Phospholipid Composition of Canola Oils During the Early Stages of Processing as Measured by TLC with Flame Ionization Detector[1]

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Canola oils at initial stages of processing from different crushing plants were analyzed for phosphatides. The major phospholipid components identified and quantified in refined canola oils were phosphatidic acid and phosphatidylinositol. Phosphatidic acid was the main phosphorus component identified in solvent extracted canola oil samples. The two-dimensional separation that was used combined classical thin-layer chromatography with quantitation on chromarods in an Iatroscan with a flame ionization detector. Phosphatides quantitated with this procedure ranged from 0.1 to 20 μg with a coefficient of variation of 4.4 to 7.2%. Using the modified procedure, recoveries of better than 90% were obtained for all phospholipids analyzed.

KEY WORDS: Canola oil, Iatroscan, phospholipids, processing.

Phosphatides are present in crude plant oils at levels ranging from 0.1 to 1.8% (1). The presence of phosphatides is undesirable because they can cause losses in neutral lipids during neutralization (2,3) and contribute to discoloration of the oil during deodorization and steam distillation (4). In addition, the phospholipids chelate metals that affect the oil directly by increasing the amount of metal ions (5). The removal of phospholipids (PL) or gums also results in elimination of iron or copper which, in turn, increases the oxidative and flavor stability of the oil (5–10). Thus, oil processors need to know the level of phosphatides during the processing of crude oil.

Little attention has been paid to the effect that the initial stages of oilseed processing have on the glycerophosphatides and glycolipids remaining in the oil. The quantitation of the phosphatides obtained from biological systems has been impeded by time-consuming methods of sample preparation, identification and measurement. Conventional thin-layer chromatography (TLC) is still used with a densitometric or flame ionization detector (FID; Iatroscan, Iatron, Tokyo, Japan) for the analysis of phospholipids. Iatroscan has been shown to analyze, with acceptable accuracy, the phospholipid phosphorus content of oils between 145 and 536 ppm (11). Therefore, modification of existing techniques is needed for accurately measuring phosphatide content in super-degummed oils where phospholipid phosphorus levels are below 10 ppm (12). An alternative method available includes high performance liquid chromatography (HPLC) with a "light scattering detector". The latter is a relatively new detector for which more data are needed to determine its efficiency. According to Christie (11), this detector has a fairly linear response within the range 50–200 μg, but begins to fall off rapidly below 10 μg.

This paper reports the use of two-dimensional TLC combined with a modified FID-Iatroscan to assess the effect that early stages of processing have on the phospholipid content of canola oil.

EXPERIMENTAL PROCEDURES

Equipment and materials. An Iatroscan TH-10 analyzer type MK II (Iatron, Tokyo, Japan) was used, coupled to a Hewlett-Packard 3390A integrator (Hewlett-Packard, Palo Alto, CA). The hydrogen and air flow rates were maintained at 175 mL/min and 2L/min, respectively. Chromarods SIII (Iatron, Tokyo, Japan) were used for separation.

Merck (Darmstadt, Germany) silica gel 60 TLC plates without fluorescent indicator were used for phospholipids separation. Chloroform, methanol, ammonium hydroxide (30% solution) and glacial acetic acid were all of analytical grade. Phospholipid standards included L-α-phosphatidic acid (PA), L-α-phosphatidylethanolamine (PE), L-α-phosphatidylcholine (PC), L-α-phosphatidylglycerol (PG), L-α-phosphatidylinositol (PI), L-α-phosphatidylserine (PS), L-α-lysophosphatidylcholine (LPC), L-α-lysophosphatidylethanolamine (LPE), digalactosyldiglyceride (DGDG) and monogalactosyldiglyceride (MGDG), were obtained from Sigma Chemical Co. (St. Louis, MO).

Oil samples. Canola oil samples were obtained from three oil-crushing plants designated A, B and C. Plant B used a whole seeds cooking method while the other two plants utilized flaked seeds for cooking and processing. The processing parameters in all plants were similar, although different equipment was used. Degumming was carried out in the presence of citric acid in all three plants. For the purpose of this paper, the end products of pressing (expeller), solvent extraction (solvent) and degumming (degummed) of canola oil were examined.

Methods. The existing detector of the Iatroscan was modified to improve its sensitivity, linearity of response and stability. The ion collector was adjusted to 0.8 mm above the rods. A ball electrode was installed inside the ion collector and polarized as a detector cylinder. The ball electrode has a diameter 35% smaller than the inner diameter of the ion collector cylinder. The bottom of the installed ball was 10 mm up from the lower edge of the cylinder.

A stainless steel frame, which holds the rods during manipulation (spotting, developing, running through the detector) was cut out in order to prevent shorting of the electrometer. The cutting of the frame also eliminated any vertical and horizontal movements of the rods, although they could still be turned. In order to eliminate the effect of rod alignment on the detector response (13) the frame was bolted to a moveable table each time prior to running it through the detector—usually the frame holding the rods is out of shape. A set of chromarods was left in 10 N sulfuric acid overnight, washed free of acid with distilled water and dried at 100°C for 30 min. After cooling them to ambient temperature, the rods were immersed in 5% copper (II) sulfate solution for 30 min (14). The rods were then removed and shaken to dislodge any droplets adhering to them and dried at 100°C for 30 min.

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The dried rods were then scanned twice at scan speed of 3.1 mm/sec (gear No. 30) prior to use, the rods should turn brown.

The oils were dissolved in a chloroform/methanol mixture (9:1) to yield a 30% (v/v) solution. This solution was then spotted in volume of 20 μL on the TLC plates, and the solvent was removed by a stream of nitrogen. Plates were developed in chloroform/methanol/ammonia (65:30:4). The spots were visualized with dichlorofluorescein (Sigma), scraped off and vacuum transferred to a column prepared from a Pasteur pipette. The individual components were eluted with chloroform/methanol (2:1) containing an internal standard of 20 μg/mL (C_{30} hydrocarbon). The eluate was evaporated to dryness under nitrogen at ambient temperature and diluted with 20 and 100 μL of chloroform/methanol (2:1) for degummed and crude oils, respectively. Samples (1–5 μL) were then spotted on the chromarods. During spotting, the rods were turned while nitrogen was blown on the spotted area. After spotting, the rods were developed in the following solvent systems: For PC, PA and LPC, chloroform/methanol/acetic acid/water (80:14:14:3); for PE and PI, chloroform/methanol/ammonia (65:25:2). Separation of the analyzed spots is shown in Figure 1.

To permit quantitation of the individual phospholipids in the oils, standard solutions containing from 0.1 to 20 μg of phospholipids were analyzed. A standard curve of peak area vs. phospholipid concentration was obtained for each individual phosphatide. Each point on the standard curve represented the mean of 8–10 replicate analyses. The method was further tested by simulating oil with a mixture of the phospholipid standards at concentration levels of 0.5, 1.0 and 5 μg/mL for each phospholipid. The simulated oils were prepared by diluting the phospholipid standards in purified canola oil (14). Each result in the recovery study represents the mean of 12–18 replications. For comparison, all oil samples were analyzed for total phosphorus content by the ashing-spectrometric method (15). Percent phospholipids was calculated by using a conversion factor 25, multiplied by the phosphorus content, measured colorimetrically (16). The phosphorus content for individual phospholipid peaks was calculated using means of a modified formula published by Du Plesis (12). Total phosphorus content in oil samples represents the summation of the individual phospholipid components.

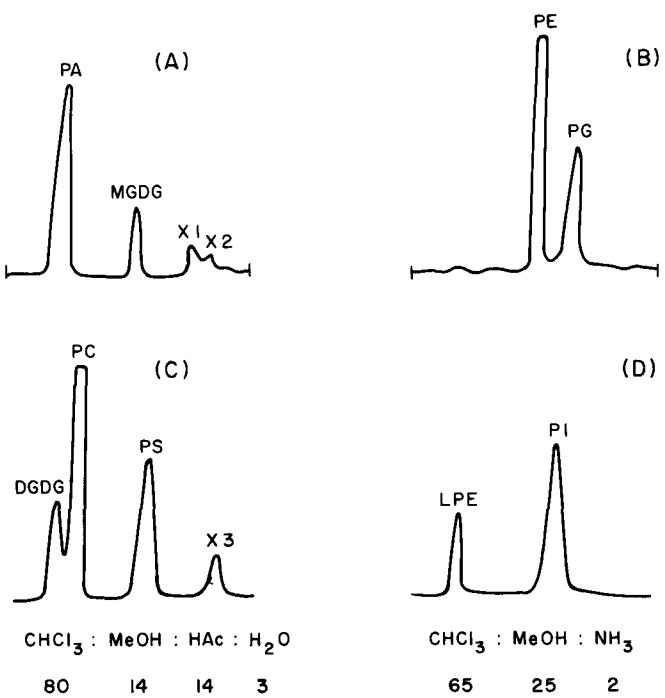

FIG. 1. Chromatograms of the TLC components separated on chromarods. Spot identification: (A) phosphatidic acid; (B) phosphatidylethanolamine; (C) phosphatidylcholine; and (D) phosphatidylinositol.

RESULTS AND DISCUSSION

Equipment. The flame detector of the Iatroscan was modified as described in the Experimental Procedures section. All the modifications improved the linearity of the detector response and the stability of the baseline. This was accompanied by a ten-fold increase in detector response as compared to the signal from an unmodified apparatus. This enhanced sensitivity reduced the amount of total lipids required to less than 10 μg per analysis, even 5 μg was readily analyzed. Kramer *et al.* (17) found that 5 mg of sample was needed when using colorimetric and GC methods for phospholipid analysis, compared to only 33 μg for the Iatroscan.

Phospholipid standards. For PS and PI, the detector response was low when compared to other standards at equivalent concentrations. When different quantities of phospholipid standards were analyzed there was a strong association between peak area and phospholipid content (Fig. 2). The linear least squares regression equations for the individual curves are presented in Table 1.

The detector response for PE and PC was not statistically different, which is consistent with earlier studies (12,18). However, the values for PS and PI presented here are far better than those reported by Du Plessis *et al.* (12). The improved response for PS and PI was due in part to better evaporation and combustion in the flame. The

TABLE 1

Simple Correlation of Individual Phosphatide Peaks Area vs. Concentration

Component	Constant (c)[a]	Coefficient (m)[a]	r[b]	r^2[c]	Standard[d] error of estimation
PC	0.2495	1.7934×10^{-4}	0.9697	0.9403	0.1680
PE	0.1103	1.85356×10^{-4}	0.9799	0.9602	0.1116
PI	−0.0141	3.1724×10^{-4}	0.9496	0.9017	0.1932
PA	0.8779	2.5642×10^{-4}	0.9593	0.9203	0.2621
PS	0.0298	3.6361×10^{-4}	0.9995	0.9990	0.2208
LPE	−0.2255	2.9346×10^{-4}	0.9697	0.9384	0.4099
LPC	1.0975	2.4751×10^{-4}	0.9598	0.9212	0.1339
PG	−0.0769	1.9848×10^{-4}	0.9398	0.8832	0.1342
MGDG	0.4036	2.0533×10^{-4}	0.9698	0.9405	0.1360
DGDG	−0.5086	2.4187×10^{-4}	0.9496	0.9017	0.1791

[a] $y = mx + c$.

[b] r = Correlation coefficient, significant at $p < 0.01$ d.f. = 58.

[c] r^2 = Coefficient of determination.

[d] Significant at $p < 0.01$ d.f. = 58.

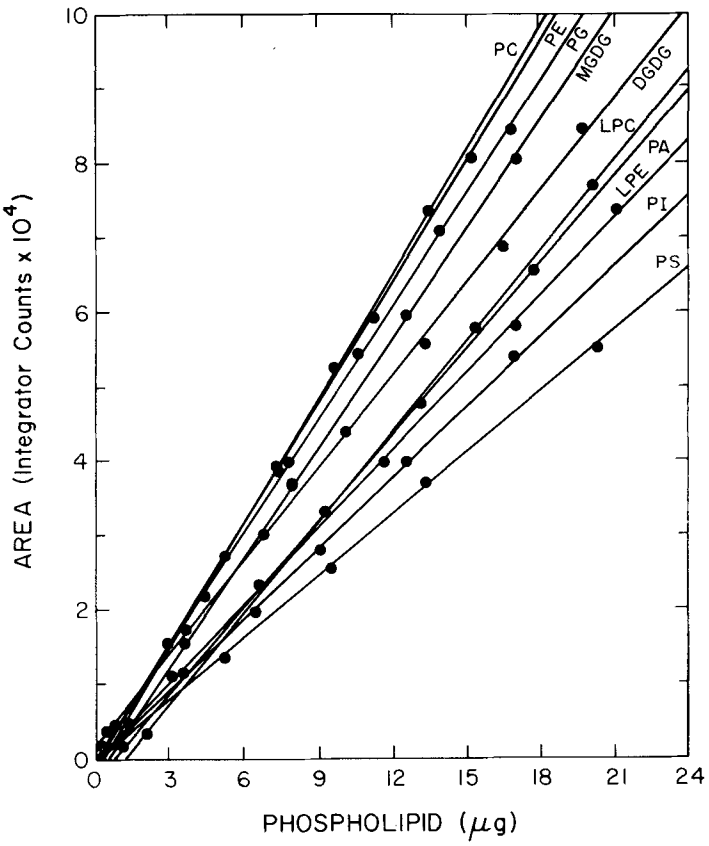

FIG. 2. Calibration curves for individual phospholipid standards.

response of compounds in the flame ionization detector is complex and depends on the ratio of carbon and oxygen atoms in the molecule (19,20). With copper (II) sulfate-impregnated silica gel (Chromarods), the detector behaves similarly to a hydrogen atmosphere flame ionization detector, with the result that response is a function of mass and not of the structure of the compounds being analyzed (21). Because the slopes are not constant for the different phospholipid components examined, the detector response, even with copper treatment of the rods, is not just a simple function of mass of the compounds, but also the structure of the compounds analyzed. This is particularly evident in the case of PI and PS which, in a true mass detector, should have slopes similar to other phosphatides of equivalent mass such as PC, PE, MGDG and DGDG.

Recoveries of greater than 90% were evident for all phospholipids analyzed, and relative standard deviations averaged 5.7% (Table 2). Thus, a combination of two-dimensional thin-layer chromatography and use of the modified flame ionization detector resulted in a marked improvement in accuracy as compared to previously published methods (12,17,18). These modifications resulted in an improved separation, a stable baseline and a much greater sensitivity than achieved previously. The overall effect was a marked improvement in quantitation of a wide range of phospholipid types.

Canola oil samples. Phospholipid analyses for the different canola oil samples obtained from three processing plants is shown in Table 3. In these oils, the lower limit of reliable determination of phosphorus and phospholipids was in the nanogram range. The values reported here are more reliable and much lower than that published previously by Du Plessis *et al.* (12). A fair agreement was found between total phosphorus determined by the Iatroscan method and the spectrophotometric method for all oils analyzed. The Iatroscan method, however, gave slightly lower results in most oils examined. This difference may be due to the possibility that the spectrophotometric method may be measuring non-phosphorus or other minor phospholipid and phosphorus compounds not investigated in this study. Goh and co-workers (5) also reported that the total phosphorus content of palm oil was considerably higher than the phospholipid phosphorus. They attributed this difference to the presence of inorganic phosphates. It is known that phytin occurs in seeds (22), and compounds such as these may account for the differences in total phosphorus content and phospholipid phosphorus. Studies in our laboratory have shown canola seeds to be rich in phytin, which is rapidly hydrolyzed by phytase to inorganic phosphorus under moist germination conditions (23,24).

The total amount of phospholipid in crude oil (expeller plus solvent extracted) was less than 2% of the total lipids, which is in agreement with work published by McKillican (25), who used hexane to extract lipids from the seed as in normal industrial practice. The total amount of phospholipids in the seeds is usually twice as high when chloroform/methanol is used as extractant (26,27).

Individual phosphatides were identified by comparison with known standards (Table 3). Several unidentified peaks were also detected and are referred to as "other". The major phosphatides detected in canola oil samples were PC, PE, PI, PA and PS, which account for over 90% of the total phosphatides. Data published to date has generally reported PC, PE and PI as the major phospholipids present. In this study, the latter accounted for 25, 18 and 17% of the phospholipids in canola oil samples. Weenink (28) found that high erucic rapeseed contained 22, 15 and 18% of PC, PE and PI, respectively. The proportions of PC, PE and PI in high erucic acid rapeseed oil were reported by Zajac (29) to be 40, 16 and 32% as compared to 49, 8 and 16% by Sosulski (30) in both high and low erucic acid rapeseed oil.

The amount of phosphatides in solvent extracted canola oil from Plant C was substantially lower as compared to solvent extracted oils from the other two plants. The total phosphatide content in the solvent and expeller oils from Plant B, wholeseed cooking method, were 15 and 23% higher as compared to the corresponding oils from Plants A and C. Following degumming, the phospholipid content in the oil from Plant B was 3–5 times lower compared to the degummed oils from Plants A and C, respectively.

The amount of hydratable phospholipids in canola oil was higher in samples from the plant using the whole seeds. The hydratable phospholipids are believed to be mainly phosphatidylcholine and phosphatidylethanolamine (29,31). The combined total amount of these phospholipids in the expeller and solvent extracted oils obtained from Plant B were 2 and 4 times higher than from Plants A and C, respectively.

The major phospholipid detected in the degummed oils was phosphatidic acid. Larsson *et al.* (32) showed that the

TABLE 2

Percentage Recovery of Phospholipids[a]

	PC	PE	PI	PA	PS	LPE	LPC	PG	MGDG	DGDG
Recovery	98.5	97.8	94.3	97.9	93.6	98.7	103.2	99.1	101.4	100.0
Relative standard deviation (%)	4.6	5.2	6.1	6.8	7.2	5.6	4.4	6.9	5.2	4.9

[a]Mean of 12-18 replicates.

TABLE 3

Composition of Phospholipids and Glycolipids in Canola Oils

Plant oil sample	Phosphorus content (ppm)		Phospholipid (%)	Composition of phospholipids[a] (%)										
	Iatroscan	Colorimetric		PC	PE	PI	PA	PS	LPE	LPC	MGPG	DGDG	DG	Other
Flaked A														
Solvent	489[b] ± 5	506 ± 5	1.27[c]	25.9	18.6	19.1	23.2	3.5	0.3	0.2	0.3	0.8	1.5	6.6
Expeller	172 ± 3	184 ± 3	0.46	6.7	15.2	5.2	65.2	4.7	0.4	0.3	0.1	0.3	0.4	1.5
Degummed	40 ± 2	42 ± 2	0.11	1.8	15.7	19.5	46.9	12.8	0.1	0.1	0.0	0.1	0.2	2.8
Wholeseed B														
Solvent	529 ± 6	540 ± 5	1.35	31.2	18.8	19.7	21.6	3.1	0.4	0.4	0.2	0.8	1.8	1.8
Expeller	242 ± 4	250 ± 3	0.63	34.3	16.1	18.7	20.3	4.5	0.2	0.1	0.2	0.3	0.8	4.5
Degummed	12 ± 2	12 ± 2	0.03	2.8	10.8	28.9	38.4	14.6	0.1	0.2	0.4	0.2	0.3	3.3
Flaked C														
Solvent	386 ± 4	398 ± 3	0.99	15.1	17.8	10.3	48.9	4.6	0.3	0.4	0.2	0.7	1.2	0.5
Expeller	238 ± 3	242 ± 2	0.61	4.7	16.2	6.4	67.2	3.8	0.2	0.3	0.2	0.1	0.6	0.3
Degummed	82 ± 2	84 ± 2	0.21	3.1	15.2	7.8	61.8	10.4	0.1	0.0	0.2	0.4	0.0	1.0

[a]Mean value of four replications. [b]Mean value of six replications. [c]Calculations described in the Methods section.

phosphatidic acid content in an oil was dependent on the processing conditions used. They found a five-fold increase in phosphatidic acid in an oil produced by industrial processing as compared to that prepared in a laboratory. This increase in phosphatidic acid was attributed mainly to hydrolysis of phosphatidylcholine during processing (32). The highest content of phosphatidic acid in oils from Plant C, together with a significant reduction of PC, may be due to factors stimulating PC hydrolysis during processing. The presence of phospholipase D in corn germ, a thermostable enzyme, was attributed for the formation of phosphatidic acid from PC, PE and PI during corn oil processing (31-33).

The levels of the major nonhydratable phospholipids, particularly PA, PS and PI, were reduced 36-, 16- and 44-fold, respectively, when solvent and expeller oils were compared to the degummed oil. A decrease of 12-, 5-, 13- and 7-, 3- and 8-fold was observed in the amounts of the same phosphatides in oils obtained from Plants A and C, respectively. Oil samples with a high content of hydratable phosphatides had the lowest amount of phospholipid phosphorus after degumming. Also, the amount of PA in total phospholipids was much lower than in the other two samples. This might suggest that hydratable phospholipids play a role in removing nonhydratable phosphatides. Alternatively, using whole seeds for cooking might inhibit the decomposition of some phospholipids to phosphatidic acid.

The method described in this paper provides an effective and reliable procedure for separating and quantitating phospholipids in canola oil in the submicrogram range. The advantages of this method, such as the small amount of sample required, as well as the universality and sensitivity of the flame ionization detector provides an effective tool for analyzing mixtures of lipids. Analyzing the individual phospholipids in the expeller and solvent extracted oils can be used to predict the quality of the final product. More research is still required to correlate the presence of phospholipids with processing conditions.

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