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High-performance liquid chromatographic analysis of wheat flour lipids using an evaporative light scattering detector

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ABSTRACT

A high-performance liquid chromatographic method which utilized an evaporative light scattering detector for separation of starch and non-starch lipids of unbleached soft red winter wheat flour is described. Separation of the major starch and non-starch lipids was achieved in 60 min using a Lichrosorb Si-60 silica cartridge system and ternary gradient system. The evaporative light scattering detector gave a flat stable baseline, reproducible results and also eliminated the "solvent fronts" in which peaks of interest would have co-eluted. The technique was found useful in identifying the lipids and their relative mass percentages that were present in the flour.

INTRODUCTION

The analysis of cereal lipids is a complex and detailed procedure which is time consuming and in many cases insensitive as well as non-reproducible. In the past thin-layer chromatography (TLC) and quantification for fatty acid methyl esters by gas chromatography (GC) analysis have been the methods of choice for detection of non-starch and starch lipids in wheat flour [1,2]. The introduction of evaporative light scattering detection (ELSD) by Christie [3] to separate lipid classes in animal tissues by high-performance liquid chromatography (HPLC) demonstrated that this type of detector is very useful for analyzing lipids. ELSD gives a very stable baseline and is insensitive to solvent changes and gradients. One of the difficulties encountered with the evaporative light scattering detector is that the detector response is linear in the range of 10–200 μg and drops off drastically below 10 μg making it difficult to quantify lipid classes present in this lower range. With proper calibration curves, consistent instrument set up, and sufficient sample size, direct quantification is possible [4,5]. Only a few applications of HPLC of lipids in cereals have been reported [6,7]. Christie and Morrison [7] reported a method using HPLC to separate polar lipid classes in cereal grains using an evaporative light scattering detector. The method failed to separate all of the lipid classes present in the flour and some difficulty was encountered with the separation of the glycolipids and less polar phospholipids. In addition, individual simple lipids were not resolved and emerged together at the start of the analysis when a complex lipid extract from wheat flour was analyzed. This made it necessary to extract out the glycolipid fraction prior to HPLC analysis. Moreau [8] introduced a method which separated the major classes of plant lipids from corn coleoptiles by HPLC using ELSD adapted from a procedure which originally used flame ionization as a means for detection [9]. Flame ionization detection has also been reported elsewhere for detection of phospholipids [10–12].

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Since flame ionization detectors are no longer commercially available, ELSD has presently become the detection method used most often in separation of lipids by HPLC [5]. It seemed possible that the method described by Moreau [8] could be adapted to separate the major plant lipid classes in cereal grains. The objectives of this research were: (1) to develop an HPLC procedure using ELSD which would successfully separate all the lipid classes present in soft wheat flour without prior fractionation of the individual glycolipids and phospholipids, (2) to identify each lipid class present, and (3) to acquire a relative mass percent of each lipid present.

EXPERIMENTAL

Extraction

Lipids were extracted from unbleached soft red winter wheat flour with water-saturated 1-butanol (WSB) [1,2]. The flour sample contained 1.5% lipid by soxhlet determination for percent fat [13]. Non-starch lipids were extracted from 3 g of flour with 30 ml of solvent at 20°C in a 50-ml teflon tube for 15 min mixed at 5-min intervals, centrifuged at 5900 *g* for 15 min and the supernatant transferred to a 250-ml evaporator flask. The solvent was then removed using a rotary evaporator under nitrogen at 60°C. After complete removal of all the water and solvent the residue was redissolved in 700 μl of chloroform–methanol (2:1). The flask was rinsed down several times and the residue was filtered through a 0.45-μm PTFE filter prior to HPLC analysis. The non-starch extract was then placed in a vial, loaded in the autosampler, and 12 μl injected. The flour pellet was re-extracted three times with WSB to remove the interstitial lipid solution, and these extracts were discarded before proceeding with the extraction of the starch lipids.

Starch lipids were removed by adding 16 ml of WSB to the washed pellet, heated in a boiling water bath (90–100°C), changing the solvent every hour for 3 hours (three extractions) and the combined hot extracts were taken for analysis. The extracts were centrifuged and the solvent was removed with a rotary evaporator. The residue was redissolved in 700 μl of chloroform–methanol (2:1), and filtered as described above. An aliquot of 15 μl was injected into the HPLC system.

Materials and reagents

Pure reference standards of monogalactosyldiglyceride, digalactosyldiglyceride, lysophosphatidylcholine, lysophosphatidylethanolamine, sterylglucoside, and acylated sterylglucoside were purchased from Matreya (Pleasant Gap, PA, USA). All other standards were purchased from Sigma (St. Louis, MO, USA). Standards were stored frozen in the dark and made up fresh daily to contain 50–100 μg of each lipid class standard in chloroform–methanol (2:1). PTFE tubes (50 μl) were used in the extraction process and HPLC-grade solvents were purchased from Fisher Scientific (Raleigh, NC, USA).

Chromatographic conditions

Lipid extracts were separated on a 100 × 3 mm Chromsep 7 Micron Lichrosorb Si-60 Silica Cartridge System (Chrompack, Raritan, NJ, USA). The guard column was integrated in the Chromsep cartridge holder. The gradient system in Table I was adopted as optimal for separation of the major lipid classes.

TABLE I

TERNARY GRADIENT SYSTEM FOR LIPID CLASS SEPARATION

Time (min)	Flow-rate (ml/min)	Composition of mobile phase[a]		
		% A	% B	% C
0	0.5	100	0	0
5	0.5	95	5	0
10	0.5	85	15	0
15	0.5	40	60	0
33	0.5	40	51	9
48	0.5	40	51	9
53	0.5	40	60	0
58	0.5	100	0	0
80	0.5	100	0	0

[a] A = Hexane–tetrahydrofuran (99:1), B = isopropanol, C = water.

All solvents were degassed and filtered prior to analysis. Helium was used as a sparge gas at 20 ml/min during the analysis.

Instrumentation

The HPLC system consisted of a Waters 600E controller with dual pumps, Waters 700E Wisp auto-sampler (Waters Assoc., Milford, MA, USA), and NEC Powermate Data acquisition controller with analysis software. The ELSD apparatus was obtained from Varex (Burtonville, MD, USA). The drift tube temperature was set at 60°C (40°C exhaust temperature). Nitrogen was used as the nebulizing gas at a flow of 45 mm (10 psi).

Calibration curves

Solutions of known concentrations of PE, LPE, LPC, MGDG, and DGDG (for abbreviations see Table II) were analyzed by the described HPLC procedure. Calibration curves were prepared using 10–200 μg of each standard by plotting the concentration *versus* the peak area response of the ELSD.

Statistical analysis

Data were analyzed by the statistical analysis system [14]. Results were reported as the means of three observations and standard deviation for each non-starch and starch lipid class present in the sample. Linear regression was used to determine correlation coefficients (r) for each calibration curve of the individual standards.

RESULTS

Solvent systems such as chloroform–methanol, 2-propanol, and water-saturated 1-butanol were tested for the extraction of both the non-starch and starch lipids. Chloroform–methanol and 2-propanol did not yield consistent results from extraction to extraction. Water saturated 1-butanol demonstrated to be the best solvent in producing the most consistent results with complete extraction. It has been previously cited that

TABLE II

RELATIVE MASS PERCENTS FOR EACH LIPID CLASS

Each value is the mean of three observations ± standard deviation.

Peak No.	Lipid class	Abbrev.	Non-starch	Starch
1	Steryl ester + triglycerides	SE + TG	11.5 ± 0.2	4.7 ± 0.6
2	Triglycerides	TG	12.2 ± 0.6	0.4 ± 0.0
3	Free sterol	ST	4.1 ± 0.2	ND[a]
4	Free fatty acid	FFA	31.7 ± 0.8	6.0 ± 0.3
5	Unknown	UNK	3.5 ± 0.3	ND
6	Acylated sterylglycoside	ASG	2.5 ± 0.2	ND
7	Monogalactosyldiglyceride	MGDG	6.4 ± 0.4	ND
8	Monogalactosylmonoglyceride	MGMG	3.4 ± 0.3	ND
9	Sterylglycoside	SG	1.8 ± 0.1	ND
10	Digalactosyldiglyceride	DGDG	11.7 ± 0.8	ND
11	N-Acylphosphatidylethanolamine	NAPE	1.8 ± 0.1	ND
12	N-Acyllysophosphatidylethanolamine	NALPE	0.1 ± 0.0	ND
13	Phosphatidylethanolamine	PE	3.5 ± 0.1	ND
14	Phosphatidylglycerol	PG	2.0 ± 0.0	ND
15	Lysophosphatidylethanolamine	LPE	0.8 ± 0.0	10.2 ± 0.5
16	Phosphatidylcholine	PC	0.7 ± 0.1	1.0 ± 0.1
17	Lysophosphatidylglycerol	LPG	0.0	0.1 ± 0.0
18	Lysophosphatidylcholine	LPC	2.3 ± 0.2	77.6 ± 0.6

[a] ND = None detected.

water-saturated 1-butanol is generally considered the best solvent for extraction of wheat lipids [15].

The extracted residue was redissolved in chloroform–methanol (2:1) since the digalactosyldiglyceride was not soluble in such solvents as hexane–chloroform (1:1) or chloroform alone. The digalactosyldiglyceride peak was either very broad or sometimes not present when either hexane–chloroform (1:1) or chloroform were used. Lipid extracts when redissolved in chloroform–methanol (2:1) resulted in sharp, well resolved peaks.

Different duration times of the ternary gradients were attempted to shorten the HPLC run. The results indicated variation in retention times between each analysis. With strict adherence to the conditions described in Table I these problems were eliminated.

Standards were injected individually as well as in a mixture to determine retention times and resolution of peaks. Retention times did not vary after 200 injections, nor was there any increase in backpressure. Loss of resolution occurred most often due to fluctuations in backpressure from dirty pump seals or the necessity to clean the nebulizer and drift tube on the detector.

The non-starch lipid fraction consisted of seventeen major peaks (Fig. 1) when analyzed by HPLC–ELSD. The exhaust and tube temperatures were kept at a minimum to prevent the free fatty acid peak from being volatilized [16]. The non-starch lipids (Table II) included: (SE + TG), TG, ST, FFA, UNK, ASG, MGDG, MGMG, SG, DGDG, NAPE, NALPE, PE, PG, LPE, PC, and LPC. The identity of each peak was confirmed from the retention time of each corresponding lipid class standard and the enhancing technique of the lipid class peak. Data was in agreement with previous research [15,17] on the composition of lipids in wheat flour. Research has demonstrated that samples dissolved in polar solvents would cause the triacylglycerols to sometimes elute as a double peak with a portion of the TG co-eluting with SE [3]. Since it was found essential to dissolve the non-starch and starch residues in a polar solvent such as chloroform–methanol (2:1) it may be concluded that the first peak in the chromatographs contain both SE and TG.

Seven major peaks were resolved when the starch lipids were separated (Fig. 2). They included (Table II): SE + TG, TG, FFA, LPE, PC, LPG and LPC. Research [17,18] has shown that the starch lipid of wheat flour contains approximately 6–10% FFA, 77% LPC, 10% LPE and very little or no triglycerides, diacyl lipids and monoacyl lipids. The starch lipids shown in Fig. 2 were extracted with hot WSB

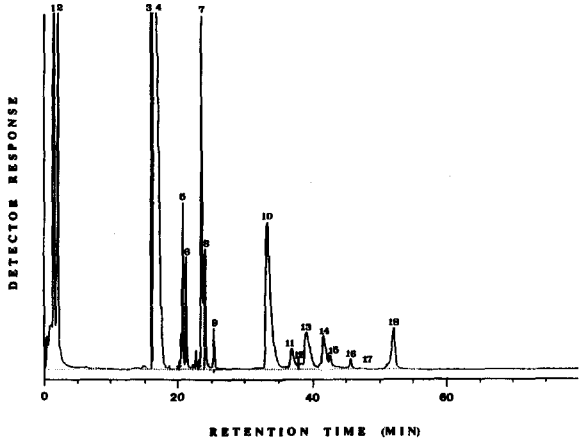

Fig. 1. Separation of non-starch lipids in soft wheat flour on a Lichrosorb Si 60 silica column by HPLC–ELSD. See text for conditions. See Table II for identification of each numbered peak.

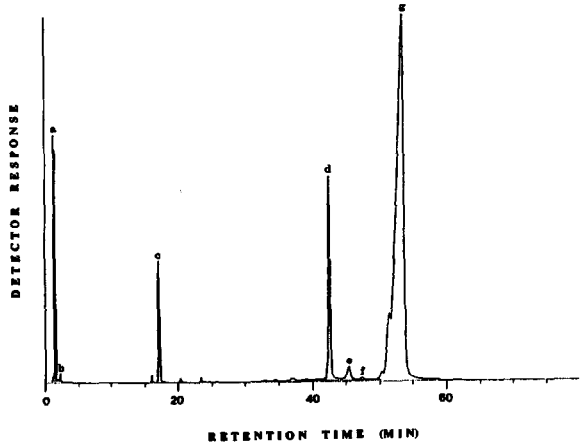

Fig. 2. Separation of starch lipids from soft red winter wheat flour on a Lichrosorb Si 60 silica column by HPLC–ELSD. (a) SE + TG; (b) TG; (c) FFA; (d) LPE; (e) PC; (f) LPG and (g) LPC.

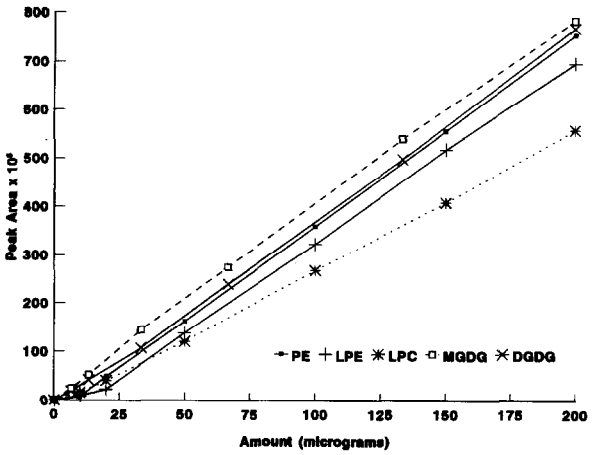

Fig. 3. Calibration curves for phospholipids and glycolipids by HPLC with an evaporative light scattering detector.

after first removing the non-starch lipids. The starch lipids may contain some residual non-starch lipids since it is not protein-free starch. This may explain the presence of the first peak (SE + TG) and the high percentage of the FFA. Extensive research has concluded that the FFA and other monoacyl lipids are non-starch lipids absorbed into the starch lipid granules, therefore, forming surface lipid artifacts [17].

Calibration curves of PE, LPE, LPC, MGDG, and DGDG are shown in (Fig. 3). Linear relationships ($r > 0.99$) between peak area in integrator counts and amounts of phospholipids and glycolipids were found in the 10–200 μg range. The sigmoidal relationship holds and tails off for component amounts below 10 μg [3]. The response of the glycolipids resembles those for phospholipids.

DISCUSSION

The proposed HPLC–ELSD method for separation of non-starch and starch lipids in wheat flour was found to have the following advantages over conventional methods: better resolution and efficiency, a stable baseline, high sensitivity, detection limits in the 10–200 μg range, and good reproducibility. This HPLC–ELSD method was also adaptable for direct analysis of both polar and non-polar lipids in wheat flour without prior fractionation of the glycolipids. The column cartridge system is fairly inexpensive and easily replaced. Once lipid extraction was achieved with the aid of an autosampler and data handling station the completion of the analysis was automated.

The 22-min re-equilibration between the end of one gradient and the injection of the next sample is crucial to eliminate variations in the retention times and adequate separation of peaks. Use of a ternary gradient clearly resolved the major peaks in both the non-starch and starch extracts.

The lower tube and exhaust temperature allowed detection of the free fatty acids without volatilization. Cleaning of the pump seals, detector nebulizer and drift tube were essential for good reproducibility of day to day runs.

In order to use this HPLC method for quantification it is necessary to prepare different calibration curves for each component in the sample. It is also essential to set up the detector parameters as well as the elution profile exactly the same way each day to maintain reproducibility.

Although it has been reported that starch lipids can be extracted from flour after first removing the non-starch lipids in practice it is best to extract them from pure protein-free washed starch to avoid contamination with any residual non-starch lipids in the flour protein [15].

The lipid data presented in Table II is a representation of only relative mass percents. The possibility of using this application for quantification of the lipid classes present in wheat flour is presently being explored.

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