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Advances in phospholipid quantification methods

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This mini review summarizes most of the available methods for phospholipid (PL) quantification. The methods for total PL measured as phosphorus, PL class and fatty acid composition, fatty acyl stereospecific distribution and molecular species composition are reviewed. The limit of detection for each method and limitations for some of the advanced instrumental methods are described. Critical references are given to direct the readers for more in-depth investigation on the topics of interest.

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Introduction

Phospholipids (PL), or lecithin as a term for commercial product, can be obtained from many commercial sources such as soybeans, sunflower, rapeseed, dairy, and egg yolk [1]. PL is used in food, beverage, pharmaceutical, and cosmetic industry as an emulsifier to provide desired properties, such as smooth texture, better dispersion, and stable emulsion. It is also used as nutritional supplement. Lecithin used in food industry accounts for majority of overall demand in the global market, followed by pharmaceutical industry and industrial purpose [2].

The importance for an accurate quantification of total PL in source materials, lecithin products, or final products is unquestionable. Depending on application needs and specific purpose, PL can be analyzed on several other compositional and structural levels, including the composition of PL classes and their fatty acid composition, the fatty acyl stereospecific distribution, and the molecular species composition within each PL class. This review provides a summary of the commonly used and more advanced methods for PL assay and discuss certain limitations and challenges for quantification.

Quantification of total PL by phosphorus assay

Total PL can be quantified by determining the total phosphorous (P) content. Two methods of the American Oil Chemists' Society (AOCS) Official Methods are used. The turbidity measurement, i.e. the nephelometric method (AOCS Ca 19-86), is based on the insolubility of PL in acetone. Based on turbidity generated when a sample of oil is dispersed in the solvent, the turbidity is determined. A soybean or corn oil sample of 0.3–8.4 g quantity containing a minimum of 8 μg P is needed for the assay [3]. The more commonly used method is the colorimetric method for P quantification after ashing the oil (about 3 g) with zinc oxide, and the residual is reacted with sodium molybdate to form a blue phosphomolybdic complex that can be quantified spectrophotometrically using a standard curve (AOCS Ca 12-55). The content of P multiplied by a factor of 304 is the equivalent total PL content. The limit of detection (LOD) of this method is about 3 μg of P.

PL class composition quantification by TLC and GC

The most traditional, visual, and direct method for PL class quantification is using TLC for class separation, then the bands of individual PL are collected for GC quantification of the fatty acids. Lipid extract is streaked on a preparative TLC plate. A few PL standards are spotted on one side of the plate. A polar solvent mixture suitable for polar lipid separation is used to develop the plate. The PL bands are identified according to the migration of the standards and silica bands are scraped off the plate. The PLs bound to silica are converted to fatty acid methyl esters (FAME) using a base or acid catalyzed transesterification reaction. With an internal standard, the total FAME can be quantified. The FAME composition can be used to calculate the average molecular weight of the fatty acid for a specific type of PL. Then, using the molar quantify of FAME quantified by GC and the calculated MW of the specific PL, the quantity of the PL can be calculated. Although this method is old-fashioned, it is extremely simple, and both the fatty acid composition and PL class composition data can be obtained. Since the quantification is done by GC, it has high sensitivity and can be very accurate assuming TLC handling is quantitative and the internal standard added can account for the completeness of transesterification reaction and FAME recovery. Oxidation of lipids spread on silica particles may be a concern for certain applications. The LOD for GC with a flame ionization detector (FID) is about 0.1 to

0.4 ng of PL [4]. Yao et al. [5] fractionated total lipid extracted from microalgae into neutral lipid, free fatty acid, and polar lipid by TLC. Fatty acid composition of all lipid classes was determined by GC analysis of FAME using ~10 mg lipid extract.

The method of combining TLC and FID has been developed recently for PL class quantification. A quartz reusable rod coated with a thin layer of silica or alumina is used for PL separation. It is then advanced at a constant speed through the flame of the FID. The separated PLs are ionized and the ions are quantified as done in GC-FID. Sinanoglou et al. [6] reported the use of Iatroscan TLC-FID method to quantify of neutral and polar lipids extracted from foods, and the LOD for PL was 20 ng. Another simple method for PL class quantification, particularly when an analytical instrument is not available, is the 2-dimensional TLC method of the AOCS Ja 7-86. This two-step TLC development in mobile phases of different polarity allows the collection the separated PL spots. An acid digestion and reaction with molybdate are performed to measure total P spectrophotometrically for each separated PL. This method has the LOD of 1 μg P.

PL class composition determination by HPLC

PL class composition analysis is most commonly performed using HPLC with an evaporative light scattering detector (ELSD) or more recently a charged aerosol detector (CAD) [7•]. The eluent from the LC column is nebulized using a stream of nitrogen, and the resulting aerosol is transported through a heated drift tube where the volatile components and solvents are evaporated. In the ELSD detector, the desolventized solid particles go through a detection cell of laser light beam. The detector measures the number of photons scattered from the solid particles, and the signal intensity is proportional to the mass of the particles. In CAD, the dried particle stream is charged with a secondary stream of nitrogen that has passed through a high-voltage platinum wire to obtain a positive charge by corona discharge. The resulting charged particle flux is measured by an electrometer. The response of both detection methods can be fitted to a power function. UV detector or refractive index (RI) detector is not suitable for PL quantification because UV signal is dependent on the number and configuration of the double bond in the PL molecules, and RI response is sensitive to changes in temperature and solvent gradient. The HPLC method is sensitive, and can be fast if it is fully developed, i.e., the separation and detection conditions are fully optimized. HPLC-ELSD is an AOCS standard method (Ja 7c-07) for PL class quantification, and it recommends using a standard mixture of various classes of PLs at 3 concentration levels and the LOD is estimated as 0.6 μg. However, it has been commonly encountered in practice that the ELSD detector response can be variable or unstable, and the non-linear responses change with time. Therefore, extensive and frequent quantitative calibration with multiple standards has to be performed.

CAD is a newer detector compared to the ELSD. It has been proven to have a greater sensitivity and better precision than that of the ELSD [7•,8,9]. The LOD of CAD determined in the study of Barry et al. [10] is 2–60 ng for various classes of PL. In a study of comparing the performance of CAD and ELSD for the analysis of PL classes by normal phase HPLC [7•], CAD was found to be more sensitive at the lowest mass range than ELSD and it had wider linear response range. The LODs for CAD and ELSD are between 15 and 249 ng and 71 and 1195 ng, respectively, for various classes of PL of a microorganism's membrane [7•]. Kielbowicz et al. [9] reported a similar high sensitivity (12–58 ng LOD) for dairy PL analysis using CAD detection. Practical linear responses in the injection range of about 10–2000 ng PL was reported for egg yolk lipid quantification using HPLC-CAD [11]. The performance of ELSD and CAD was also compared to another universal detector, the ion trap mass spectrometer with atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) sources (APCI-MS and ESI-MS) [8]. The CAD showed a wider linear range than ELSD, but ESI-MS and APCI-MS had much lower LOD, 1–4 ng and 0.03–0.05 ng, respectively. It suggests that the relative intensity of peaks obtained with the CAD is more representative of the relative abundance of the ceramide than ELSD, and CAD again performed better than ELSD at low concentration range.

PL class composition by NMR

^{31}P NMR spectroscopy is a fast and accurate quantitation method for PL classes with a single quantitative internal standard. NMR is intrinsically quantitative, non-destructive and requires less sample preparation than the more delicate methods such as HPLC and MS. The area under the peak of a specific signal is strictly proportional to the number of nuclei generating this signal. Thus, there is a linear relationship between the molar amount of the corresponding compound and signal strength. The quantitative ^{31}P NMR method can be used for analyzing PL extracts without prior separation, however, the number of PL class that can be quantified separately and accurately is critically dependent on experimental conditions. The strategy for optimization of PL ^{31}P NMR spectra was reviewed by Lutz and Cozzone [12••], and the critical experimental parameters are reported as extract concentration, quantity of chelating agent, pH of the treatment system, and measurement temperature. Figure 1 shows an example of using ^{31}P NMR for PL class quantification of soy lecithin samples treated with phospholipases, an unpublished work from author's laboratory.

The use of ^{1}H NMR spectrometry in PL analysis has been hindered by strong signal overlaps, broad solvent peaks and difficulties in spectral interpretation due to a