	12.28	9.56	20.61	20.63	20.01	8.46
Reproducibility limit (R) ($2.83 \times S_R$)	4.39	3.16	2.72	7.10	7.19	1.73

Phosphatidylethanolamine

Sample reference	1	2	3	4	5	6
Number of laboratories	11	11	11	11	11	11
Number of laboratories retained after elimination of outliers	11	10	11	10	10	11
Mean value (g/100 g sample)	14.04	15.80	10.24	9.45	9.49	11.18
Repeatability standard deviation (S_r)	0.17	0.15	0.23	0.09	0.09	0.17
Repeatability relative standard deviation	1.18	0.98	2.29	0.98	1.00	1.54
Repeatability limit (r) ($2.83 \times S_r$)	0.48	0.42	0.65	0.25	0.25	0.48
Reproducibility standard deviation (S_R)	0.74	1.10	0.88	0.77	0.59	1.19
Reproducibility relative standard deviation	5.29	6.98	8.55	8.10	6.18	10.65
Reproducibility limit (R) ($2.83 \times S_R$)	2.09	3.11	2.49	2.18	1.67	3.37

Phosphatidylcholine

Sample reference	1	2	3	4	5	6
Number of laboratories	11	11	11	11	11	11
Number of laboratories retained after elimination of outliers	11	11	10	10	10	11
Mean value (g/100 g sample)	17.24	19.01	14.44	9.68	9.33	14.74
Repeatability standard deviation (S_r)	0.20	0.49	0.35	0.13	0.41	0.40
Repeatability relative standard deviation	1.19	2.60	2.44	1.40	4.35	2.68
Repeatability limit (r) ($2.83 \times S_r$)	0.57	1.39	0.99	0.37	1.16	1.13
Reproducibility standard deviation (S_R)	0.97	1.12	0.86	0.22	0.66	0.83
Reproducibility relative standard deviation	5.61	5.89	5.95	2.25	7.04	5.66
Reproducibility limit (R) ($2.83 \times S_R$)	4.39	3.16	2.72	7.10	7.19	1.73

Phosphatidylinositol

Sample reference	1	2	3	4	5	6
Number of laboratories	11	11	11	11	11	11
Number of laboratories retained after elimination of outliers	9	10	9	9	10	8
Mean value (g/100 g sample)	11.60	12.43	7.67	7.84	8.86	9.16
Repeatability standard deviation (S_r)	0.49	0.52	0.27	0.32	0.56	0.19
Repeatability relative standard deviation	4.20	4.22	3.52	4.08	6.28	2.11
Repeatability limit (r) ($2.83 \times S_r$)	1.39	1.47	0.76	0.91	1.58	0.54
Reproducibility standard deviation (S_R)	1.07	1.47	1.55	0.85	3.45	0.63
Reproducibility relative standard deviation	9.20	11.80	20.18	10.88	38.89	6.89
Reproducibility limit (R) ($2.83 \times S_R$)	3.03	4.16	4.39	2.41	9.76	1.78