Analysis of Cereal Digalactosyldiacylglycerol Molecular Species by High-Performance Liquid Chromatography

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ABSTRACT: An optimized procedure for the high-performance liquid chromatographic resolution of molecular species of digalactosyldiacylglycerols from cereals has been developed. The optimal conditions were found by a three-step optimization strategy by taking eluent composition, eluent profile, system temperature, and flow into consideration. Separation of seven molecular species was achieved for oats and wheat-kernel digalactosyldiacylglycerols. Molecular species with the same partition number were eluted in the order of increasing unsaturation when utilizing eluent systems based on alcohols and water. This is reversed as compared to what is found when using eluent systems that contain acetonitrile, in which the elution order of the molecular species with the same partition number is according to decreasing unsaturation.
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KEY WORDS: Cereals, digalactosyldiacylglycerol, experimental design, HPLC, oat, reversed-phase liquid chromatography.

Digalactosyldiacylglycerol (DGalDG) is frequently found in the grains of cereals (1) and in the photosynthetic membranes of higher plants, algae, and cyanobacteria (2). The fatty acid composition can differ markedly between sources, and the isolation and characterization of DGalDG molecular species from different natural sources are therefore of interest. Molecular species separations have been done by reversed-phase high-performance liquid chromatography (RP-HPLC), silver-impregnated thin-layer chromatography, and high-temperature gas–liquid chromatography. Photosynthetic tissues (3–12) have a high degree of unsaturated fatty acids and thus have a different molecular species profile than seeds from cereals (13–15). Oat is a source of DGalDG, and its fatty acid composition has been investigated (1,16,17), revealing palmitic, oleic, and linoleic acid as the major fatty acids. Especially oleic acid is present in high amounts, compared to other cereals. The molecular species pattern of oats, however, has not been determined. The elution order of molecular species depends strongly on the choice of eluent type. If, for example, the eluent contains acetonitrile, the elution order of molecular species is altered, compared to that in an eluent system based on methanol and water.

Monitoring of the molecular species from DGalDG with ultraviolet (UV) detection has been done (3,4,6,7,10,13,14). The UV-detection technique is, however, dependent on UV-absorbing groups. The refractometer (5,9,15), which is not dependent on the presence of such chromophores, is unfortunately not suitable for gradient elution. The HPLC–flame-ionization detector (FID), although universal (8), is not compatible with salts or solvents with high boiling points. The evaporative light-scattering detector is not dependent upon chromophores and can be used for detection and quantitation of intact molecular species of DGalDG.

The discipline of chemometrics is becoming widely accepted in many areas of chemistry, chromatography being an especially prolific area (18). We have developed a pragmatic liquid chromatography-optimization strategy that allows the analyst to find optimal separation conditions on the basis of literature values or previous scouting runs and the physical properties of the solvents (19,20).

The goal of the present study was to develop and optimize an RP-HPLC system for the analysis of molecular species of DGalDG from oats with chemometrical methods. Another natural DGalDG source, wheat flour, was used to monitor the viability of the system.

EXPERIMENTAL PROCEDURES

Chemicals and materials. HPLC-grade eluents were obtained from Merck (Darmstadt, Germany). Purified DGalDG from oat and wheat were obtained by an extraction procedure described elsewhere (21). Samples injected on HPLC had a concentration of 5 mg/mL.

HPLC. HPLC was done with a Shimadzu model LC-6 pump system (Kyoto, Japan) equipped with a Rheodyne switching valve (Cotati, CA) and a light-scattering detector from Cunow, model DDL 11 (Cergy, St. Christophe, France). The detector nebulizer was set at 1.8 bar and held at 35°C. All detector settings were subsequently optimized by using the final elution system described in the Results and Discussion section. Gradient elution was done on an ODS column from Merck (Superspher 100 RP-18, 250 × 4.6 mm, 5 μm), and for

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TABLE 1
Factorial Design of the First Experimental Set

Experiment number	Acetone (vol%)	2-Propanol (vol%)	Acetonitrile (vol%)	Methanol (vol%)	Heptane (vol%)	Water (vol%)	Temperature (°C)	Flow (mL/min)	Curve profile	Gradient time (min)
1A	24	0	37	28	0	11	60	1.4	2	20
1B	25	0	38	27	9	1	40	1	2	40
1C	40	0	6	52	0	2	40	1.4	−2	40
1D	39	0	0	50	0	11	60	1	−2	20
1E	5	45	0	22	4	24	60	1	−2	40
1F	7	33	0	0	57	3	40	1.4	−2	20
1G	16	32	0	52	0	0	40	1	2	20
1H	23	32	0	35	9	0	60	1.4	2	40
1I	26	4	10	27	33	0	50	1.2	0	30

the ruggedness test, an ODS column from Eka Nobel (Kromasil 100-5C18, 250 × 4.6 mm, 5 µm; Bohus, Sweden) was used. The column was thermostatted with a waterbath at 55°C. All experimental data were collected and analyzed on a Kontron PC integration pack (Milan, Italy).

Gas chromatography. Molecular species of DGalDG were isolated *via* a stream splitter, dried under a stream of nitrogen, and derivatized to fatty acid methyl esters (FAME) by an alkaline methanolysis procedure described elsewhere (22). Analysis of the FAME was done on a Varian 3500 gas chromatograph, equipped with an on-column injector (Walnut Creek, CA). The analytical column was a DB-WAX, 30 m × 0.25 mm (i.d.) from J&W (Folsom, CA). A retention gap (0.5 m) was installed between the injector and the column. A multiramped temperature program was applied: 130°C held for 2 min, increased by 50°C/min to 150°C, increased by 3.3°C/min to 235°C, and held for 5 min. Detection was done with an FID set at 250°C. The detector signal was monitored by the Gynkosoft Chromatography Data System (Gynkotek, Germering b., München, Germany).

Multivariate methods. Partial least squares (PLS)-modeling, described in detail in the literature (23–25), was done in CODEX® (SumIT System AB, Solna, Sweden). Nonlinear mapping (26) was done in a routine developed in MATLAB (Mathworks Inc., Natick, MA) by using the Euclidean distance. Statistical experimental design, described in detail in the literature (27,28), was used in the different steps of the strategy for each of the studies. These designs were either reduced fractional factorials (2^{4-1}, etc.) or Plackett–Burman designs. The designs were generated in CODEX®.

The chromatographic system was developed and optimized according to a multivariate optimization three-step procedure, developed in our laboratory:

Step I. Solvent system compositions (vol%), collected from the literature or from initial scouting runs, were used to target the experimental design in a nonlinear map of solvent physical properties. This was accomplished by using the volumetric proportions for the solvent systems from a scouting run and utilizing a PLS-model developed on the tabulated physical data of pure solvents and measured data on a set of mixtures to calculate the physicochemical properties. These were thereafter mapped into the nonlinear map of pure solvents and solvent mixtures to define the search area. This step is presented in detail in Kaufmann *et al.* (19).

Step II. A three-factor two-level factorial design was generated and laid out in the targeted area in the map with the reduced map axes as design factors (Table 1), thus enabling the selection of relevant solvents and solvent combinations. The chromatograms corresponding to the different systems were run, and the system that exhibited best chromatography was chosen as the basis for the third step.

Step III. A training set was generated by using the solvent system from the best run as the basis for a second factorial design, where the levels of the chosen solvents are varied (Table 2). The chromatograms from this new set of runs were evaluated quantitatively, and the results, together with results

TABLE 2
Factorial Design of the Second Experimental Set

Experiment number	Acetone (vol%)	2-Propanol (vol%)	Acetonitrile (vol%)	Methanol (vol%)	Heptane (vol%)	Water (vol%)	Temperature (°C)	Flow (mL/min)	Curve profile	Gradient time (min)
2A	5	48	0	18	2	27	55	1	2	20
2B	5	40	0	26	2	27	65	0.8	2	40
2C	5	47	0	18	3	27	65	1	−2	40
2D	5	39	0	26	3	27	55	0.8	−2	20
2E	5	54	0	18	2	21	55	0.8	−2	40
2F	5	46	0	26	2	21	65	1	−2	20
2G	5	50	0	18	6	21	65	0.8	2	20
2H	5	45	0	26	3	21	55	1	2	40

TABLE 3
Fine Tuning Design

Experiment number	Acetone (vol%)	2-Propanol (vol%)	Methanol (vol%)	Temperature (°C)	Flow (mL/min)	Salt (yes/no)
3A	9	38	24	55	1	0
3B	5	42	24	55	1	0
3C	5	38	28	55	1	0
3D	1	42	28	55	1	0
4A	5	38	28	55	1	1
4B	5	38	28	50	0.9	0

for a fine-tuning design (Table 3), were used as a training set in a PLS-model to predict optimal chromatographic conditions. Steps II and III are described by Kaufmann (20).

RESULTS AND DISCUSSION

The goal of this work was to resolve as many molecular species of DGalDG from a cereal source (oats) as possible by using a multivariate optimization strategy. Unfortunately, RP-HPLC cannot separate positional isomers that may be present in DGalDG. Theoretically, the number of molecular species from DGalDG that can be separated by RP-HPLC are $(n^2 + n)/2$, where n denotes the number of fatty acids. For oats, three fatty acids (palmitic, oleic, and linoleic) comprise about 95% of the fatty acids, with only small amounts of stearic and linolenic acids (17), indicating 15 possible DGalDG molecular species, of which six are major ones. Small amounts (<1%) of 24:0 were also detected in oats. However, none of the collected fractions contained significant amounts of this fatty acid. Other cereals, such as barley and rye, contain a higher degree of linolenic acid and a lower degree of oleic acid (17).

In developing the HPLC system, the B-eluent composition was held constant throughout the optimization process at a sufficiently nonpolar level to elute the most apolar molecular species. The B-eluent was composed of solvents initially found by the targeting procedure, i.e., acetone/2-propanol/methanol/heptane/water (5:47:37:2:9). The levels of these solvents were varied according to a Plackett–Burman design (Table 1) to generate the different A-eluents. The experiments from the first factorial design were either poorly retained on the ODS-column or well retained but with poor selectivity (Fig. 1). Experiment 1E (Table 1 and Fig. 1E), however, showed both good retention behavior and fair selectivity with partial separation of individual molecular species as a result. The first experimental set thus indicated that experiment 1E has the best potential to resolve all DGalDG molecular species. In the second design step (Table 2 and Fig. 2), experiments 2C–G gave partially resolved molecular species, although compressed in time. In experiments 2A, 2B, and 2H, the molecular species were retained longer on the ODS column, and they were well resolved. Optical inspection of the chromatograms gave experiment 2B (Fig. 2B) as the best of the second block. Fine-tuning of the solvent system was done by varying acetone, 2-propanol, and methanol content in experiment 2B in a new reduced design (Table 3). Experiment 3C (Table 3 and Fig. 3) gave slightly more distinct peaks for the late-eluting molecular species and was considered the best. This experiment was tested both with salt addition (experiment 4A, Table 3) and with a lower linear flow (experiment 4B, Table 3). Chromatographic data (number of resolution pairs, total analysis time, individual resolution elements, peak skew) from the experiments performed in the second design, the fine-tuning design, and the additional designs were used to predict the optimum conditions. The predicted separation conditions were almost exactly identical to those for experiment 3C (Table 3 and Fig. 3), and this experiment was therefore considered as optimal for the separation of the DGalDG.

To monitor the robustness of the eluent system, a ruggedness test was performed with experiment 3C as centerpoint and by altering the variables as shown in Table 4. This design was done with independent replicates, and the relative deviation of the retention time, taken over all peaks in the chromatogram, was used as the response criterion. Although experiment 3 in Table 4 had the best response factor, the total analysis time was too extended to be acceptable, and therefore experiment 3C was still considered the best.

The detector settings were investigated with respect to the signal-to-noise (S/N) ratio and magnitude of the response, *via* the three-level factorial design in Table 5. Unfortunately, the S/N ratio increases concomitantly with the detector response, although 1–2 orders of magnitude lower. The optimal detector response and S/N ratio could therefore not be found simultaneously, so the detector response was chosen to be maximized within an acceptable S/N ratio interval (Fig. 4).

Fractions taken of the major molecular species found in DGalDG from oats showed 18:2/18:2 (37.1%), 18:2/16:0 (31.7%), and 18:2/18:1 (12.9%) in abundance. Wheat kernel contains a higher proportion of 18:2/18:2 (68.4%). In fraction 5 of both oats and wheat kernels, small quantities of 18:3 were detected, probably due to the combination 18:3/18:0 coeluting with 18:1/16:0. Other major molecular species were 18:1/16:0, 18:1/18:1, and the 18:2 combination with stearic acid. The 16:0/16:0 molecular species was not detected, probably because of the high preference of palmitic acid to the 18:2 combination. The expected elution order of 16:0/16:0 would be just ahead of the 16:0/18:1 molecular species. The molecular species distribution is listed in Table 6.

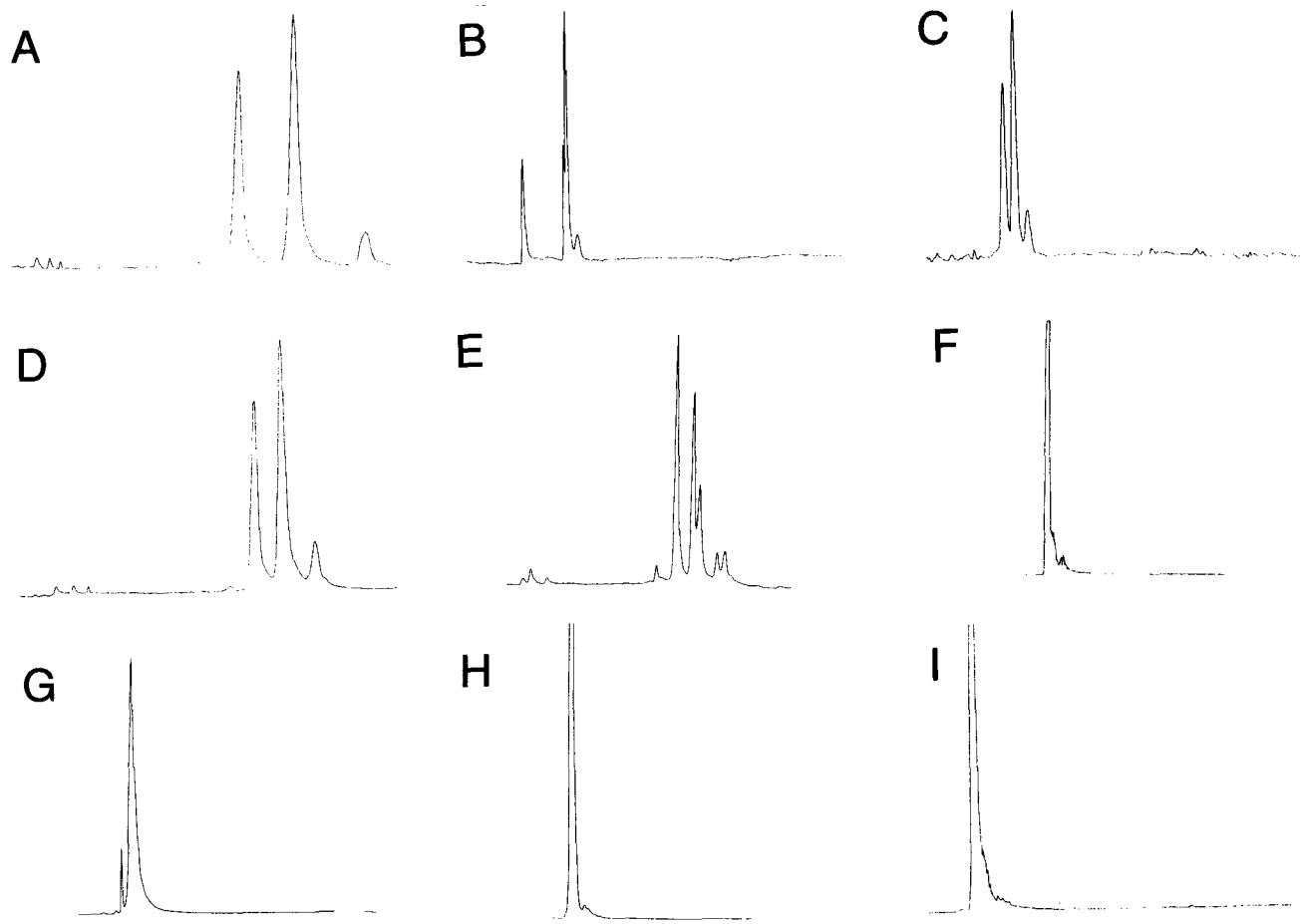

FIG. 1. Reversed-phase high-performance liquid chromatography chromatograms that represent experiments 1A–I in the first design. For chromatographic data, see Table 1. Detection: evaporative light-scattering detection.

In reversed-phase systems, the molecular species elute roughly in the order of increasing partition number (PN). The PN-value can be obtained from the simple relationship PN = CN – 2*n*, where CN is the number of carbons in the aliphatic residues, and *n* is the number of double bonds per molecule (29). Different molecular species with the same PN will elute close to each other, or will even co-elute. If, however, the eluent contains a high proportion of a nitrile component, such as acetonitrile (3,14,30), the molecular species with the highest number of double bonds within a given PN group will elute ahead of the others. This is probably due to higher affinity of the more unsaturated molecular species for the mobile phase. If the molecular species have a polar head group attached to the glycerol, i.e., they are not triacylglycerols or nonpolar derivatives, addition of acetonitrile in the eluent is not needed to achieve separation. The elution order of molecular species with the same PN is then reversed, compared to the acetonitrile eluent systems, with the molecular species with the low-

TABLE 4
Ruggedness Test Design

Experiment number	Acetone (vol%)	Methanol (vol%)	Heptane (vol%)	Water (vol%)	2-Propanol (vol%)	Temperature (°C)	Column brand[a]	Response
1	3.5	26	1	29	40.5	57	Nobel	0.8071
2	5.5	26	1	27	40.5	57	Merck	0.6557
3	3.5	28	1	27	40.5	51	Nobel	0.0680
4	5.5	28	1	29	36.5	51	Merck	1.2460
5	3.5	27.5	1.5	29	38.5	51	Merck	0.2086
6	5.5	27.5	1.5	27	38.5	51	Nobel	0.2271
7	3.5	28	3	27	38.5	57	Merck	0.6814
8	5.5	30	1	29	34.5	57	Nobel	0.3267

[a]Nobel, Bohus, Sweden; and Merck, Darmstadt, Germany.

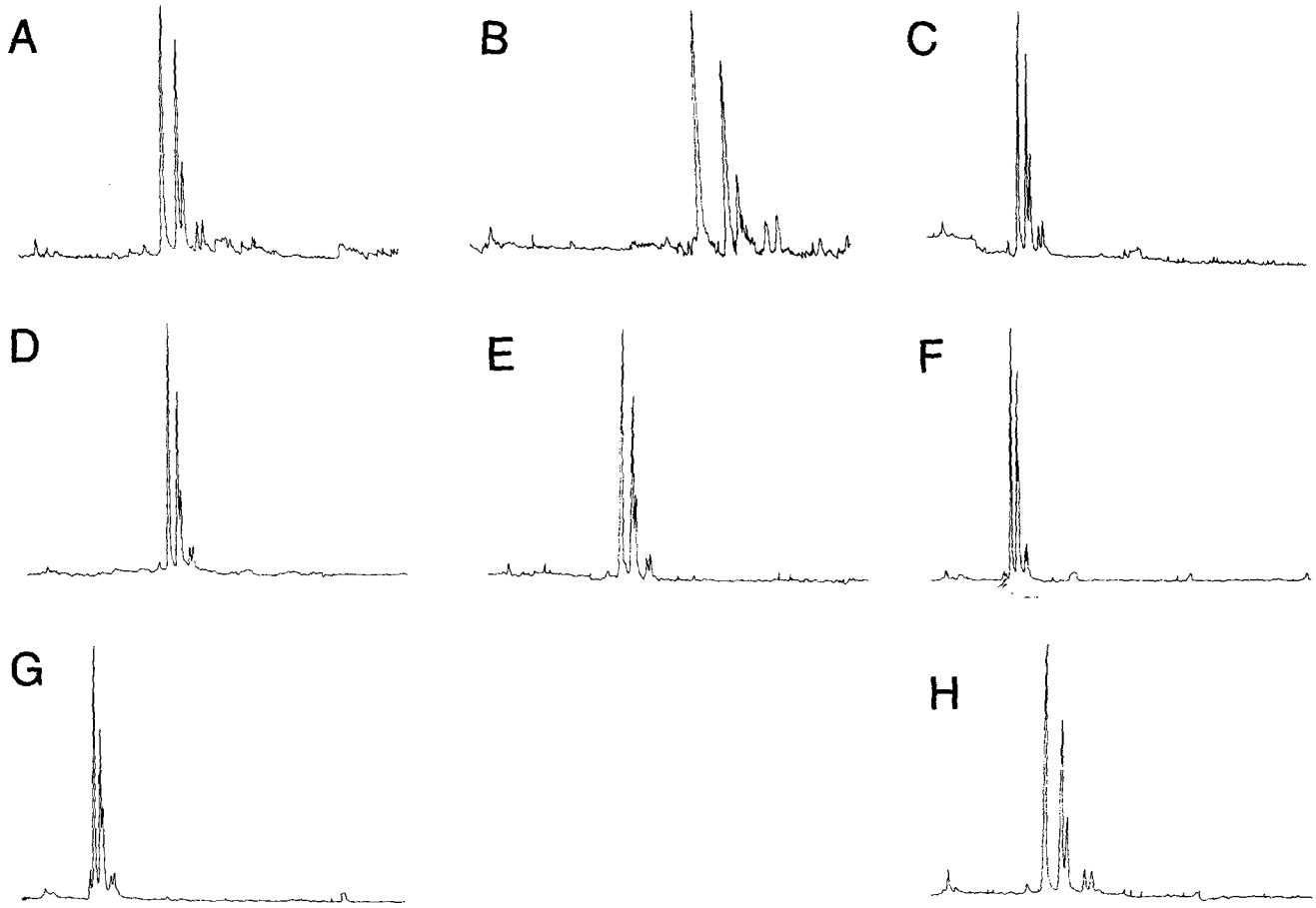

FIG. 2. Reversed-phase high-performance liquid chromatography chromatograms that represent experiments 2A–H in the second design. For chromatographic data, see Table 2. Detection: evaporative light-scattering detection.

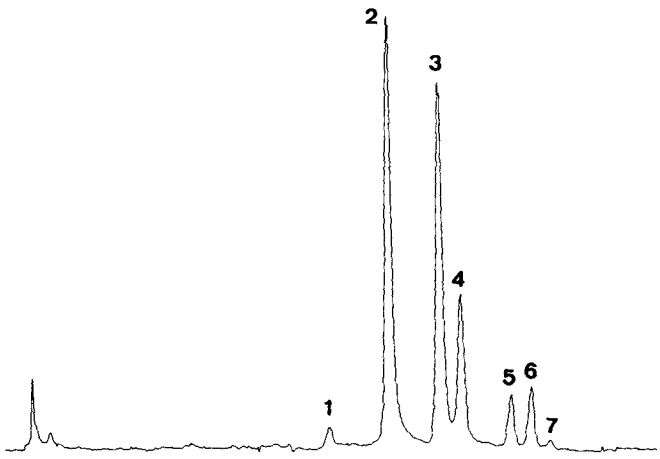

FIG. 3. Reversed-phase high-performance liquid chromatography chromatogram of experiment 3C. Mobile phase: acetone/2-propanol/methanol/heptane/water (5:38:28:2:27) to acetone/2-propanol/methanol/heptane/water (5:47:37:2:9), exponential (2) gradient over 40 min. Detection: evaporative light-scattering detection. Peaks: 1 = 18:3/18:2, 2 = 18:2/18:2, 3 = 18:2/16:0, 4 = 18:2/18:1, 5 = 18:3/18:0 + 18:1/16:0, 6 = 18:1/18:1, and 7 = 18:2/18:0.

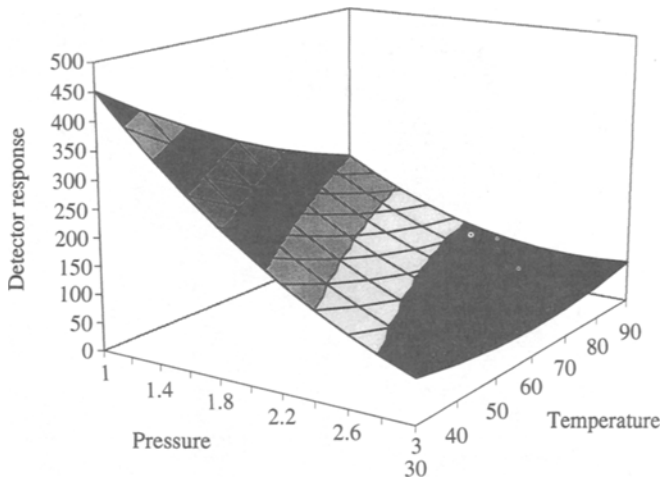

FIG. 4. Response surface plot for detector optimization. Optimum conditions: 35°C and 1.8 bar. For detector parameter settings, see Table 5.

TABLE 5
Detector Optimization Design

Experiment number	Temperature (°C)	Pressure (bar)	Response	Signal/noise
1	35	1	429	47.7
2	60	1	294	28.0
3	85	1	217	22.8
4	35	1.8	244	37.6
5	60	1.8	114	20.7
6	85	1.8	108	9.8
7	35	2.6	126	16.8
8	60	2.6	83	9.2
9	85	2.6	66	5.7

est degree of unsaturation eluting first. The effect might be due to double bond interactions with underivatized silanol groups on the column stationary phase. PN overlap, such as that of 18:3/18:0 (PN 30) and 18:1/16:0 (PN 32), indicates that the separation power of these systems is greater than that of the acetonitrile systems.

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TABLE 6
Fatty Acid Composition of Digalactosyldiacylglycerol from Oat and Wheat

	Fatty acids (wt%)							Molecular species[b]	Area%	PN[c]
	16:0	18:0	18:1[a]	18:2	18:3	24:0	Others			
Oat										
Total[d]	21.6	1.5	23.0	50.6	2.0	0.7	0.6			
Peak 1	3.2	0.4	2.0	54.7	30.4		9.3	18:3/18:2	0.9	26
Peak 2	3.5		2.0	89.4	3.2		1.9	18:2/18:2	37.1	28
Peak 3	45.6		0.7	52.3	0.1		1.3	18:2/16:0	31.7	30
Peak 4	8.1	0.2	41.4	48.1			2.2	18:2/18:1	12.9	30
Peak 5	43.2	0.7	46.6	4.2	0.4		4.9	18:3/18:0+	8.4	30+
								18:1/16:0		32
Peak 6	2.2		88.4	3.4			6.0	18:1/18:1	6.5	32
Peak 7	3.2	32.5	10.2	39.1			15.0	18:2/18:0	1.8	32
Wheat										
Total	14.0	1.0	6.7	73.3	3.8		1.2			
Peak 1	9.8	2.8	3.8	43.4	33.7		6.5	18:3/18:2	1.9	26
Peak 2	1.8	0.1	0.3	94.3	2.7		0.8	18:2/18:2	68.4	28
Peak 3	43.9		1.5	53.0	0.2		1.4	18:2/16:0	23.1	30
Peak 4	8.9		39.4	49.3	0.1		2.3	18:2/18:1	4.7	30
Peak 5	33.7	1.4	37.7	15.3	1.7		10.2	18:3/18:0+	0.8	30+
								18:1/16:0		32
Peak 6	4.4		34.1	11.5			50.0	18:1/18:1	0.2	32
Peak 7	3.4	29.6	2.8	37.2			27.0	18:2/18:0	0.4	32

[a]Both 18:1n-9 and 18:1n-7 isomers.
[b]Fatty acids are given in arbitrary order.
[c]PN, partition number.
[d]Values in area%; discrepancies are noted between the observed amounts of fatty acids by gas chromatography and the calculated amounts from the molecular species profile, probably due to different modes of detection.

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