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Analytical Methods Section

Two simplified approaches to the analysis of cereal lipids

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Two simple methods were adapted for analyzing complex cereal lipids. Direct separation of lipids extracted from quinoa seed was performed with the complete separation of components within lipid classes. Different solvent mixtures were used for the sequential development of neutral, galacto lipid and polar lipid components. Utilization of flame ionization detector with TLC permitted quantification of lipid components at levels of total lipids as low as a few micrograms. Optimization of lipid classes separation was achieved with aminopropyl silica with pure fractions analyzed for fatty acids. Lipid separation by solid phase extraction produced a sufficient amount of fractions for quantitative analysis of lipid components and for fatty acid evaluation.

INTRODUCTION

Accurate quantification of cereal lipids is difficult and laborious because of their complexity. In addition, some components may be inaccessible to normal lipid extraction procedures. Usually up to 20 different lipid components are separated from cereal grains and this represents a major challenge to cereal chemists (Morrison *et al.*, 1980). Cereal lipids are generally analyzed by thin-layer chromatography (TLC) using charring and densitometry as the detection and quantification method, respectively (Opute, 1979; Osagie & Kates, 1984). TLC is time- and solvent-intense and lacks accuracy and sensitivity (Geiss, 1987; Morrison, 1988). A combination of TLC with gas chromatography (TLC–GC) has been used for quantification of lipids classes by utilizing multiplication factors to convert total fatty acids into their lipid classes content. TLC–GC is time-consuming, involving multi-dimensional development for TLC in order with accuracy to separate analyzed components requiring a skilled operator (Morrison *et al.*, 1980; Morrison, 1988). A further complication is that separation by thin-layer chromatography with silica gel is highly dependent on moisture so that laboratories without proper humidity control can only perform analysis with this method for part of the year (Geiss, 1987).

This paper describes two methods for the separation and quantification of cereal lipid classes. The first is a direct, rapid and accurate procedure utilizing thin-layer chromatography with flame ionization detector (TLC–FID; Iatroscan). The second method described is a simple and efficient procedure for isolating individual lipid classes based on solid phase extraction (SPE) using aminopropyl silica from which fractions can be used for further fatty acid characterization by GC.

MATERIALS AND METHODS

Materials

Solvents used in this study were of chromatographic purity and purchased from Burdick and Jackson Laboratories (McGaw, IL). Lipid standards stearyl palmitate (WE), cholesterol oleate (STE), triolein (TG), diolein (DG), monoolein (MG), oleic acid (FFA), campestrol (ST), monogalactosyl diglyceride (MGDG), digalactosyl diglyceride (DGDG), phosphatidic acid (PA), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), phosphatidyl inositol (PI), lysophosphatidyl ethanolamine (LPE), phosphatidyl choline (PC) and lysophosphatidyl choline (LPC) were all obtained from Sigma Chemicals (St Louis, MO) and Serdary (London, Ontario). Syringe cartridges packed with 500 mg of aminopropyl packing were purchased from Waters (Mississauga, Ontario). Quinoa seeds (*Chenopodium quinoa* Willd.) were provided by Mr H. Hrubniek (Rossburn, Manitoba). Chromarods SIII with silica gel used for separation were purchased from Scientific Products and Equipment (Concord, Ontario).

Lipids extraction

Quinoa seed kernels were ground in a hammer mill and the lipids extracted by modification of the Bligh and

Dyer method (De la Roche *et al.*, 1973). Briefly, a 10 g portion of seeds was extracted twice with 40 ml of boiling isopropanol and twice with 40 ml of chloroform : methanol : water (1 : 2 : 0·8 v/v). To the chloroform : methanol extracts 20 ml of both chloroform and water were added and the bottom layer recovered. The isopropanol and chloroform extracts were combined and 20 ml of benzene added to remove water. Samples were evaporated to dryness on a rotary evaporator under a nitrogen blanket. The dry residue was dissolved in a known volume of chloroform : isopropanol (10 : 1, v/v). All lipid extracts were stored under nitrogen at −30°C and analyses performed within a few days.

Thin layer chromatography

Lipids were separated on a sintered silica gel (SSG) on quartz rods and detected with a flame ionization detector (FID) (Iatroscan Mark IV, Iatron, Japan). Three successive solvent mixtures (v/v)—(1) dichloroethane : chloroform : acetone : acetic acid (70 : 12 : 1·7 : 0·4); (2) acetone : acetic acid : water (70 : 1·2 : 1·5) and (3) chloroform : methanol : water : acetic acid (50 : 27 : 3·0 : 0·5)— were used to separate neutral lipids (NL), galactolipids (GL) and polar lipids (PL), respectively. The total amount of each extract applied was less than 10 μg of total lipids per chromarod. Each sample was applied in small portions to keep the band as narrow as possible and to prevent fractions from spreading during development. After development, chromarods with frame were placed in an oven at 120°C for 5 min to remove solvents. Prior to use, chromarods were activated by passing them through detector flame twice at the beginning of a day, and once after each sample run. When an excessive detector signal background was observed, rods were soaked in 6 N nitric acid overnight to clean them. Each chromarod was placed in a separate, specially designed test tube to prevent mechanical damage during cleaning (Ackman, 1981). On the following day they were washed with distilled water until a neutral pH was obtained, dried in an oven at 120°C for 1 h and further activated as described previously. Calibration curves were obtained using the standard mixtures which underwent the identical full separation procedure (Przybylski & Eskin, 1991).

Solid phase extraction

Lipid extracts were separated into classes using solid phase extraction as described by Kaluzny *et al.* (1985), with the following modifications. Up to 20 mg of total lipids was applied in chloroform or chloroform : isopropanol (10 : 1, v/v) on to an aminopropyl cartridge, and sequentially eluted using the three solvents described in Table 1, method A. The neutral lipid fraction was evaporated to dryness and three 1 ml portions of hexane were added with evaporation to dryness after each addition. The residue was then dissolved in hexane and applied to a new cartridge. Separation of the neutral lipids into fractions was performed with the solvent mixture sequence described in Table 1, method B. In both separations a optimal flow rate of 2–3 ml/min was maintained by using pressure from compressed nitrogen. The recovery and performance of the separations were determined using polar and non-polar lipids standard mixtures, at concentrations ranging over 1–10 mg/ml.

RESULTS AND DISCUSSION

Lipids separation

Lipids extracted from quinoa seeds were separated by TLC with sintered silica gel combined with flame ionization detector. The authors' studies indicate that isopropanol can replace methanol for lipid extraction with the same extraction efficiency and appropriate separation (data not presented). The characteristics of the SSG used in the FID detection system are quite different from the same materials used on TLC plates. The solvent mixtures used for lipid separation with one technique cannot be directly transferred to the other method (Ranny, 1987). Solvent mixtures established for separation of the different lipid classes are described in the materials and methods section. A method of triple successive solvent developments was established in the authors' laboratory for separating individual lipids within each lipid class (Fig. 1). Samples were spotted once on the chromarods and sequentially developed using solvent mixtures to separate individual compo-

Table 1. Fraction isolation from quinoa seed lipids by solid phase extraction with aminopropyl silica

Method	Solvent mixture (v/v)[a]	Volume (ml)[b]	Components eluted
	Hexane	6	Column activation
A	Chloroform : 2-propanol (2 : 1)	6–11–14	Neutral lipids
A	Diethyl ether : acetic acid (95 : 5)	8–11–14	Free fatty acids
A	Methanol	10–13–16	Phospholipids
B	Hexane	6–9–12	Waxes, sterol esters
B	Hexane : methylene chloride : ethyl ether (89 : 10 : 1)	8–14–20	Triglycerides
B	Hexane : ethyl acetate (95 : 5)	6–9–12	Sterols
B	Hexane : ethyl acetate (85 : 15)	8–12–16	Diglycerides
B	Chloroform : 2-propanol (2 : 1)	8–12–16	Monoglycerides

[a] Solvent mixture in volume units.
[b] Volume of solvent mixture is component amount dependent.
A, Separation of lipids on main classes; B, separation of neutral lipids fraction.

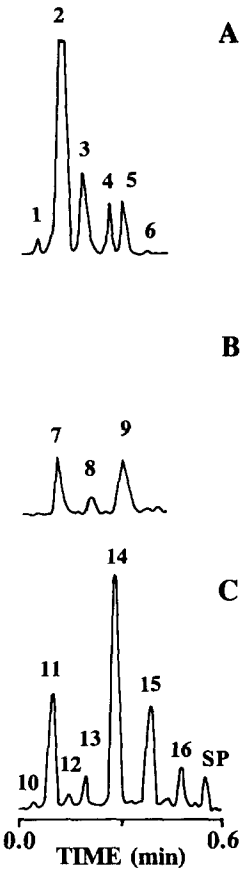

Fig. 1. TLC–FID chromatograms of quinoa lipids separation. (A) Neutral lipids; 1, wax esters (WE) + esterified sterols (STE); 2, triglycerides (TG); 3, free fatty acids (FFA); 4, 1,3 diglycerides (1,3DG); 5, 1,2 diglycerides (1,2DG); 6, free sterols (ST). (B) Monoglycerides and galactolipids: 7, monoglycerides (MG); 8, monogalactosyl diglycerides (MGDG); 9, digalactosyl diglycerides (DGDG). (C) Phospholipids: 10, phosphatidic acid (PA); 11, phosphatidyl ethanolamine (PE); 12, phosphatidyl serine (PS); 13, phosphatidyl inositol (PI); 14, lysophosphatidyl ethanolamine (LPE); 15, phosphatidyl choline (PC); 16, lysophosphatidyl choline (LPC); SP, sample application area.

nents based on the differences in polarity of the compounds. Solvent mixture 1 was used to separate NL with the chromarods partially run through the detector to identify and quantify the neutral lipid components (Fig. 1(A)). The run through the detector was stopped after the sterol peak was completed. The part of the rod where neutral lipids were separated was reactivated without affecting the more polar lipid components which were still present in the original spotting area (Fig. 1(C), SP). A second development was carried out with solvent mixture 2 to separate the galactolipids and monoglycerides which were then identified and quantified with FID as described above (Fig. 1(B)). After reactivation of the chromarods, the phospholipids were then separated with solvent mixture 3 (Fig. 1(C)). Baseline separation was achieved for almost all components within neutral, glyco- and phospholipid classes. Waxes and sterol esters were not separated due to their similar polarity. Using the chromarod system, multi-samples can be run simultaneously as one set of chromarods in a frame contains 10 individual rods. With these separations calibration and sample separation can be performed concurrently. Eight samples and two concentrations of the standard mixtures were established as the optimal set-up for this procedure. Carrying out both calibration and sample runs at the same time eliminated the effect of slight changes in parameters between runs (Geiss, 1987).

Using the sequential development technique described, only 5–10 μg of total lipids was needed to analyze fully the sample (Przybylski & Eskin, 1991). A particular advantage of TLC on SSG is the ability to perform multiple separations of a single sample on the same rod (Ackman, 1981). The beauty of this method is that when components are detected, the rods are simultaneously cleaned and activated when passed through the flame detector. A further advantage is that SSG is not sensitive to moisture as are TLC plates so that separation using this procedure can be performed throughout the year in laboratories lacking humidity control (Ranny, 1987). The flame ionization detector has particularly high sensitivity and can be used with virtually all organic compounds. The response of this detector is dependent on the number of carbon atoms present in the molecules and is only slightly affected by presence of heteroatoms such as oxygen or nitrogen (Ackman, 1981; Ranny, 1987). From the present authors' experience the detection limit for lipid compounds was in the range of a few dozen nanograms including proper integration of peak area at a signal to noise ratio of 5:1.

Solid phase extraction was adopted to simplify thin-layer and column chromatographic procedures which are often used for separating and quantifying cereal lipids. Column chromatography is often used to quantify lipid classes using gravimetric or densitometric methods of detection. Because gravimetric detection lacks accuracy, it is unsuitable when very small amounts of lipids are analyzed. Additionally, column chromatography requires large amount of solvents to be used for separation. Charring with cupric acetate after TLC separation is not reproducible, even if a densitometer is utilized, because different lipids can produce black or grey spots with unequal intensity for the same concentration (Geiss, 1987; Morrison, 1988). Alternatively a combination of TLC–GC is used to quantify individual lipids content. After TLC the separated spots are isolated and used for characterization of fatty acids composition by GC. Lipid component contents are then quantified indirectly from fatty acids using multiplication factors based on the molecular structure of the lipid compounds (Morrison *et al.*, 1980). TLC separation with silica gel is very sensitive to changes in humidity which alters silica gel activity. On thin-layer plates, large areas of gel are directly exposed to air moisture. It is not always easy to control humidity in a typical laboratory, and this can cause significant differences in separation or even prevent separation from occurring at all (Geiss, 1987). Solid phase extraction, on the other hand, using bonded silica packing, is unaffected by humidity, is very fast and requires only

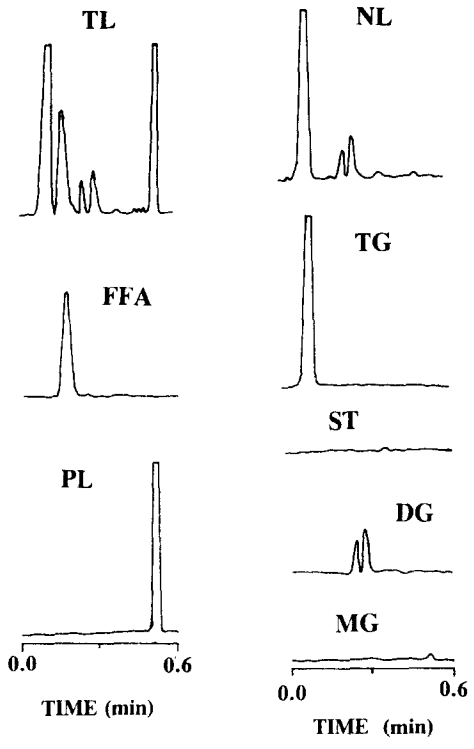

Fig. 2. TLC–FID chromatograms of quinoa lipids fractions obtained from solid phase extraction (SPE) separation.

small volumes of solvents to perform full separation (Table 1). The total capacity of the SPE packing, as specified by the manufacturers, is about 5% of it weight. Consequently, the load of 500 mg of packing in a syringe cartridge can tolerate approximately 25 mg of lipids. However, standards applied in amounts as high as 35 mg were separated by this packing load without affecting the efficiency of fractionation (Fig. 2). Using the SPE procedure, the maximum load of cereal lipid extracts used had to be reduced by 30–50% to prevent overloading, due largely to the presence of other components extracted along with the lipids (Christie, 1986). As little as 10 mg of sample was sufficient to perform effectively in duplication the fatty acid analysis by capillary GC, purity of the fractions and lipid components quantification by TLC–FID. The amount of quinoa lipid extract used for separation was lower than maximal load for the aminopropyl cartridge to prevent overloading with components which include those of non-lipids which are extracted with solvents. It was possible to use this lower amount because of the very high sensitivity of the FID used in both GC and Iatroscan. As discussed above, only nanograms of lipids were required to detect and quantify lipid content by TLC–FID. Both methods, GC and Iatroscan, required only a limited amount of samples because chromarods and capillary column have restricted capacity to perform efficient separations.

Efficiency of SPE separation was examined using mixtures of standards containing non-polar and polar lipids directly as pure mixtures and as part of the quinoa lipid extract. A recovery of over 90% was achieved for most of the standards analyzed, as shown

Table 2. Recovery of lipids components during separation on classes with solid phase extraction method (% ± SD)[a]

Lipid component	Standards[b]	Extract[c]
Stearyl palmitate	98·2 ± 2·1	96·2 ± 3·2
Triolein	99·4 ± 2·2	98·6 ± 2·6
Diolein	97·8 ± 3·2	94·8 ± 2·7
Monostearin	96·4 ± 2·7	92·7 ± 3·4
Oleic acid	95·2 ± 2·7	90·9 ± 3·7
Campestrol	90·9 ± 3·1	94·7 ± 2·8
Monogalactosyl diglyceride	92·2 ± 2·6	91·6 ± 3·6
Phosphatidyl choline	95·4 ± 2·5	90·8 ± 3·9
Phosphatidic acid	94·4 ± 3·7	88·4 ± 3·3
Phosphatidyl inositol	92·2 ± 3·3	90·4 ± 3·6
Phosphatidyl ethanolamine	90·6 ± 3·7	88·5 ± 3·8
Lysophosphatidyl ethanolamine	97·3 ± 2·9	89·8 ± 4·1
Lysophosphatidyl choline	96·3 ± 3·7	90·3 ± 4·5

[a] All results are averages from each three runs at concentrations of 1 and 10 mg/ml.
[b] Mixture of standard used.
[c] Standard mixture was added into lipid extract from quinoa seeds.

in Table 2. Reproducibility was good, as evident by the very low standard deviation (Table 2). Even in the presence of quinoa lipid extract, the added standards showed excellent recoveries (Table 2). The presence of other components in the quinoa extract did not influence either recovery, separation or reproducibility (Table 2).

Neutral lipids as a group were further separated on a second cartridge (Table 1, method B) and had to be dissolved in pure hexane to prevent the effect of even traces of isopropanol. Traces of ethanol, used as chloroform stabilizer, must also be removed as it can significantly affect separation of neutral lipids. This was particularly important as NL separation is based on a very fine tuning of strength and polarity of the applied solvents so that even small differences are critical (Kaluzny *et al.*, 1985). Consequently, it is important to check the purity of the isolated fractions each time. The major limitation in lipid detection is the lack of a suitable chromophore group which will allow a non-destructive detector such as a spectrophotometer to be used. Utilization of the highly sensitive and universal FID is irreplaceable, even though this detector is destructive. The fractions obtained by SPE separation contained a few milligrams of lipids but using TLC–FID technique quantification of components and purity checking of the fractions can still be easily accomplished. After this analysis was performed approximately 80–90% of the sample still remained and fatty acid characterization by GC was easily carried out. Flow rate and volume of solvents used significantly affect separation and purity of fractions obtained from SPE. It is necessary to perform preliminary runs to develop elution protocol, whereas utilization of TLC–FID can simplify this approach. Separation of neutral lipids from a sample is sufficient to predict required volume of solvents for lipid isolation. The area percentage of lipid components, as evaluated by TLC–FID, can be used to determine the volume of solvent needed

for complete elution of a particular fraction. As presented in Table 1, a high solvent volume should be used when a component's contribution was 40% and over, medium volume when contribution was between 20 and 40%, and a low volume of solvent when the contribution was below 20%. This information is very useful and can save a considerable amount of time normally spent to adapt the procedure for a particular sample. In order to obtain a proper and reproducible separation, the flow rate of the solvent through the cartridge must be strictly controlled within the range of 2–3 ml/min. While a faster separation can be achieved at a higher flow rate, the possibility of contamination is much greater. The SPE system behaves exactly as an HPLC column so that even if the solvent is optimally chosen, the movement of other components can also occur. Increasing the flow rate above 3 ml/min causes irreproducible separation and limited purity of the fractions.

In conclusion, the methodology described for measuring cereal lipids is simple and accurate. These methods dramatically reduce the amount of sample and solvents required, from mg to μg and from several hundreds to a few milliliters, respectively. The time for separation is also significantly reduced as it is possible to perform full separation with SPE in less than 1 h compared to up to 1 day with column chromatography. This approach has been successfully applied in a number of studies on different plant lipids including the analysis of cow cockle and quinoa lipids (Mazza *et al.*, 1992; Biliaderis *et al.*, 1993; Przybylski *et al.*, 1994)

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