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Purification of galactolipids by high-performance liquid chromatography for monolayer and Langmuir–Blodgett film studies

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ABSTRACT

In order to obtain pure galactolipids usable for monolayer work at the nitrogen–water interface, and also for fluorescence intensity measurements in Langmuir–Blodgett films when mixed with chlorophyll *a*, purification procedures for mono- and digalactosyldiacylglycerol were established using high-performance liquid chromatography. The rapid and efficient methods described were applied to commercial samples and enriched extracted fractions of lipids obtained by preparative liquid chromatography. Surface pressure–area isotherms at the nitrogen–water interface of purified and unpurified samples are also reported. The fluorescence spectra of chlorophyll *a* mixed with purified and unpurified galactolipids in Langmuir–Blodgett films clearly show the necessity for the purification.

INTRODUCTION

An important factor controlling the activity of the photosynthetic apparatus of plants and algae is the lipid composition of membranes in which the photosynthetic proteic complexes are embedded [1–4]. In thylakoids of higher plants, galactolipids comprise *ca.* 80% of the total lipid content [5]. Interestingly, this lipid class is found almost exclusively in the photosynthetic membranes. A useful technique in the investigation of the physico-chemical properties of pure galactolipids is the determination of surface pressure–area isotherms in monolayers [6].

Before monolayer work can be performed, it is necessary to establish a rapid and efficient method to purify thoroughly monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), the two most abundant galactolipids in thylakoids [5]. Two of the most common methods used to purify these lipids are thin-layer chromatography (TLC) [6–9] and liquid chromatography (LC) [6,10]. These techniques are generally time consuming and therefore inconvenient. Moreover, with TLC, it is difficult to avoid the degradation of lipid fatty acids, which are easily oxidized by oxygen present in the air and/or dissolved in solvents.

This paper presents rapid and simple high-performance liquid chromatographic (HPLC) purification procedures for MGDG and DGDG. Surface pressure–area

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isotherms are also presented and compared with those reported in the literature. These measurements are useful for cross-checking the separation before using the lipids in monolayer fluorescence experiments in a mixture with chlorophyll *a*. To emphasize the significance of the purification of galactolipids, the excitation fluorescence spectrum of a pheophytin *a*–phospholipid mixture in a Langmuir–Blodgett film is compared with that of monolayers of chlorophyll *a* mixed with purified and unpurified MGDG.

EXPERIMENTAL

Glassware cleaning procedure

As the purified lipids were to be analyzed using surface pressure–area isotherms, great care was taken to ensure clean glassware throughout the experiments. All glassware was cleaned according to an adapted version [11] of the method published by Tancrède *et al.* [6]. Quartz slides were cleaned according to the method published by Munger *et al.* [12].

Samples and reagents

Commercial samples of MGDG extracted from whole wheat flower were purchased from Serdary (London, Canada). These samples were used to establish the HPLC method prior to the purification of the extracted MGDG.

The extraction of galactolipids from barley leaves (*Hordeum vulgare* L.) and the preparative LC procedures to obtain enriched fractions of MGDG and of DGDG are described elsewhere [11]. For LC, a Sepharose CL-6B gel (Pharmacia, Uppsala, Sweden) saturated with *n*-hexane was used. The elution scheme, reported in Table I, started with *n*-hexane, which was then gradually enriched in isopropanol (IPA). This

TABLE I

ELUTION SCHEME AND GALACTOLIPID-ENRICHED FRACTIONS COLLECTED IN LIQUID CHROMATOGRAPHY

Column, 11 × 2 cm I.D.; stationary phase, Sepharose CL-6B.

Eluent	Proportions (v/v)	Volume (ml)	Collected fractions
n-Hexane		50	Pigments
n-Hexane–IPA	90:10	100	Pigments
n-Hexane–IPA	85:15	100	Pigments
n-Hexane–IPA	80:20	100	Pigments
n-Hexane–IPA	75:25	100	MGDG
n-Hexane–IPA	70:30	100	MGDG
n-Hexane–IPA	60:40	100	MGDG
n-Hexane–IPA	50:50	100	–
n-Hexane–acetone	50:50	100	–
Acetone		100	DGDG
Acetone–methanol	50:50	100	DGDG, SQDG and PG[a]
Methanol		300	PC[b]

[a] Phosphatidylglycerol.
[b] Phosphatidylcholine.

permitted the collection of most of the pigments. The MGDG emerged from the column when the concentration of IPA was between 25 and 40% (v/v). The removal of *n*-hexane, leaving acetone as the mobile phase, permitted the elution of DGDG. Sulfoquinovosyldiacylglycerol (SQDG) was recovered by the addition of 50% methanol to the acetone mobile phase. All solvents used in these procedures were of chromatographic or spectrophotometric grade.

Chlorophyll *a* from spinach was extracted and crystallized using the dioxane method of Iriyama *et al.* [13]. It was then purified by LC according to the method reported by Omata and Murata [14,15]. Pheophytin *a* was obtained by an acid treatment of the previously purified chlorophyll *a* [16].

Dioleoylphosphatidylcholine (DOPC) was purchased from P.L. Biochemicals (Milwaukee, WI, U.S.A.).

TLC procedures

All extraction, preparation and purification steps were verified by TLC. The precoated silica gel 60 plates, 50 × 200 × 0.250 mm (BDH, Toronto, Canada, or E. Merck, Darmstadt, Germany) were washed according to Tancrède *et al.* [6]. Two different elution systems were used to prevent misinterpretation for the case of DGDG. The first was acetone–benzene–water (91:30:8, v/v/v), as described by Pohl *et al.* [7]. The R_F of MGDG and DGDG are 0.56 ± 0.06 and 0.24 ± 0.05, respectively. The second migration mixture was chloroform–methanol–water (70:30:4, v/v/v), as described by Siebertz *et al.* [8]. MGDG and DGDG were then identified by the presence of spots, using iodine, at R_F values of 0.7 ± 0.1 and 0.43 ± 0.06, respectively.

HPLC procedures

The HPLC system used was purchased from Waters Assoc. (Milford, MA, U.S.A.). It was composed of two Model 510 pumps, a Model 680 automatic gradient controller, a Rheodyne (Cotati, CA, U.S.A.) Model 7126 injector equipped with a 200-μl loop and a Model 490 programmable UV–visible detector set at 205 nm. Two similar columns were used. The Altex Ultrasphere-Si silica gel column (Beckman, San Ramon, CA, U.S.A.), with dimensions 250 × 4.6 mm I.D., was used to purify MGDG. The column used to separate DGDG measured 250 × 10 mm I.D. The gel consisted of particles with an average diameter of 5 μm. No guard column was used.

All solvents used for the procedure were of HPLC grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). Mixtures of solvents used in the mobile phase were filtered through Millipore (Bedford, MA, U.S.A.) membrane filters with pores of 0.2 μm and degassed under vacuum prior to their introduction into the HPLC system. The mobile phases were composed of mixtures of *n*-hexane, IPA and water. The optimum proportions found for isolating MGDG, were 85:15:0.4 (v/v/v), with a flow-rate of 4.00 ml/min, and for DGDG 70:30:2 (v/v/v), with a flow-rate of 9.00 ml/min. All elutions were isocratic.

The concentration of lipid solutions prepared for injection into the HPLC system was *ca.* 0.2 mg/ml. The dry-weighed lipid was dissolved in the elution mixture. Because of the low solubility of the lipid in the eluent, 200 μl of the lipid solution were injected into the HPLC column, corresponding to *ca.* 40 μg of lipid. As a precautionary measure, all lipids to be used in monolayer work were purified twice.

GLC procedure

The determination of the molecular weight of the galactolipid prior to surface pressure measurements is very important. This was done, in this work, by methylation of the lipid fatty acids, followed by analysis by gas–liquid chromatography (GLC). The complete methylation procedure is reported elsewhere [11]. The Model 3700 GLC system from Varian (Sunnyvale, CA, U.S.A.) was equipped with a flame ionization detector and a 2.4-m GP 10% SP2330 on 100–120-mesh Chromosorb W AW column (Supelco, Bellefonte, PA, U.S.A.). The column temperature programme was as follows: 2 min at 170°C, increased to 235°C at 3°C/min, held at 235°C for 10 min. The injector and detector temperatures were 260 and 300°C, respectively. The nitrogen carrier gas flow-rate was 20 ml/min. Standard mixtures of methylated fatty acids (Supelco Canada, Oakville, Canada) were used to identify the peaks. A Shimadzu (Kyoto, Japan) C-R3A Chromatopac integrator was used to analyze the chromatograms.

Langmuir trough

The laboratory-built aluminum trough, 52.0 cm long × 14.0 cm wide × 4.4 cm deep, was covered with an adhesive PTFE film (Fluorocarbon Dielectrix Division, Lockport, NY, U.S.A.). An in-wall, closed water circuit was set at 20°C using a Lauda Model k-21R thermostated bath from Brinkmann Instruments (Rexdale, Canada). An in-wall, open circuit of nitrogen was flushed over the buffer surface at low flow-rates, to prevent oxygen from reaching the spread molecules on the interface. The on and off positions of the mobile barrier were set by the position of two switches and barrier movement was controlled by an electric motor activated by an external control box. The surface pressure was detected by a Milar float from DuPont (Montréal, Canada). The float was attached to a torsion wire of 0.05 cm diameter (Fender Musical Instruments, Fullerton, CA, U.S.A.). This wire was connected to a metallic bar which was free of movement inside a magnetic transducer (Model 7 DCDT-050, Hewlett-Packard, Boeblengen, Germany). The displacement of the float was converted by the transducer into a voltage detected by a multimeter (Model 4060, Brunelle Instruments, St. Elie d'Orford, Canada). The sensitivity of the Langmuir balance, calibration and recording of the isotherms were calculated by an in-house program adapted for an Apple IIc computer.

The subphase used for the surface pressure–area isotherms and the Langmuir–Blodgett film preparation was a purified 10^{-3} *M* phosphate buffer adjusted to pH 8.00. The water used to prepare the subphase was doubly distilled in quartz; its specific resistivity and surface tension were 18 MΩ cm and 71 mN/m (as determined by Du Nouy's method), respectively. Purification of the anhydrous Na_2HPO_4 (ACP Chemicals, Montréal, Canada) was made possible by six repetitive washes of the salt in chloroform (Anachemia Accusolv, Montréal, Canada), agitated each time for 10–15 min. The fresh subphase was flushed with nitrogen before it was put in the trough. A waiting period of 60 min was applied to the buffer standing in the trough, so that all surface-active contaminants might reach the interface. The surface was cleaned by suction before deposition of the lipid.

The concentration of the lipid solution used to measure surface pressure–area isotherms was in the range $3 \cdot 10^{-4}$ *M*. This concentration was found to be optimum for the spreading of $1.8 \cdot 10^{16}$–$2.2 \cdot 10^{16}$ molecules in 80–100 μl of solution. Benzene

used as the deposition solvent was freshly distilled and free from gaseous oxygen by flushing with a stream of nitrogen or argon. The solution was kept in a 3.5-ml glass vial closed by a screw-capped PTFE Mininert valve from Pierce (Rockford, IL, U.S.A.). This permitted removal of a fraction of the solution without exposing it to excessive evaporation.

To prepare Langmuir–Blodgett films for fluorescence measurements, cleaned quartz slides were dipped into the subphase on which a mixture of pigment and lipid (in a 1:100 molar ratio) had been spread. The vertical movement was controlled by a hydraulic system described elsewhere [12]. The surface pressure was kept at 20 mN/m throughout film deposition on the quartz slide.

Spectrofluorimeter

The apparatus used to measure the excitation fluorescence spectra of pigments mixed with lipids was a Fluorolog II, Model 1870 (Spex Industries, Metuchen, NJ, U.S.A.). The emission wavelength was set at 678 nm and the excitation ranged from 350 to 500 nm. The two slits at excitation were set at 4 mm, with the two others at the emission adjusted to 1.5 mm. Emission was polarized in order to avoid Wood's anomaly. The monochromators were controlled by a Spex Datamate computer. The spectra were corrected for lamp emission and sensitivity of the water-cooled photomultiplier tube (Model R 928/115, Products for Research, Danvers, MA, U.S.A.) [17].

RESULTS AND DISCUSSION

MGDG was established to be best isolated using *n*-hexane–IPA–water (85:15:0.4, v/v/v). The chromatograms obtained for commercial and extracted lipids are shown in Fig. 1. Isocratic elution was chosen because of some difficulties when the column was not in complete equilibrium with the mobile phase. This was probably due to the presence of water, necessary in the elution solvents, forming a hydration coating surrounding the gel particles. MGDG was eluted from the column in less than 5 min. The injections could therefore be done in a 10-min sequence. As the capacity factors (k') of MGDG and the following product were in a good range, selectivity was within the desired margin of 1.5–2.0. Resolution was also acceptable.

In the chromatograms, the products eluted in the first 2 min are the porphyrin pigments, such as chlorophyll *a* and *b* and pheophytin *a* and *b*. During the next 2 min, some carotenoids are eluted, characterized by their yellow-orange color. As indicated below, the peak following MGDG is believed to be a saturated lipid.

To evaluate the approximate yield of the purification, the weight of the lipid sample before and after purification was measured. The injection of 1.901 mg of crude material gave 0.572 mg of light-colored MGDG, indicating the presence of pigments. This corresponds to a 30% yield. This value is surprisingly low when compared with the calculated yield obtained from the area ratio of the MGDG peak to the total chromatogram, which gave 59%. Therefore, one of the eluted products, which is believed to be of similar molecular weight, has a very small absorption coefficient, and the area under its peak is not proportional to the amount eluted. It is possibly the product following MGDG which behaves as a lipid, but has not been identified. This eluted component is likely to be a more saturated derivative of MGDG or of another lipid, as the absorption at 205 nm is related to the unsaturated bonds in the fatty acids.

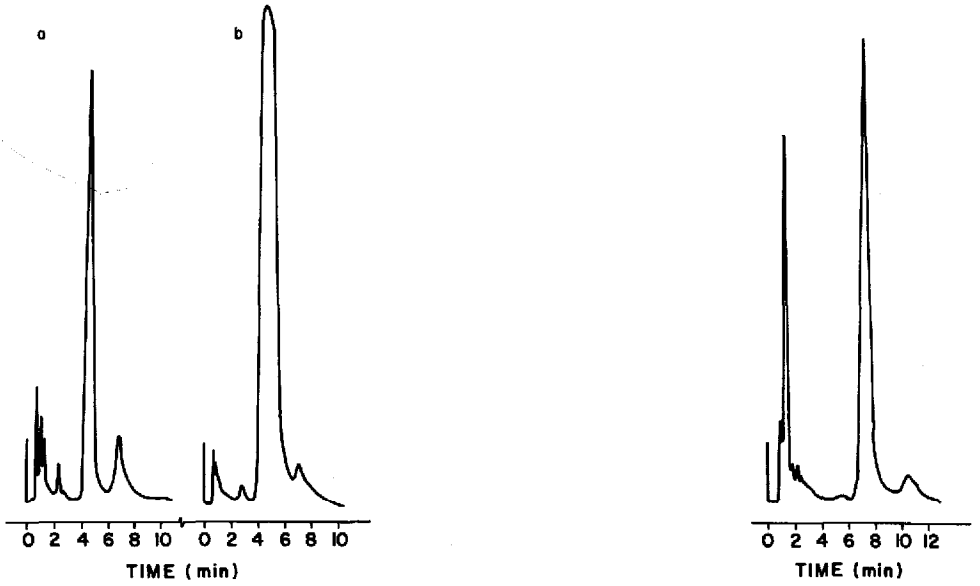

Fig. 1. HPLC of MGDG using an Altex Ultrasphere-Si column (250 × 4.6 mm I.D.). Mobile phase, *n*-hexane–IPA–water (85:15:0.4, v/v/v); flow-rate, 4.00 ml/min. (a) Commercial sample (retention time, t_R = 4.9 ± 0.2 min); (b) extracted sample (t_R = 4.6 ± 0.2 min).

Fig. 2. HPLC of extracted DGDG using an Altex Ultrasphere-Si column (250 × 10 mm I.D.). Mobile phase, *n*-hexane–IPA–water (70:30:2, v/v/v); flow-rate, 9.0 ml/min. t_R = 7.2 ± 0.1 min.

The best mobile phase found for purifying DGDG was *n*-hexane–IPA–water (70:30:2, v/v/v), giving the chromatogram shown in Fig. 2. During the first 4 min, pigments, carotenoids and MGDG (the second strongest peak in the chromatogram) are eluted. The small peak eluting before DGDG is an unknown lipid, and that following DGDG is probably SQDG. The conditions permitted a new injection every 15 min. As there is half as much DGDG as MGDG in the extracts, it is evident that the DGDG peak is less concentrated with regard to the amount of contaminants present in the sample.

Other methods that involve the use of HPLC to identify and quantify galactolipids have been published. Of these reported techniques, reversed-phase columns were employed to determine the fatty acid composition of one particular isolated type of lipid [18–22]. Therefore, these methods were not applicable for the separation of an individual class of galactolipid from a complex pigment–lipid matrix. The other published procedures for determining the galactolipid content of plant tissues used polar columns. In two of these reports, the mobile phase contained small amounts of acid [23,24]. This is undesirable, as acids may degrade lipids. Furthermore, traces of acid inevitably destroy the chlorophyll *a* pigments that are in the pigment-purified lipid mixture. In the third procedure [22], total lipid extracts were injected on to a silica gel column and, using a gradient elution scheme, all the major lipid components in the samples could be separated. Unfortunately, this method did not adequately resolve MGDG from pigments and so was unsuitable for MGDG

purification. Moreover, DGDG was not isolated from SQDG, so this analytical method cannot be used to purify DGDG either. Finally, an HPLC method has been published that used a gradient elution extending up to 50 min [25]. This method was excellent for separating MGDG and DGDG, although SQDG was indefinitely adsorbed on the column. However, as the total galactolipid extracts obtained from barley contained appreciable amounts of pigments and phospholipids, it became advantageous to include preparative liquid chromatography in the experimental procedure. This step separated the sample into enriched fractions of MGDG, DGDG and SQDG. The sequential separation into individual classes of galactolipids permitted the development of HPLC purification procedures for each lipid that were much more rapid than the method involving a lengthy gradient elution [25].

Table II presents the fatty acid composition of each sample studied using surface pressure–area isotherms. The lipid fatty acid composition is a very important factor controlling the reproducibility of surface pressure measurements. The differences between the commercial and extracted MGDG are due to their different origins. The isotherms reflect these differences (see Fig. 3, curves a and b). However, the similarity of fatty acids in the purified and unpurified extracted MGDG is evident. The largest difference in the chromatograms (not shown) is the presence of a contaminant peak in unpurified MGDG. This contaminant is due to the esterification of the phytol side-chain of chlorophyllic pigments. This contaminant peak was calculated in the total chromatogram area of the unpurified MGDG, lowering the relative amount of 18:3 fatty acid in the sample compared with the purified lipid that does not show this peak.

TABLE II

FATTY ACID COMPOSITION AND MOLECULAR WEIGHT OF COMMERCIAL MGDG AND EXTRACTED MGDG AND DGDG

Column, GP 10% SP 2230 on 100–120-mesh Chromosorb W AW; Column dimensions, 2.4 m × 6.0 mm I.D., carrier flow-rate, 20 ml/min.

Lipid	Fatty acid (%)				Molecular weight (g/mol)
	16:0	18:1	18:2	18:3	
MGDG, Serdary	21	16	50	10	770
MGDG, extracted:					
Unpurified	Trace	–	5.9	88	773
Purified	Trace	–	4.4	95	775
DGDG, extracted	14.5	2.5	4.0	78.9	931

The first surface pressure–area isotherm presented in Fig. 3 was measured with the purified commercial MGDG. The principal characteristics of these measurements are shown in Table III. The first isotherm was almost identical with that published by Tancrède *et al.* [26], who used the same experimental conditions. The only difference was the origin of their commercial MGDG sample. However, they did not verify the fatty acid composition of their sample.

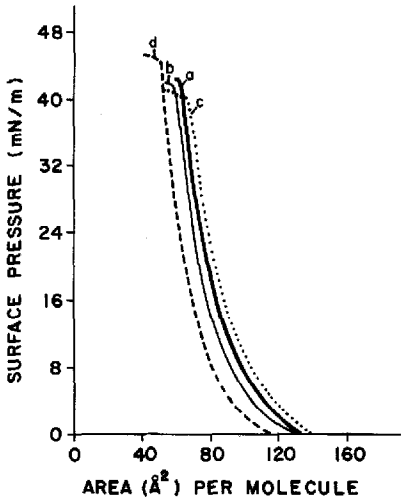

Fig. 3. Surface pressure–area isotherms of galactolipids using 10^{-3} *M* phosphate buffer (pH 8.00). (a) Purified commercial MGDG; (b) purified extracted MGDG; (c) unpurified extracted MGDG; (d) purified extracted DGDG.

The second and third measured isotherms were from the purified and unpurified extracted MGDG. The purified lipid isotherm was identical with that reported by Tancrède *et al.* [6] for MGDG purified by TLC. If their lipid source was actually from spinach, which is rich in linolenic acid (18:3), this similarity clearly shows that our reported HPLC purification procedures are as effective as the TLC method. Compared with the purified sample, the unpurified material has a lower collapse value, and the curve is observed to be displaced to the right, indicating the presence of contamination, mostly pigments that occupy more space than lipids at the interface. As pointed out by Tancrède *et al.* [6], the highest collapse of the purified MGDG is always relative to the highest purity.

The last isotherm shown in Fig. 3 is of the purified, extracted DGDG. This curve

TABLE III

MOLECULAR AREA AT THREE DIFFERENT SURFACE PRESSURES AND COLLAPSE PRESSURE OF GALACTOLIPIDS

Subphase, 10^{-3} *M* phosphate buffer (pH 8.00).

Lipid	Molecular area (Å²/mol) at			Collapse pressure (mN/m)
	10 mN/m	20 mN/m	30 mN/m	
MGDG, Serdary, purified	94	78	70	42
MGDG, extracted:				
Purified	87	73	66	42
Unpurified	100	83	74	40
DGDG, extracted, purified	77	65	58	44

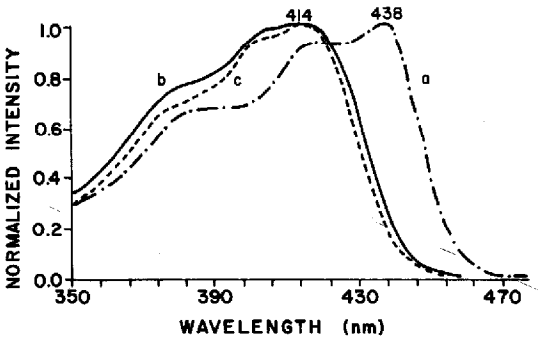

Fig. 4. Excitation fluorescence spectra of pigment–lipid mixtures in Langmuir–Blodgett films (molar ratio 1:100), with detection at 678 nm. (a) Chlorophyll *a*–purified MGDG; (b) chlorophyll *a*–unpurified MGDG; (c) pheophytin *a*–DOPC.

(d) is similar to that published by Trosper and Sauer [27], who used identical experimental conditions. However, the collapse of the DGDG isotherm in Fig. 3 was 4 mN/m higher than that reported in the literature. As mentioned previously, this higher value is usually interpreted as an indication of a greater lipid purity.

From a comparison of their isotherms, it can be seen that DGDG uses less molecular area at the interface than MGDG. Although the polar head of DGDG is twice as large as that of MGDG, this does not increase the occupied space at the interface. On the contrary, a combination of a higher degree of interaction between the DGDG polar heads, favored by the increased number of hydroxyl groups, and a lower unsaturation index in the fatty acids which are more easily ordered, may be responsible for that effect.

The importance of the purification of the galactolipids was evident in the excitation fluorescence spectra shown in Fig. 4. When purified MGDG was used in the chlorophyll *a*–galactolipid mixture, the spectrum showed a maximum at 438 nm, characteristic of the chlorophyll *a* pigment [17]. However, the use of unpurified MGDG shifted the maximum to 414 nm. This value is usually interpreted as a pheophytinization of the pigment, which has lost the central Mg atom in the process. To verify this interpretation, the measurement of a mixture of pheophytin *a* and DOPC was performed. The close resemblance of the two curves is unmistakable. The unpurified MGDG contained a contaminant that was capable of destroying the chlorophyll *a* pigment in the monolayer. Therefore, the proposed MGDG purification method was shown to be successful in eliminating all contaminants that might produce the degradation of chlorophyll *a*.

CONCLUSIONS

The rapid isocratic purification procedures described in this paper were efficient for isolating MGDG and DGDG, prior to their characterization by the surface pressure–area isotherms at the nitrogen–water interface. These measurements were in good agreement with those found in the literature. The procedures reported in this work employed no acid, and minimized all contacts between the lipids and oxygen,

a source of lipid degradation often encountered when purifying lipids by TLC or LC. Finally, the excitation fluorescence spectra in Langmuir–Blodgett films of different mixtures of pigment and lipid showed the importance of a good purification procedure. The unpurified MGDG induced the degradation of the chlorophyll *a* pigment, characterized by the disappearance of the 438-nm band, shifting the maximum to 414 nm.

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