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Determination of glycoglycerolipids by Chromarod thin-layer chromatography with Iatroscan flame ionization detection

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Abstract

A Chromarod thin-layer chromatography separation procedure was developed for polar lipids in spinach, the flagellate *Isochrysis galbana*, and the toxic dinoflagellate, *Gymnodinium* sp. Monogalactosyl diacylglycerol, digalactosyl diacylglycerol and sulphoquinovosyl diacylglycerol were separated from each other, as well as from chlorophyll *a*, carotenoids, monoacylglycerol, phosphatidyl ethanolamine and other phospholipids. Quantitation of 0.5–10 μg loads of individual glycoglycerolipids and pigments was performed by scanning the rods through the flame ionization detector of an Iatroscan. Polar lipid class proportions in spinach and *I. galbana* were within 7% of those in the literature, and summed classes are 84% of total lipids by gravimetry.

Keywords: Glycoglycerolipids; Glycerolipids; Lipids

1. Introduction

Glycoglycerolipids are glycosyllipids or glycolipids in which one or more monosaccharide residues are linked by a glycosyl linkage to a lipid moiety containing a glycerol residue [1]. This type of glycolipid is found predominantly in the plant kingdom, in association with chloroplasts. There is currently considerable interest in glycolipids derived from microalgae, as a source of biologically active substances [2,3].

The analysis of glycoglycerolipids is complicated by the variety of structures represented among the polar components of lipid extracts, by their instability and by the lack of standards. There is however, in the literature, a column chromatographic technique that has been applied to glycolipids in freshwater diatoms [4]. In this study, we sought to develop Chromarod thin-layer chromatography (TLC) separation procedures in conjunction with the silica gel cartridge procedures of Yongmanitchai and Ward [4], with the aim of developing a quantitative technique for plant glycolipid determinations. Organic material separated on Chromarods is quantified by passing them through the flame ionization detection (FID) system of an Iatroscan. This FID system is not as sensitive, precise or linear as found in gas chromatographs, but it does provide rapid μg quantitation without derivatization or clean-up.

Chromarod TLC with Iatroscan FID is used routinely in the quantitative analysis of lipids from marine microorganisms [5], but usually the glycolipids are eluted with monoacylglycerols (MGs) and pigments in a group for which the term "acetone-

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mobile polar lipid" (AMPL) has been coined [6]. The focus of the present study was to separate out the AMPL components and to quantify them in an Iatroscan. This would also permit a reevaluation of the suitability of standards used for the quantitation of the AMPL mixture. A MG or a monogalactosyl diacylglycerol (MGDG) is often used to calibrate this peak, but it is known that chlorophyll is a high response compound [7]. A comparison of the responses of the various components should ensure correct calibration of AMPL peaks in the future.

2. Experimental

2.1. Standards and solvents

The principal standards used were chlorophyll *a*, (from spinach; Sigma, St. Louis, MO, USA), 1-monopalmitoyl glycerol (Sigma), galactosyl diglyceride (from whole wheat flour; Sigma), digalactosyl diglyceride (from whole wheat flour; Sigma), and dipalmitoyl phosphatidyl ethanolamine (Sigma). Chlorophyll *b* (Sigma) was also used in a few developments but was found to elute with chlorophyll *a* in the polar solvent systems tested and so was not used regularly. Likewise, diphosphatidyl glycerol and dipalmitoyl phosphatidyl choline (Sigma) were also used in a few developments but were found to elute near dipalmitoyl phosphatidyl ethanolamine in the systems tested and so were not used regularly.

In addition, two glycolipid standards were prepared from a spinach (*Spinacia oleracea*) extract. Digalactosyl diacylglycerol (DGDG) and sulphoquinovosyl diacylglycerol (SQDG) fractions were isolated by column chromatography according to Yongmanitchai and Ward [4]. TLC–FID indicated that these fractions also contained more polar and less polar lipid classes so they were further purified by preparative plate TLC. The bands were removed from the plate and extracted in chloroform–methanol (2:1, v/v). The DGDG standard gave a single FID peak in the Iatroscan, while the SQDG standard had a significant level of phospholipid (PL) contamination. A purer SQDG fraction was obtained from the alga *Gyrodinium* cf. *nagasakinese* after column chromatography, probably because of the greater proportion of SQDG in the initial lipid extract. The minor pigments were identified by comparison with values in the literature for silica gel TLC migration order [8], for colour [9] and for plant pigment composition [10].

The principal solvents used were trichloromethane (with 0.6–1% ethanol as a stabilizer: Riedel-de Haen, Seelze, Germany), acetone (Merck, Darmstadt, Germany) and formic acid (98–100%, Merck).

2.2. Chromarod thin-layer chromatography

After application of samples and standards to precleaned Chromarods-SIII, the material was focused at the origin by developing twice in acetone, and then conditioned for 5 min over a saturated sodium chloride solution. The frame was then placed in a paper-lined tank containing 50–51 ml of solvents for development. Conditioning and developments in chloroform, or acetone-based solvent systems, were performed at a temperature of 23–25°C. Rods were acid-cleaned (50% HNO_3) daily.

2.3. Iatroscan flame ionization detection

After developing, the rods were dried for 5 min and then scanned in an Iatroscan MK IV analyzer (Iatron, Japan). The signal from the FID was processed using Boreal software (Flotec, La Queue lez Yvelines, France). In most cases, rods were scanned in a two-step procedure involving a partial scan after the first development and a complete scan after a second development. When this occurred, the two scans were combined using Boreal to give a single scan in which all the peaks appeared.

2.4. Plate thin-layer chromatography

Analytical and preparative separations of samples and standards were also performed on silica gel 60 thin-layer plates with concentration zones. Identification of lipid classes and confirmation of their purity was carried out on prechanneled CF_{254} plates (Merck 13144, 0.25 mm). The plates were prewashed with acetone, activated for 1 h at 105°C and stored in a desiccator prior to use. After development in chloroform–methanol–water (25:10:1, v/v), the plate was sprayed with α-naphthol–sulphuric acid reagent, which caused the carbohydrate moieties of the

glycolipid fractions to appear as purple bands. The R_F ratio of the two galactolipids and the sulpholipid, MGDG–DGDG–SQDG was 0.92:0.68:0.45, which is similar to the ratio of 0.9:0.47:0.36 previously reported [4].

Glycolipid fractions were purified on semi-preparative plates (Merck 11845, 0.25 mm) after development in chloroform–methanol–water (25:10:1, v/v). Most of each band was removed from the plate and extracted in chloroform–methanol (2:1, v/v). The remainder was sprayed with α-naphthol reagent and then heated at 100°C to confirm the presence of the glycolipid and its location.

2.5. Lipid extracts

Plant material from four sources was extracted first in isopropanol and then in chloroform and methanol. To obtain large quantities of glycolipids, 600 g of store-bought frozen spinach was blended in 40 ml of isopropanol and then sonicated with 400 ml of chloroform–methanol (2:1, v/v). Samples from three different microalgal cultures were also obtained, one from a mass culture of *Isochrysis galbana* (Clone T-Iso), sampled at the end of the exponential phase at a cell density of $8 \cdot 10^6$–$10 \cdot 10^6$ cells/ml, the others from several batch cultures of two potentially toxic marine dinoflagellates, *Gymnodinium* cf. *nagasakiense* and *Gymnodinium* sp., also sampled at the end of the exponential phase.

The *I. galbana* sample consisted of 3 l of cells plus medium which were extracted together after addition of 20 ml of isopropanol. Lipids were extracted three times with a total of 600 ml of chloroform–methanol (95:5, v/v) with sonication. For the latter two extractions, the pH of the solution was changed to pH 2 with HCl. The *Gymnodinium* samples were gravity filtered onto pre-combusted Whatman GF/F glassfibre filters and stored at −20°C in isopropanol until extraction in chloroform–methanol (2:1, v/v) and analysis.

3. Results and discussion

The separation of plant glycolipids from phospholipids and pigments requires a solvent system that minimizes the mobility of the phospholipids and that selectively moves glycolipids and pigments. To fulfil this purpose, acetone was a major component of all solvent systems tested. Acetone was used by Rouser et al. [11] to separate plant and animal glycolipids from phospholipids by silica gel column chromatography. Several solvent systems, consisting of chloroform, acetone, formic acid and water, were tested in 40 min developments with standards and samples.

3.1. Standard separations

In the solvent systems tested with standards, chloroform and acetone ranged from 40–60% (v/v), and formic acid and water ranged from 0–2% (v/v). The systems chosen were based, in part, on those used by Yongmanitchai and Ward [4] for silica cartridge separations of algal lipids. Solvent systems containing more chloroform and less acetone and formic acid gave better separations of chlorophyll *a* (CHL), MG and MGDG (Fig. 1a), while those containing more acetone and less chloroform gave better separations of DGDG from phosphatidyl ethanolamine (PE: Fig. 1b). Chloroform–acetone (1:1, v/v) was a good compromise solvent system for separating the major glycolipid classes in a single 40 min development (Fig. 1c). As found by Rouser et al. [11] in column chromatographic separations of glycolipids, the addition of water to the solvent systems did not seem to confer any greater selectivity to the glycolipid separations.

Correlation analysis was used to confirm these observations. CHL, MG, MGDG and DGDG showed significant correlations with the proportions of chloroform and acetone. The scan times of none of the lipids correlated with the proportion of formic acid.

3.2. Sample separations

The separation of glycolipids in plant extracts differs from that of glycosphingolipids [12], in that it is complicated by the presence of various pigments with different chemical structures and mobilities. Separation of the principal glycolipids from all the pigment bands proved difficult to do in a single step and so a two-step procedure was used (Fig. 2). The first step involved a 40-min development in chloroform–acetone (3:2, v/v) followed by a partial scan to a point immediately following the MGDG peak (PPS 37 on the Iatroscan). The rods were then

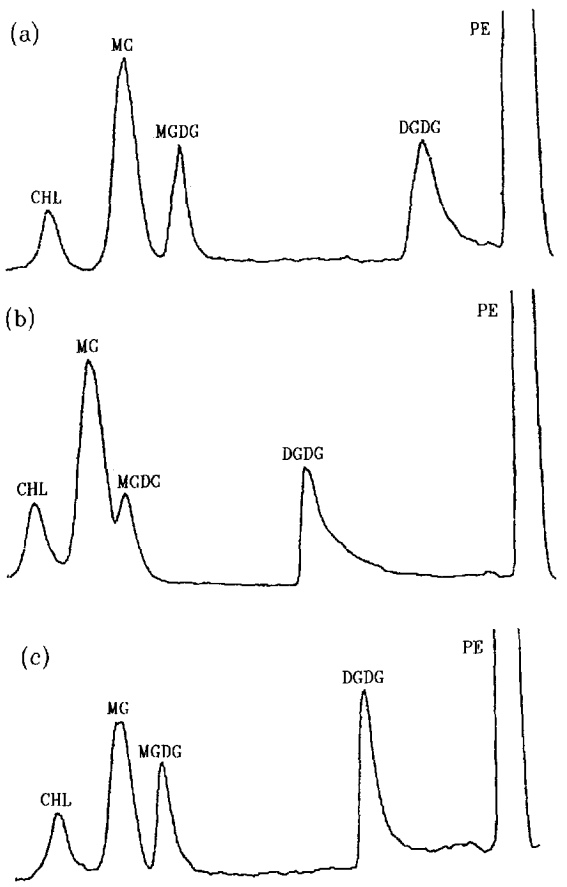

Fig. 1. Separation of standard solutions (1–4 μg/μl) of chlorophyll *a* (CHL), monoacylglycerol (MG), monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG) and phosphatidyl ethanolamine (PE) on Chromarods-SIII with loads of 1–4 μg. All developments were for 40 min after focusing twice at the origin. (a) Chloroform–acetone (3:2, v/v); (b) acetone–chloroform (3:2, v/v) and (c) chloroform–acetone (1:1, v/v).

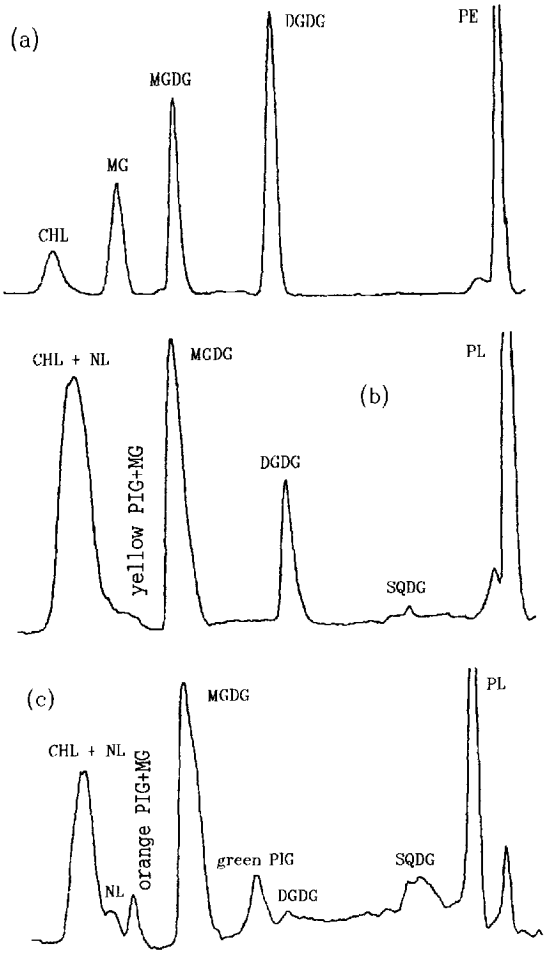

Fig. 2. Separation of standards and samples in a two-step procedure: (1) 40 min development in chloroform–acetone (3:2, v/v) followed by a partial scan until after the MGDG peak (PPS 37 on the Iatroscan) and (2) redevelopment for 30 min in acetone–formic acid (49:1, v/v) followed by a full scan. Sample peaks were identified by co-spotting with authentic standards, where available, by comparison of the position of coloured bands on the rods with peak scan times, and by comparison with fractions obtained from silica gel column chromatography. Note that the response scales differ for each chromatogram. (a) Standards as in Fig. 1; (b) spinach extract and (c) *Isochrysis galbana* (Clone T-Iso) extract.

redeveloped for 30 min in acetone–formic acid (49:1, v/v) and scanned for their entire length.

While the proportion of formic acid was less important in determining the separation of lipid class standards, it was quite important in determining the mobility of the pigment bands and of glycolipids more polar than DGDG on the Chromarods. Addition of formic acid to the first solvent system in the two-step procedure caused the migration of the light green pigment band to the position occupied by MGDG, and so it was not included in this step. Development in chloroform–acetone alone resulted in the light green band remaining at the origin. In 100% acetone this band did migrate, but in this development the inclusion of formic acid was found to be necessary to maximize the migration of the more polar glycolipids from the origin. An attempt at developing these glycolipids in chloroform–metha-

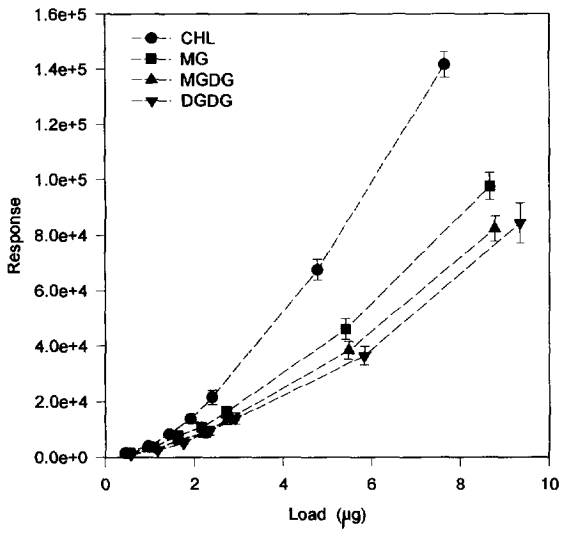

Fig. 3. Calibration curves for acetone-mobile polar lipids. Error bars are standard deviations for responses from four to eight rods. CHL, chlorophyll *a*; MG, 1-monopalmitoyl glycerol; MGDG, galactosyl diglyceride; DGDG, digalactosyl diglyceride.

nol (9:1, v/v), based on the SQDG separation of Yongmanitchai and Ward [4], resulted in an inferior separation, as DGDG was not eluted so far down the rod and PE started to elute from the origin. Use of automated multiple development [13] would undoubtedly further enhance Chromarod separations.

3.3. Quantitation

The AMPLs showed a range of responses (Fig. 3), but all followed the same basic form. Regression analysis with the raw calibration data indicated that power law equations ($y=ax^b$) gave better fits ($r=$ 0.99–1.00) than did linear equations ($y=a+bx$, $r=$ 0.98–0.99). This is because of the curvilinear response of FID [14]: power law exponents ranged from 1.50±0.03 for MG to 1.62±0.03 for CHL.

Peaks from the plant extracts (Table 1) were quantified using standards purchased from Sigma (Table 2). Using these standards, spinach MGDG, DGDG and SQDG were within 1–5% of proportions calculated for spinach chloroplast membranes [15]. Also, the ratio of MGDG to DGDG is 1.9 which is identical to that described for spinach chloroplasts [16], and SQDG is present in much smaller proportions, as is typical of plant leaves [17]. In *I. galbana*, AMPL and PL amounted to 81% of the total lipids, which is within the range described by others [18–20] who did not subfractionate their polar lipids. Summed polar lipid proportions (Table 2) were within 7% of most of the literature values for *I. galbana* that was grown under a variety of conditions. As was found by Stern and Tietz [21], one of the glycolipids, MGDG, is in fact the major lipid class in *I. galbana*. The second most prominent

Table 1
Ranges of mean lipid class retention times for spinach, *Isochrysis galbana*, *Gymnodinium* cf. *nagasakiense* and *Gymnodinium* sp.

Lipid class	Abbreviations	Scan time (min)
Chlorophyll *a* + neutral lipid	$CHL+NL_a$	0.04–0.07
Neutral lipid	NL_b	0.07–0.12
Fucoxanthin[a] + monoacylglycerol	orange[a] PIG_a+MG	0.12–0.14
Polar lipid	polar LIP_a	0.16–0.18
Monogalactosyl diacylglycerol	MGDG	0.19–0.23
Chlorophyll *c*	green PIG	0.27–0.30
Digalactosyl diacylglycerol	DGDG	0.33–0.36
Light orange pigment	orange PIG_b	0.39±0.01[b]
Polar lipid	polar LIP_b	0.40±0.02[c]
Sulphoquinovosyl diacylglycerol	SQDG	0.49–0.52
Phospholipid	PL	0.64–0.67

Extracts were spotted on Chromarods-SIII and then subjected to a 40-min development in chloroform–acetone (3:2, v/v) followed by a partial scan until after the MGDG peak (PPS 37 on the Iatroscan), and then redevelopment for 30 min in acetone–formic acid (49:1, v/v) followed by a full scan. Chromatograms were then combined to give a total scan time of 0.72 min.

[a] Yellow carotenoid (leutin) in spinach.

[b] Mean±S.D., $n=3$, *Gymnodinium* sp. only.

[c] Mean±S.D., $n=8$, *Gymnodinium* cf. *nagasakiense* only.

Table 2
Lipid class composition (% total lipid) of spinach (mean±S.D., $n=7$) and of *Isochrysis galbana* ($n=5$).

Lipid Class	Calibration standard	Spinach	*I. galbana*
$CHL+NL_a$	chlorophyll *a*	25.9±1.2	15.7±1.7
NL_b	1-monopalmitoyl glycerol	–	3.6±0.6
orange[a] PIG+MG	1-monopalmitoyl glycerol	6.9±3.1	4.7±0.9
MGDG	galactosyl diglyceride	32.1±1.6	31.6±3.0
green PIG	chlorophyll *a*	–	5.7±1.0
DGDG	digalactosyl diglyceride	16.9±1.4	4.5±0.8
SQDG	galactosyl diglyceride	2.9±0.6	20.6±3.9
PL	phosphatidyl ethanolamine	16.5±2.1	13.5±3.4

Separation procedures and abbreviations as in Table 1.
[a] Yellow carotenoid (leutin) in spinach.

class, SQDG, was also the second most prominent polar lipid in the diatom *Phaeodactylum tricornutum* [22].

The coefficient of variation for five to eight analyses of samples was, on average, around 15%. This is a little higher than can normally be expected for neutral lipids determined by TLC–FID [5–7] and probably reflects the greater lability of the AMPLs.

In a comparison between gravimetry and Iatroscan-determined lipid classes for *I. galbana* samples, summed lipid classes were 84±5% ($n=5$) of gravimetric values. This value is almost identical to that determined for samples from the Great Lakes [6]. The discrepancy between the methods for determination of total lipid may reflect an inappropriate choice of calibration standard, or the fact that TLC–FID is only quantitative for non-volatile compounds. Use of chlorophyll *a* to calibrate the CHL+neutral lipid peak would only be appropriate if there was little neutral lipid present, since neutral lipids have a response closer to that of MG. Otherwise the high response of CHL (Fig. 3) would cause a significant underestimation of the total content of this peak. Likewise, PE may not be appropriate for all PLs. To evaluate the total lipid content properly, the two-step procedure used here could be combined with other steps for neutral lipids [6] and for phospholipids [5]. However, TLC–FID usually gives a slightly lower total lipid content in any case, because more volatile compounds may evaporate from the rod immediately before they would pass through the flame of the detector [6].

The large difference in response between CHL and the other AMPLs suggests that some caution is necessary in calibrating single AMPL peaks in which CHL is a major contributor. Where this is not the case, MG would seem to be a reasonable compromise for calibration of plant AMPL. However, in situations where the proportion of chlorophyll *a* is unknown, this type of development (Fig. 2) should be performed after separating the neutral lipids [6] in order to obtain pure chlorophyll peaks.

Acknowledgments

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