Accumulation of Phospholipids and Glycolipids in Seed Kernels of Different Sunflower Mutants (*Helianthus annuus*)

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ABSTRACT: Phospholipids are essential components of the oil bodies present in seeds, and they are also the main components of the commercial seed lecithins used in many food formulas. In the present study, we analyzed the characteristics of the polar lipid fraction of seeds from different sunflower FA mutants. In sunflower seeds the accumulation of polar lipids reaches a maximum 25 d after anthesis before diminishing during the final stages of maturation and desiccation. We have developed an HPLC method, using ELSD, that produces optimal separation of all polar seed lipids. This method improves the results that could be obtained with previous HPLC methods and hence, we have used it to analyze the polar lipid fraction of sunflower seeds. We show that this fraction comprises phospholipids and glycolipids, of which PC is the most abundant species. Moreover, we found that the relative polar lipid content in control and mutant seeds is similar, suggesting that the mutant traits do not affect polar lipid synthesis. The degradation of polar lipids in isolated seeds was also examined and we found that the PC and PE present in developing sunflower seed kernels were rapidly degraded owing to the activity of D-type phospholipases.

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KEY WORDS: Evaporative light scattering detector, freeze-induced degradation, glycolipids, *Helianthus annuus*, HPLC analysis, phosphatidylcholine, phosphatidylethanolamine, phospholipase D, phospholipids, sunflower mutant.

Phospholipids are the main lipid components of nonphotosynthetic plant cell membranes (1). They fulfill an important role in the storage and packaging of TAG in seed oils, participating in the formation of oil bodies in association with oleosin proteins (2). Oilseed phospholipids are also the major component of lecithins, which are a by-product isolated during the extraction of edible oils and which are important ingredients of many industrial food formulae (3). The most commonly occurring polar lipids in seed tissues are phosphatidylcholine (PC), phosphatidylethanolamine (PE),. and phosphatidylinositol (PI; 4,5). Both the nature of the acyl moiety and the relative quantity influence the functions fulfilled by such plant phospholipids. Therefore, it is commonly accepted that cell membranes should maintain a balance of saturated/unsaturated FA to remain functional under any given condition (6).

The synthesis of phospholipids and TAG is closely related in seeds. Moreover, changes in the oil composition can affect the incorporation of FA into functional membrane lipids, in turn affecting seed development and viability. Indeed, such a phenomenon has been studied in mutants of sunflower that display different degrees of oil saturation (7). The degree of saturation of seed polar lipids was affected by changes in the acyl composition of TAG, although these changes do not appear to affect either the function or the viability of the seeds. Furthermore, the lipid fraction from vegetative tissues of the fully developed plants also was not affected by changes in acyl lipid composition (8). However, the possible modifications in the relative amounts of the different phospholipid classes were not taken into consideration in these studies, an analysis that to date has been obstructed by some methodological difficulties. Hence, it was not possible to resolve certain phospholipid species in pioneering studies using TLC, and quantitative data about the compounds that were separated could not be extracted.

As well as TLC, HPLC techniques have been used to analyze phospholipids. However, the most commonly used detection techniques (UV-vis and refraction index) are hindered by the poor absorbance of these compounds, and thus their sensitivity is poor (9). In the present study, the separation and analysis of different lipid classes have been tested using different HPLC methods, assessing their suitability to separate polar and neutral lipids from sunflower seeds. The most efficient combination for lipid analysis involved the combined fractionation in silica gel cartridges and ELSD. Chromatography was performed in normal and diol-bound phases, using a variety of solvent gradients. As a result, the method that was finally adopted permits the identification and quantification of the polar lipids present in the developing kernel of several sunflower mutants during the process of oil accumulation. The accumulation of both polar lipids and TAG was compared in distinct mutants and, accordingly, PC was identified as the most abundant polar lipid in the mature seeds. Furthermore, high levels of phospholipid-degrading activities were identified in the developing sunflower endosperm that should be taken into account when studying the lipid composition and metabolism in this organ. Finally, these results highlight the potential use of these phospholipids as a source of lecithin, presenting an alternative to the soybean.

EXPERIMENTAL PROCEDURES

Plant material. Sunflower plants were cultivated in growth chambers at 25/15°C (day/night), with a 16-h photoperiod, a

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photon flux density of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and with fertirrigation lines. Plants from the standard sunflower line CAS-6 and plants displaying high-oleic (CAS-9; 10), high-stearic (CAS-3; 11), and high-palmitic (CAS-12; 12) traits were used in this study.

Lipid extraction. Total lipids were extracted using a modified version of the method described by Hara and Radin (13). Freshly harvested sunflower kernel endosperm (0.05 to 0.5 g) was ground to a fine powder in 5 mL of hexane/2-propanol 3:2 in a glass tube using a glass bar and sea sand. The tissue was heated to 80°C for 10 min, 2.5 mL of 6.7% sodium sulfate was added to the tubes, and then they were shaken vigorously. The organic supernatant was separated by centrifugation at 1500 × *g* for 5 min and transferred to a clean tube. The aqueous residue was again extracted with 3.75 mL of hexane/2-propanol 7:2 (vol/vol), and the resulting organic phase was combined with that obtained in the preceding step. The lipids were then fractionated from the crude organic extracts.

Lipid fractionation. Total lipid extracts were evaporated under nitrogen and the residue was dissolved in 5 mL of chloroform. The resulting solution was fractionated in a Lichrolut 0.5 g silica gel cartridge (Merck) using a vacuum manifold and then equilibrated with 2 mL of chloroform as described by Nash and Frankel (14). The solution of total lipids was loaded on the column, which was then washed with another 15 mL of chloroform to elute neutral lipids from the column. Subsequently, the column was washed with 10 mL of methanol to recover the polar lipids quantitatively. A 1-mL aliquot of the neutral lipid fraction was evaporated under nitrogen, then dissolved in hexane. Next the TAG were quantified. The polar lipid fraction was first evaporated to dryness under nitrogen, then dissolved in 1.5 mL of hexane/2-propanol 3:2. Next, the polar lipids were analyzed and quantified.

HPLC system. Separation by HPLC was carried out in a Waters 2695 Module (Milford, MA) equipped with a Waters 2420 ELSD. Polar and neutral lipids were separated at 30°C using a Lichrospher 100 Diol 254-4 (5 μm) column (Merck) or a normal phase Lichrocart 250-4 (5 μm) column (Merck). In all cases the flow rate was 1 mL/min, and the samples were dissolved in the same solvent as that used to equilibrate the column at the time of injection. The data were processed using Empower software, and the ELSD was regularly calibrated using commercial high-purity standards for each lipid.

RESULTS AND DISCUSSION

Optimization of HPLC. To achieve efficient separation of polar lipids from sunflower kernels, it was necessary to develop an optimal method of HPLC-ELSD. In most of the previous studies of animal or plant polar lipids, these species have been separated using normal-phase silica gel columns with gradients based on acetonitrile, chloroform, or hexane/2-propanol. Here, acetonitrile-based methods were ruled out, as they required the use of sulfuric or phosphoric acid, which could damage the ELSD (15,16). Moreover, chloroform/methanol/ammonium hydroxide-based methods (17,18) display poor resolution and multiple peaks for the most common phospholipids PC and PE.

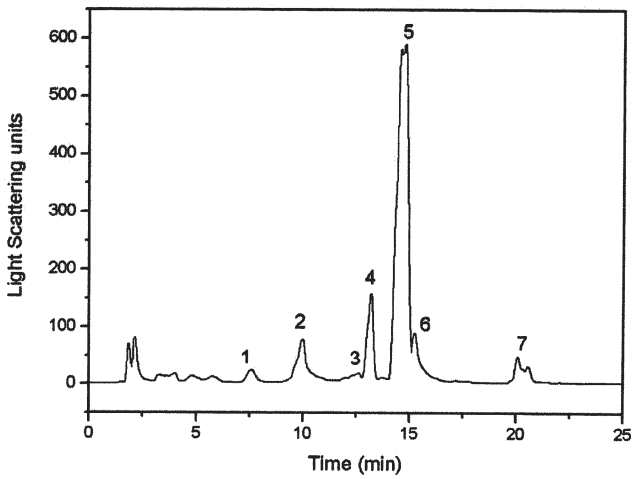

FIG. 1. HPLC separation of polar lipids from sunflower seeds, as illustrated by the chromatogram corresponding to polar lipids from standard CAS-6 seeds harvested 25 d after anthesis. The details of the elution conditions are described in the Results and Discussion section. The peaks correspond to: monogalactosyl monoglyceride (1), phosphatidic acid (2), phosphatidylglycerol (3), phosphatidylethanolamine (4), phosphatidylcholine (5), digalactosyldiglyceride (6) and phosphatidylinositol (7).

More promising results were obtained with hexane/2-propanol although when buffered solutions were used, the ELSD signal was saturated. Indeed, the method described by Picchioni *et al*. (19) based on hexane/2-propanol/water gradients efficiently separated the main phospholipids present in sunflower kernel (PE, PC, and PI). While this technique provided stable base lines and good peak shapes, it did not fully resolve phosphatidic acid (PA) from digalactosyldiglyceride (DGDG) and phosphatidylglycerol (PG) from PE. Moreover, to obtain reproducible retention times a lengthy column equilibration was required.

Diol-bound columns have recently been shown to produce more reproducible and more efficient separation of polar lipids than normal-phase columns. Indeed, a method that efficiently separated PC, PE, and PI from salmon muscle on a diol-bound column has been developed in which these lipids are eluted with a binary gradient of hexane/2-propanol solvents buffered with acetic acid and triethylamine (20). In this system, solvent A was hexane/2-propanol/acetic acid/triethylamine (82:17:1:0.08) whereas solvent B consisted of 2-propanol/water/acetic acid/triethylamine (85:14:1:0.08). In this way, it was possible to achieve good resolution when separating the most commonly occurring plant phospholipids such as PC, PE, PI, PG, and phosphatidylserine (PS); the galactolipids monogalactosyl diglyceride (MGDG) and DGDG and the leaf sulfolipid sulfoquinovosyl diglyceride (SQVDG). However, this process failed to achieve good separation of the PC and DGDG pair and it almost reached the limit of backpressure for our system. Therefore, we introduced several modifications to this procedure, including lowering the working temperature from 45 to 30°C and modifying the gradient. Additionally, separation was initiated with 90% of solvent A and 10% of solvent B before increasing the amount

TABLE 1
Response Coefficients of Different Glycerolipids in the Analytical System Described

Lipid	Response coefficient		
	Retention time (min)	(V·s/μg)	R^2
Phosphatidic acid	10.00	0.25	0.9926
Phosphatidyl glycerol	12.62	0.30	0.9969
Phosphatidyl ethanolamine	13.21	0.32	0.9972
Phosphatidyl choline	14.80	0.34	0.9989
Phosphatidyl inositol	20.06	0.30	0.9931
Phosphatidyl serine	19.12	0.32	0.9911
Monogalactosyl diglyceride	7.59	0.39	0.9916
Digalactosyl diglyceride	15.25	0.40	0.9986
Sulfoquinovosyl diglyceride	18.15	0.17	0.9912
Triolein		0.72	0.9975
Tristearin		0.99	0.9914

of solvent B to 40% over 24 min. This composition was maintained for 1 min before the column was again equilibrated with the initial solvent composition for 9 min. These modifications produced a good separation of the polar lipids present in the sunflower kernel (Fig. 1) at a reduced maximum working pressure of 1,400 psi (9650 kPa) and over a shorter time period than that reported previously (15 min shorter).

In the original method, the same column could also be used to separate sterol esters, TAG, FFA, DAG, and free sterols. However, such a detailed characterization of the neutral lipids could not be carried out in oil-accumulating sunflower kernels. In this material, TAG account for around 99% of these lipids, hampering the rigorous quantification of the other components in this fraction. Hence, we adapted the method to perform a straightforward determination of the TAG content of the seed kernel in less than 4 min, performing an isocratic elution of the column using hexane/2-propanol/acetic acid (90:15:1).

To calibrate the system for the polar and neutral lipids, different amounts of standards were injected and the response of the detector was studied (polar lipids ranging from 1 to 80 μg and the TAG from 1 to 10 μg). Theoretical studies and experimental data demonstrated that rather than a linear response, ELSD provide sigmoidal responses at increasing amounts of a given standard (21). Nevertheless, under the conditions described, the data fitted well with linear regressions (Figs. 2 and 3). The results obtained from the phospholipids were mostly consistent with those reported previously by Silversand and Haux (20), with multiple peaks appearing for PI (Fig. 1). The response factors were similar for all of these, ranging from 0.30 to 0.34 V·s/μg, except for PA, which fell to 0.25 V·s/μg (Fig. 2A, Table 1). Furthermore, linear responses were observed for galactolipids, with slightly higher factors than those of the phospholipids, reaching values of 0.4 V·s/μg (Fig. 2B). The only sulfolipid studied here displayed a response factor lower than the galactolipids and phospholipids.

The calibration of the analytical system was completed by determining the response factor of TAG (Fig. 3) using two standards: triolein and tristearin. Not only could the nature of the glycerolipids be determined, but the influence of the FA composition on the response of the detector could also be seen. Thus, triolein presented a response factor that was about 30% lower than that of tristearin (Table 1), probably because the latter was in a solid state at the temperature of nebulization (58°C) whereas triolein was liquid. The quantification of the TAG in sunflower seeds was carried out using a triolein curve since its degree of unsaturation is more similar to that of the oil of the sunflower seeds studied.

Profiles of polar and neutral lipid accumulation. Seed development occurs over a period of about 30 d, during which oil accumulation commences 10 d after anthesis (daa) and reaches a peak about 10 d later, at the time of hull hardening. The mature seeds finally undergo a process of desiccation, completing a cycle that typically lasts about 2 mon in the field. We studied

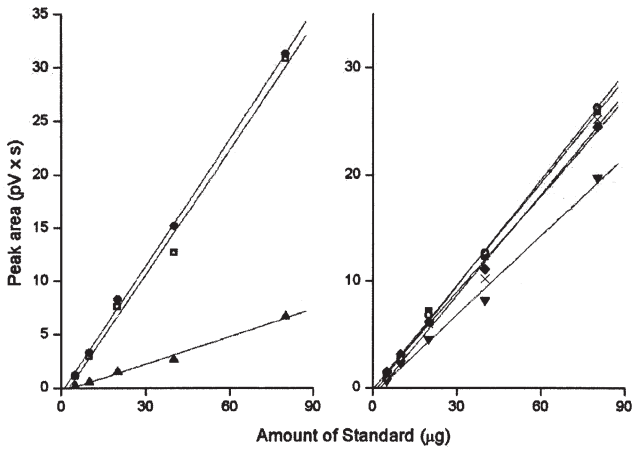

FIG. 2. Calibration curves for galactolipids and sulfolipids (A) and phospholipids (B), analyzed by HPLC with light-scattering detection. Peak area was plotted against the absolute amount of the standard injected, and the correlation was determined by linear regression analysis. The curves represent digalactosyl diglyceride (-●-), monogalactosyl diglyceride (-□-), and sulfoquinovosyl diglyceride (-▲-) (A); and phosphatidylcholine (-○-), phosphatidylethanolamine (-■-), phosphatidylglycerol (-◆-), phosphatidic acid (-▼-), and phosphatidylinositol (-x-) (B).

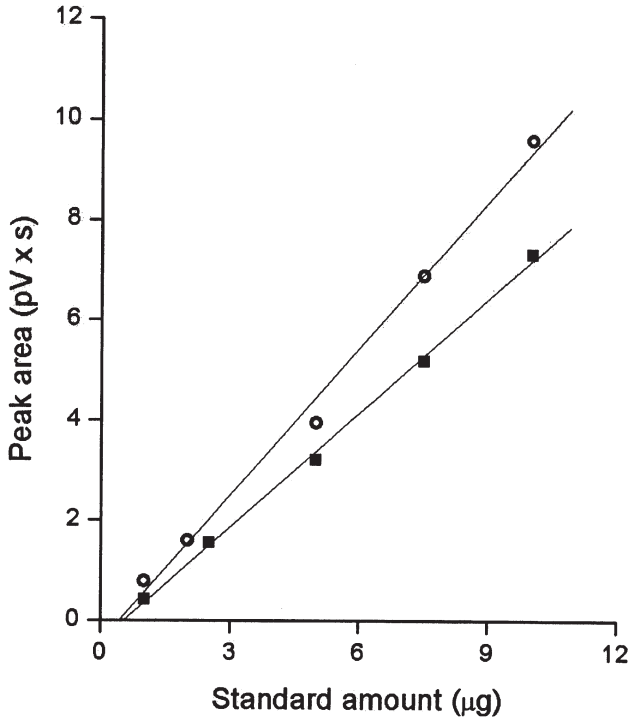

FIG. 3. Calibration curves for TAG quantified by HPLC with light-scattering detection. Peak area was plotted against the absolute amount of the standard injected, and the correlation was determined by linear regression analysis. Triolein (-■-) and tristearin are represented (-○-).

the accumulation of the different polar lipid species in sunflower kernels from 10 to 45 daa, an interval that embraces the whole period of oil accumulation and desiccation (22). The data regarding the accumulation of polar lipids were complemented by quantifying the TAG content of the seeds. Previous TLC studies on developing sunflower kernels showed that the phospholipids PE, PC, and PI can be detected, as well as the galactolipids MGDG and DGDG (7). However, the fractionation of sunflower phospholipids in normal-phase TLC does not permit the separation of some species, and the commonly occurring PA is very poorly resolved as it yields diffuse bands. In contrast, the HPLC method applied here was capable of resolving the galactolipid, sulfolipid, and phospholipid species commonly found in plants, also permitting their quantification. In this way, the presence of PA and PG in developing sunflower endosperm was confirmed, whereas neither PS nor SQVDG, a sulfolipid commonly found in leaves, was detected in any lipid extract.

In accordance with previous data regarding the phospholipid content of sunflower seed, the total polar lipid content of the lines studied accounted for 0.5 to 1.2% by weight of the mature seeds (Table 2; 23). By far the most abundant lipid found was PC (3–5 mg/g), followed by PE (0.6–1.0 mg/g) and PI (0.5–1.0 mg/g). Both PG and galactolipids were minor components, as was PA. During seed development, the profile of polar lipid accumulation was similar in all the sunflower lines examined. Thus, although the proportion of polar lipids increased during seed formation, reaching a maximum at approximately 25 daa, it decreased by 25–50% in the final steps of seed maturation (Table 2). Of the different lines studied, the high-stearic CAS-3 displayed a slightly higher polar lipid content, which showed significant differences from the common sunflower CAS-6 lines at certain stages of development. These profiles contrasted with that for TAG accumulation, which displayed sigmoidal kinetics, increasing steadily from 10 to 35 daa to reach a plateau during the final period studied (Fig. 4). Furthermore, there were clear differences between all the lines examined; the common sunflower CAS-6 line yielded a higher oil content, followed by the high-oleic CAS-9 seeds. The highly saturated CAS-12 (high palmitic-high oleic) and CAS-3 (high stearic-high linoleic) lines fell in the lower range of oil accumulation. These reductions in oil content reflected an impairment or a blockage of different steps in the FA synthesis pathway associated with each phenotype. Indeed, the CAS-3 line displays reduced stearate desaturase activity (25), whereas CAS-12 has a deficiency in the condensing activity of the β-ketoacyl-acyl carrier protein synthase II (26). Alternatively, these changes could be caused by other nonspecific modifications that occurred during the mutagenesis process, unrelated to the modification of their FA content.

Except for PA, all the polar lipids found in the sunflower kernels presented an accumulation profile similar to that found for PC. The differences displayed by PA may reflect the fact that it cannot be considered as a functional membrane lipid, but rather it is an intermediate in the biosynthesis of both neutral and polar lipids as well as an important compound in plant signal transduction pathways. Nevertheless, the ratio and the relative content of the different polar lipid species were relatively well conserved between the different mutants, which means that certain mechanisms appear to exist that prevent the composition of the polar lipid classes of the membranes from being modified.

Studies of polar lipid degradation. In earlier studies of the phospholipid fractions in sunflower kernels, certain variability has been reported in the PC content of different mutant lines. While very little phospholipid was originally found in the high-oleic lines CAS-9 and CAS-12 (7), more careful examination here failed to detect these variations (Table 2). Therefore, we addressed the possibility that differences originally observed might have been caused by a degradation artifact. Although seed phospholipids are often very stable species, they could be attacked by endogenous phospholipases during the storage or preparation processes, being degraded to DAG (phospholipase C), PA (phospholipase D), or lysophospholipids (phospholipase A; 24). Furthermore, the phospholipid PA can also be degraded to DAG through the action of the enzyme PA phosphatase.

Although the extraction methods usually used provide good protection against phospholipolysis (13), this process could occur during the storage and handling prior to extraction. Thus, we assessed the phospholipid composition following two parallel protocols. Fresh seed kernels from the same plant (CAS-6, common sunflower, 25 daa) were divided into two groups,

TABLE 2
Phospholipid and Glycolipid Composition[a] of Different Sunflower Lines During Kernel Development

		Lipid content (mg/g fresh weight)							
Line	daa	MGDG	PA	PG	PE	PC	DGDG	PI	Total
CAS-6	10	0.2 ± 0.1	0.4 ± 0.2	0.1 ± 0.0	0.3 ± 0.1	0.7 ± 0.2	0.2 ± 0.1	0.2 ± 0.2	2.1 ± 0.5
	15	0.3 ± 0.1	0.9 ± 0.4	0.3 ± 0.1	0.8 ± 0.0	2.1 ± 0.3	0.4 ± 0.1	0.6 ± 0.4	5.3 ± 1.0
	20	0.4 ± 0.0	1.3 ± 0.8	0.4 ± 0.1	1.1 ± 0.1	3.3 ± 0.6	0.4 ± 0.1	0.9 ± 0.3	7.9 ± 1.0
	25	0.4 ± 0.1	0.9 ± 0.5	0.4 ± 0.1	1.5 ± 0.1	5.7 ± 1.0	0.7 ± 0.3	1.0 ± 0.2	10.6 ± 1.4
	30	0.3 ± 0.1	0.7 ± 0.5	0.3 ± 0.1	1.4 ± 0.4	5.9 ± 1.4	0.6 ± 0.2	1.0 ± 0.3	10.2 ± 1.5
	35	0.4 ± 0.0	0.7 ± 0.3	0.3 ± 0.1	1.2 ± 0.5	5.2 ± 0.8	0.7 ± 0.3	1.1 ± 0.1	9.6 ± 1.0
	40	0.3 ± 0.1	0.5 ± 0.2	0.2 ± 0.1	0.8 ± 0.2	4.2 ± 1.2	0.6 ± 0.2	0.8 ± 0.2	7.3 ± 1.3
	45	0.2 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.7 ± 0.1	2.9 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	5.2 ± 0.3
CAS-9	10	0.4 ± 0.3	0.8 ± 0.3	0.3 ± 0.1	0.5 ± 0.2	1.3 ± 0.6	0.4 ± 0.1	0.6 ± 0.4	4.3 ± 0.9
	15	0.4 ± 0.2	0.7 ± 0.3	0.4 ± 0.1	0.8 ± 0.2	2.6 ± 0.5	0.4 ± 0.1	0.6 ± 0.2	5.9 ± 0.8
	20	0.5 ± 0.1	1.5 ± 0.7	0.5 ± 0.1	1.0 ± 0.2	3.4 ± 0.5	0.6 ± 0.1	1.0 ± 0.2	8.5 ± 1.0
	25	0.5 ± 0.1	1.3 ± 0.9	0.5 ± 0.1	1.0 ± 0.2	5.2 ± 0.9	0.7 ± 0.2	1.1 ± 0.2	10.4 ± 1.4
	30	0.3 ± 0.1	1.2 ± 0.4	0.4 ± 0.0	1.0 ± 0.1	5.1 ± 1.0	0.6 ± 0.1	1.1 ± 0.2	9.7 ± 1.1
	35	0.4 ± 0.1	1.2 ± 0.6	0.4 ± 0.0	0.8 ± 0.2	5.0 ± 0.4	0.6 ± 0.1	1.3 ± 0.1	9.5 ± 0.7
	40	0.2 ± 0.0	0.8 ± 0.5	0.3 ± 0.1	0.8 ± 0.2	3.7 ± 0.7	0.5 ± 0.3	0.7 ± 0.3	6.9 ± 1.0
	45	0.4 ± 0.2	0.7 ± 0.2	0.3 ± 0.1	0.7 ± 0.2	4.2 ± 0.6	0.5 ± 0.1	0.8 ± 0.2	7.6 ± 0.7
CAS-3	10	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	1.3 ± 0.3	0.2 ± 0.1	0.2 ± 0.1	2.6 ± 0.3
	15	0.2 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	1.2 ± 0.1	3.4 ± 0.7	0.4 ± 0.0	0.5 ± 0.1	6.5 ± 0.7
	20	0.5 ± 0.2	1.0 ± 0.9	0.5 ± 0.2	1.2 ± 0.1	4.0 ± 0.9	0.6 ± 0.1	1.2 ± 0.3	9.0 ± 1.4
	25	0.3 ± 0.1	0.8 ± 0.1	0.6 ± 0.0	1.4 ± 0.3	5.4 ± 1.3	0.8 ± 0.2	1.3 ± 0.2	10.5 ± 1.4
	30	0.5 ± 0.3	1.0 ± 0.2	0.6 ± 0.1	1.3 ± 0.1	6.0 ± 1.0	1.0 ± 0.3	1.6 ± 0.1	12.0 ± 1.1
	35	0.4 ± 0.3	1.4 ± 0.9	0.7 ± 0.4	1.2 ± 0.0	5.7 ± 1.5	1.2 ± 0.9	1.5 ± 0.6	12.1 ± 2.1
	40	0.3 ± 0.1	1.0 ± 0.7	0.6 ± 0.3	1.2 ± 0.1	5.7 ± 1.0	1.0 ± 0.7	1.3 ± 0.6	11.1 ± 1.6
	45	0.3 ± 0.2	1.0 ± 0.5	0.5 ± 0.3	0.9 ± 0.2	4.5 ± 0.2	0.8 ± 0.6	1.1 ± 0.7	9.2 ± 1.6
CAS-12	10	0.3 ± 0.3	0.7 ± 0.5	0.3 ± 0.1	0.6 ± 0.2	1.0 ± 0.5	0.3 ± 0.2	0.5 ± 0.2	4.2 ± 0.9
	15	0.3 ± 0.2	0.8 ± 0.6	0.4 ± 0.2	0.9 ± 0.5	2.2 ± 1.2	0.3 ± 0.1	0.6 ± 0.2	5.5 ± 1.5
	20	0.3 ± 0.0	0.9 ± 0.1	0.5 ± 0.0	1.2 ± 0.1	3.3 ± 0.7	0.6 ± 0.2	0.8 ± 0.2	7.5 ± 0.8
	25	0.3 ± 0.1	0.9 ± 0.4	0.5 ± 0.3	1.2 ± 0.1	4.1 ± 0.8	0.5 ± 0.1	1.0 ± 0.2	8.5 ± 1.0
	30	0.2 ± 0.0	0.9 ± 0.3	0.6 ± 0.3	1.6 ± 0.4	5.1 ± 0.7	0.7 ± 0.3	1.4 ± 0.3	10.0 ± 1.0
	35	0.2 ± 0.0	0.8 ± 0.1	0.6 ± 0.2	1.4 ± 0.5	5.1 ± 1.1	0.8 ± 0.3	1.4 ± 0.1	10.3 ± 1.3
	40	0.2 ± 0.0	0.7 ± 0.2	0.4 ± 0.1	1.1 ± 0.4	4.8 ± 1.2	0.8 ± 0.4	1.0 ± 0.3	9.1 ± 1.4
	45	0.2 ± 0.0	0.6 ± 0.1	0.3 ± 0.0	1.0 ± 0.1	4.1 ± 0.1	0.7 ± 0.3	0.6 ± 0.1	7.5 ± 0.3

[a]The data correspond to the average of four independent measurements.

the first of which was left at room temperature, while the second one was frozen at −80°C for 16 h and then thawed. The phospholipids were then extracted at different times and examined as described above. The damage produced by freezing was reflected in a rapid increase in the PA content of unprocessed seeds (Fig. 5), indicating that D-type phospholipase activity is induced during this process. Alternatively, the damage to the cell membranes induced by freezing may make the phospholipids available to pre-existing lipases. Intact kernels underwent degradation at similar rates but accumulated less PA. These activities were probably involved in keeping a high turnover of membrane phospholipids as a part of the general housekeeping processes of the seed. When seeds were detached, the *de novo* phospholipid synthesis slowed owing to the shortage of sucrose, which makes the total amount of phospholipids decrease. The phospholipids that were subjected to the highest rates of degradation were PC and PE. Both these phospholipids were almost completely degraded after 16 h at room temperature in the case of frozen seeds, only 10–15% of the initial content remaining in intact seeds.

The PA content of seeds greatly increased at the beginning of the time course but remained constant after 4 h, indicating that as well as phospholipase D, C-type phospholipases or PA phosphatases also catalyzed the degradation of these polar lipids. The absence of lysophospholipids following either protocol indicated that there was negligible phospholipase A activity. Freezing-induced degradation of phospholipids has been reported for other oil seeds where PA increases at the expense of PC after freeze/thaw cycles during the storage of peanuts (25). In addition to PC and PE, phospholipase D also attacked PI in peanuts, indicating that the enzymes expressed in that species have different substrate specificity from those present in sunflower. The relationship between the activity of these phospholipases and those related to phospholipid synthesis is probably an important factor in determining the final polar lipid content of the kernel. Hence, the general decrease of phospholipids that takes place from 25 daa in all the mutant lines was probably due to an increase of the lipase activity in the final stages of seed development. However, during the seed drying process the activity of these enzymes must have completely disappeared; otherwise, the phospholipids would be totally degraded in the harvested sunflower seeds.

Thus we have established a method to analyze the polar and neutral lipid classes in sunflower kernels. This method could

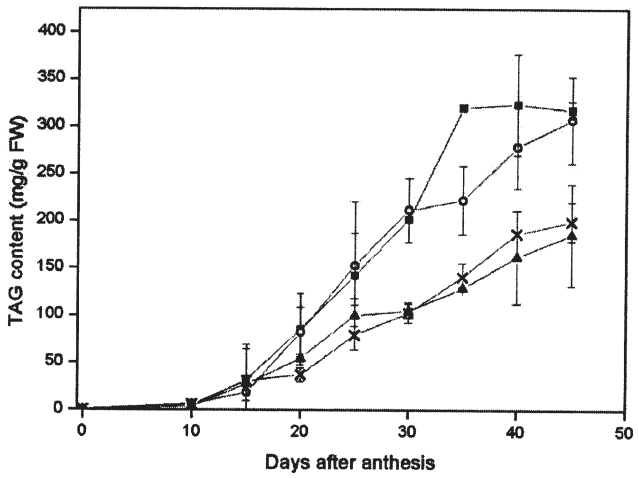

FIG. 4. Accumulation of TAG in the seed kernel of different sunflower lines. The data correspond to the common sunflower, CAS-6 (-■-); high-oleic sunflower, CAS-9 (-○-); high stearic-high oleic, CAS-3 (-▲-); and high palmitic-high oleic, CAS-12 (-x-) lines.

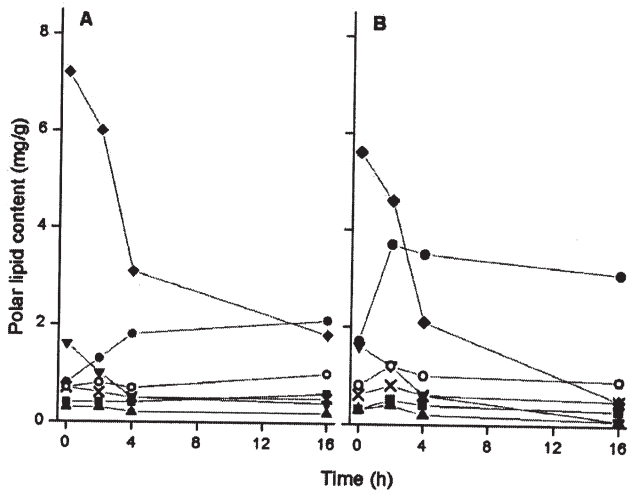

FIG. 5. Degradation of phospholipids and glycolipids at 25°C from fresh (A) and freeze/thawed (B) CAS-6 seed kernels harvested 25 d after anthesis. The data correspond to three measurements, and the SD were in the range of 15% of the measured values. The changes in phosphatidylcholine (-◆-), phosphatidylethanolamine (-▼-), phosphatidic acid (-●-), phosphatidylinositol (-○-), monogalactosyl diglyceride (-■-), phosphatidylglycerol (-▲-), and digalactosyl diglyceride (-x-) were monitored.

also be used to analyze the polar lipids in seeds from plants other than sunflower, and it has been applied to analyze plant lipids in leaf tissue (data not shown). The mutations introduced in highly saturated and high-oleate sunflower lines affect both the FA composition and the oil content. However, in these mutants neither the relative amount of the different phospholipid species nor their accumulation profile was affected. The amount of phospholipids in sunflower kernels was similar to that found in soybean (0.5–1.5%), although the PC content was higher in sunflower (50%) than in soybean (30%; 26). Since PC is the main functional component of lecithins, the phospholipid fraction of sunflower seeds is a potential substitute for this product in many food formulae. In this regard, the wide variation in the FA composition found in the polar lipid fractions of the different sunflower lines examined (7) indicates that new sunflower lecithins with novel properties might become available. On the other hand, we show that there are very active type C and D phospholipases in fresh sunflower seeds. These enzymes attack PE and PC species in the kernels, especially when membrane damage is produced by freeze/thaw cycles. This fact should be taken into account when manipulating sunflower seeds, since the content of the functional phospholipids PC and PE declined rapidly, in only a few hours.

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