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⁂Analysis of Lipid Classes and Lipofuscin Substances by High Performance Liquid Chromatography

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ABSTRACT

The fractionation and analysis of the lipid classes and fluorescent substances of animal tissues by high performance liquid chromatography (HPLC) using a combination of fluorescence and flame ionization detectors is described. The lipid classes and fluorescent substances are extracted from rat kidney and liver tissue by a new method that involves preextraction of nonlipid and aqueous-soluble fluorescent substances with hot dilute (0.05 N) acetic acid. The lipid classes and organic-soluble fluorescent substances are extracted from the residual tissue in three extractions: the first with chloroform/methanol, 1:1, v/v; the second with chloroform/methanol, 1:2, v/v; and the third with methanol. The fractionation of these compounds by HPLC is carried out with a column 0.2 × 45 cm, packed with a special adsorbent prepared by reacting silicic acid with ammonium hydroxide. The eluent is passed through a fluorescence detector, which provides a profile of the fluorescent compounds, and then to a flame ionization detector for analysis of the lipid classes. The method is demonstrated on rat blood serum, liver and kidney tissue.

INTRODUCTION

The lipid classes are generally analyzed by thin layer chromatography (TLC), either directly by charring and photodensitometry (1-5) or by application of conventional analytical methods to compounds recovered from chromatoplates (6-9). The quantification of lipofuscin substances is based on spectrophotofluorometric analysis applied directly to tissue extracts (10), after fractionation by silicic acid through column chromatography (11), Sephadex chromatography (12) or TLC (13). Although these methods are used widely for the analysis of lipofuscin substances and lipid classes, the fact that they are applied in many ramifications atests to the need for simple, more precise procedures. In this paper we report the application of HPLC to the analysis of lipid classes and fluorescent substances in animal tissues simultaneously by a combination of fluorescence and flame ionization detectors.

EXPERIMENTAL

Standards

Retinol and retinyl palmitate, which was used as a standard for retinyl esters, were purchased from Sigma Chemical Company, St. Louis, MO. The lipid class standards used in this study were available in our laboratory from the Lipid Preparation Project, in which we were active. The identity and purity of each preparation was monitored by TLC. Neutral lipids used as standards were cholesterol (CH), cholesteryl palmitate (CE), tripalmitin (TG), dipalmitin (DG), and palmitic acid (FFA). Phospholipid standards included rat liver phosphatidylcholine (PC), lysophosphatidylcholine (LPC), beef brain phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE) and phosphatidylserine (PS), egg sphingomyelin (SPH), soybean phosphatidylinositol (PI), and beef kidney diphosphatidylglycerol (DPG).

Solvents

Solvents used in the extraction of the tissues as well as for liquid chromatography, including the regeneration of the columns, must be rigorously purified to remove substances that interfere in the analyses of both the fluorescent substances and lipid classes. Purification is performed by passing each solvent; i.e., Skellysolve B, methylene chloride, chloroform and methanol through individual columns of silicic acid followed by distillation in an all-glass still.

Tissues

Blood serum, liver and kidney tissue used as specimens in this work were obtained from male Sprague-Dawley rats fed a rat chow diet. The animals were killed by exsanguination by withdrawal of the blood from the retroocular plexes; the kidneys and livers were excised and used immediately.

Tissue Extraction

A new extraction procedure that we developed for brain (14) and plant tissue (15) was modified for extraction of the lipid classes and fluorescent substances from liver and kidney tissues. Freshly excised tissue (0.5 g) is placed in a 15-ml centrifuge tube containing 5 ml of hot dilute (0.05 N) acetic acid in a boiling water bath (ca. 95 C) for 30 min. After the heat treatment, the tissues are homogenized for 1 min with a Tekmar tissuemizer, Model SDT

with SDT-100N shaft, at maximal speed, and the tubes are centrifuged at 12,500 rpm for 15 min. The supernatant is decanted and the procedure repeated at room temperature on the residual tissue. The purpose of these extractions is to destroy hydrolytic enzymes and remove impurities that ordinarily contaminate chloroform/methanol extracts. These extracts contain no lipid or lipofuscin substances and are discarded. The lipid classes and lipofuscin substances are extracted from the residual tissue in three extractions, the first with 40 ml of chloroform/methanol, 1:1, v/v; the second with 40 ml of chloroform/methanol, 1:2, v/v; and the third with 40 ml of methanol. Each extraction is done by homogenization for 1 min with a Tekmar tissuemizer. The extracts are recovered by centrifugation at 3,000 rpm, combined, and the lipid and lipofuscin substances recovered by evaporation of the solvents under reduced pressure at room temperature on a Rotovaporator.

HPLC

The tissue extracts are fractionated on a column (0.2 × 45 cm) of silicic acid treated with ammonium hydroxide. This adsorbent is prepared by stirring Spherisorb, S-GP, 8-μ (Phase Separation, Ltd., Hauppauge, NY) with concentrated ammonium hydroxide for 48 hr at room temperature. The treated silicic acid is filtered and washed thoroughly with distilled water and methanol, after which it is activated by heating at 110 C for 2 hr. Columns were packed with this adsorbent, as described by Scott and Kucera (16), using a Haskel pump, Model DHF-302A, at a pressure of 15,000 psi. The HPLC is done using an Instrument Specialties Co., Model 384 (ISCO, Lincoln, NB) pumping system at ca. 1,000 psi to give a solvent flow rate of 0.6 ml/min. The eluent is passed through an Aminco-Bowman spectrophotofluorometric detector and to a flame ionization detector fabricated in our laboratory (17). The signals from the detectors are recorded simultaneously by means of a two-pen strip chart recorder using different colored inks. Both colors became black upon reproduction. Hence, the chromatograms of the fluorescent compounds reported here are dotted to distinguish them. The fractionation of extracts is started by eluting the cholesteryl esters, triglycerides and other nonpolar components with Skellysolve B/methylene chloride, 1:1, v/v. This solvent is passed through the column for exactly 6 min. Then, chloroform/methylene chloride, 1:3, v/v, is passed through the column also for 6 min, during which cholesterol is eluted. Following elution of cholesterol, methanol containing 6% concentrated ammonium hydroxide is added to this solvent (chloroform/methylene chloride, 1:3, v/v) in a 40-min linear gradient. Finally, the column is regenerated by passing chloroform/methylene chloride, 1:3, v/v, through it for 20 min, which is followed by passing Skellysolve B/methylene chloride, 1:1, v/v, for 20 min.

The column used in this study has been in use ca. 6 months. During this time, more than 100 injections have been made, with very slight deterioration of column activity and resolution. A guard column of the same column packing, 20 mm × 2 mm, was used and was replaced twice during the 6 months.

RESULTS

The analysis of an extract of rat kidney tissue (Fig. 1) is typical of the application of the method to animal tissues. The profiles of the lipid classes and fluorescent substances are run simultaneously but the pens are offset 1 cm; therefore, the start or zero time of the chromatogram of the lipid classes is at the 2-min point relative to the chromatogram of the fluorescent substances.

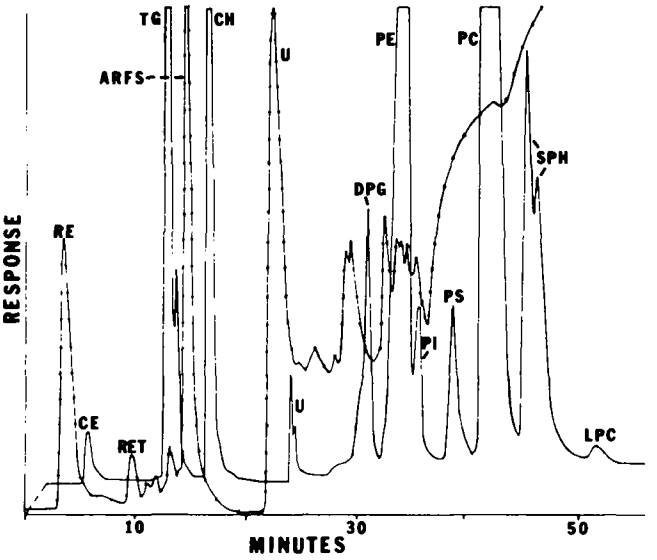

FIG. 1. HPLC of lipid classes (smooth line) and fluorescent substances (dotted line) of rat kidney determined simultaneously via flame ionization and fluorescence detectors, respectively. The chromatogram of the lipid classes is offset from that of the fluorescent compounds by 2 min. Major peaks in the fluorescence chromatogram are: RE, retinyl esters; RET, retinol; ARFS, age-related fluorescent substance; U, unidentified. Major peaks in the profile of the lipid classes are: CE, cholesteryl esters; TG, triglycerides; CH, cholesterol; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine.

Generally, the analysis is done on ca. 1 mg of tissue extract to detect the fluorescent substances because these substances are present in most animal tissue only in very small amounts.

Identification of the lipid classes was made by TLC analysis of the peaks collected from ca. two-thirds of the eluent by means of a splitter after passage through the fluorescence detector. Identification of the peaks in the chromatograms was also made by comparing retention times with authentic standards chromatographed under the standardized conditions. Retinol and retinyl esters were identified by the HPLC of standards and by spectrophotofluorometric analysis of eluent corresponding to the peaks in the chromatogram. Because the elution program is conducted in part manually, its precise reproduction from one run to another is sometimes variable. Consequently, the retention times vary from one chromatogram to another. However, identification of the peaks can usually be made from the general pattern of the elution of the components.

In the chromatogram of the fluorescent compounds a peak was designated as an age-related fluorescent substance (ARFS) on the basis that it was relatively minor in young animals and a major component in old animals (as illustrated with kidney extracts in Fig. 2).

Spectrophotofluorometric analysis of collected fractions containing ARFS showed that it had excitation and emission maxima of 360 and 420 nm, respectively, which is characteristic of lipofuscin substances. This peak is readily distinguished from retinol and retinyl esters in the chromatogram of the fluorescent compounds, but it overlaps cholesterol in the profile of the lipid classes. The same peak (ARFS) aslo was observed in the blood serum of rats (Fig. 3). The composition of the lipid classes differs greatly in kidney and serum, but the ARFS peak is easily distinguished relative to cholesterol, as well as retinol and retinyl esters.

The chromatogram of the fluorescent substances in Figure 1 indicated that animal tissues contained several other fluorescent compounds besides retinol, retinyl esters and the ARFS. However, no effort was made to identify other peaks in the chromatogram of the fluorescent compounds in this study. At about t_R 25 min and through the remainder of the analysis, the fluorescence baseline rises due to the increasing amount of methanol-concentrated NH_4OH solvent in the eluting solvent.

The course of the extraction of the fluorescent substances and the lipid classes from rat liver tissue was followed by spectrophotofluorometric and HPLC analyses of the individual extracts (Figs. 4 and 5). The spectral analyses (Fig. 4) showed that the acetic acid extract did not contain any lipofuscin substances, inasmuch as the excitation and emission maxima at 280 and 345 nm, respectively, are not characteristic of these substances. The first chloroform/methanol extraction, 1:1, v/v, contained most of the retinol and retinyl esters, as evidenced by the fluorescent spectrum which had excitation-emission maxima characteristic of these compounds. This fraction contained the bulk of the lipid classes, as shown by the HPLC analysis (Fig. 5). The HPLC also confirmed that retinyl esters were the major component of the fluorescent compounds of this fraction. The second chloroform/methanol extraction, 1:2, v/v, contained most of the lipofuscin substances, as evidenced by the shift in the excitation and emission maxima to 360 and 420 nm, respectively. The HPLC analysis (Fig. 5) showed, accordingly, that the major component of the fluorescent substances of this extract was the ARFS. The third extraction, that with methanol, contained only traces of lipid and fluorescent substances and generally can be disregarded.

A novel feature of the flame ionization detector used in this study is that it converts all compounds to hydrocarbons. Hence, it should be possible to quantify the lipid classes on the basis of the proportionalities of their peak areas corrected for carbon content or by the use of response factors. Analysis of rat kidney phospholipids by simple proportionalities of their peak areas agreed well with values reported in the literature (18) for these compounds (Table I).

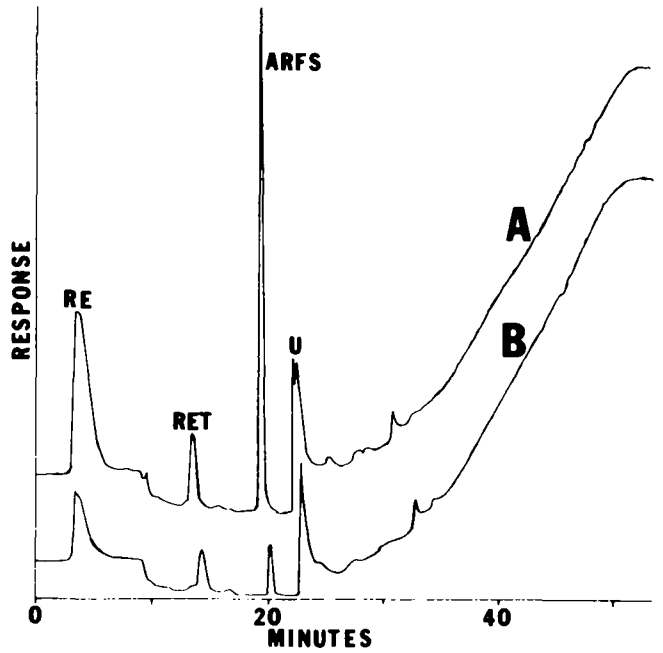

FIG. 2. Comparison of the HPLC analysis of the fluorescent compounds of the kidney extracts from A, old (77-week) and B, young (18-week) rats. Abbreviations as in Fig. 1.

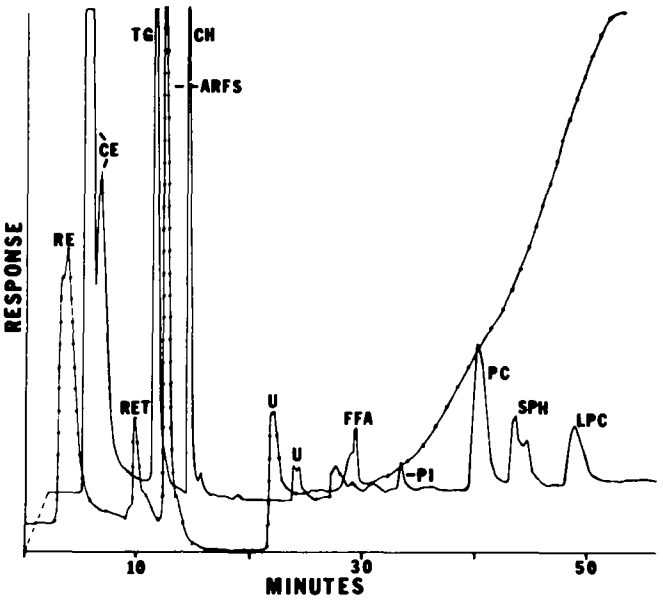

FIG. 3. HPLC of lipid classes (smooth line) and fluorescent substances (dotted line) of rat serum determined simultaneously via flame ionization and fluorescence detectors, respectively. The chromatogram of the lipid classes is offset from that of the fluorescent compounds by 2 min. Major peaks in the chromatogram of the fluorescent compounds and major peaks in the profile of the lipid classes are as abbreviated in Fig. 1 (FFA, free fatty acids).

DISCUSSION

The methodology described in this report offers an improved method for the extraction of fluorescent substances and lipid classes from animal tissues, and provides a novel approach to the analysis of these substances by HPLC. Hot dilute acetic acid does not extract any lipid from animal tissues, and inactivates hydrolytic enzymes, as demonstrated in previous work (14,15). Animal tissues treated with hot dilute acetic acid can be stored indefinitely at -20 C without deterioration of the lipid or an increase in fluorescent substances. Preextraction of animal tissues with acetic acid removes nonlipid substances that are normally

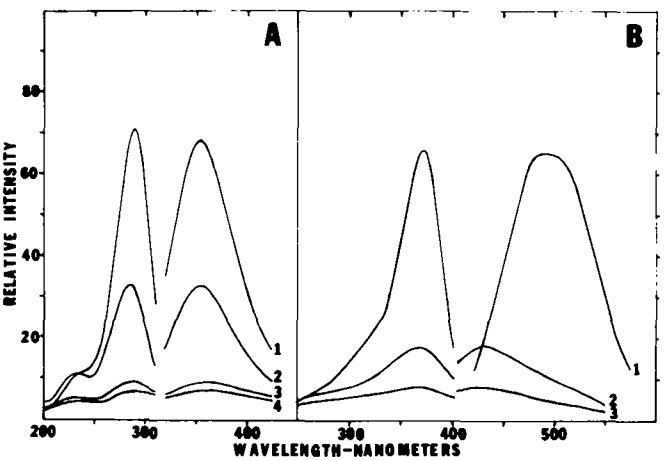

FIG. 4. Fluorescence excitation and emission scans of acetic acid extracts and subsequent organic solvent extracts of rat liver: A = 1st, 2nd, 3rd and 4th acetic acid extracts. B = 1st chloroform/methanol (C/M) extract; 2nd C/M extract; and 3rd C/M extract.

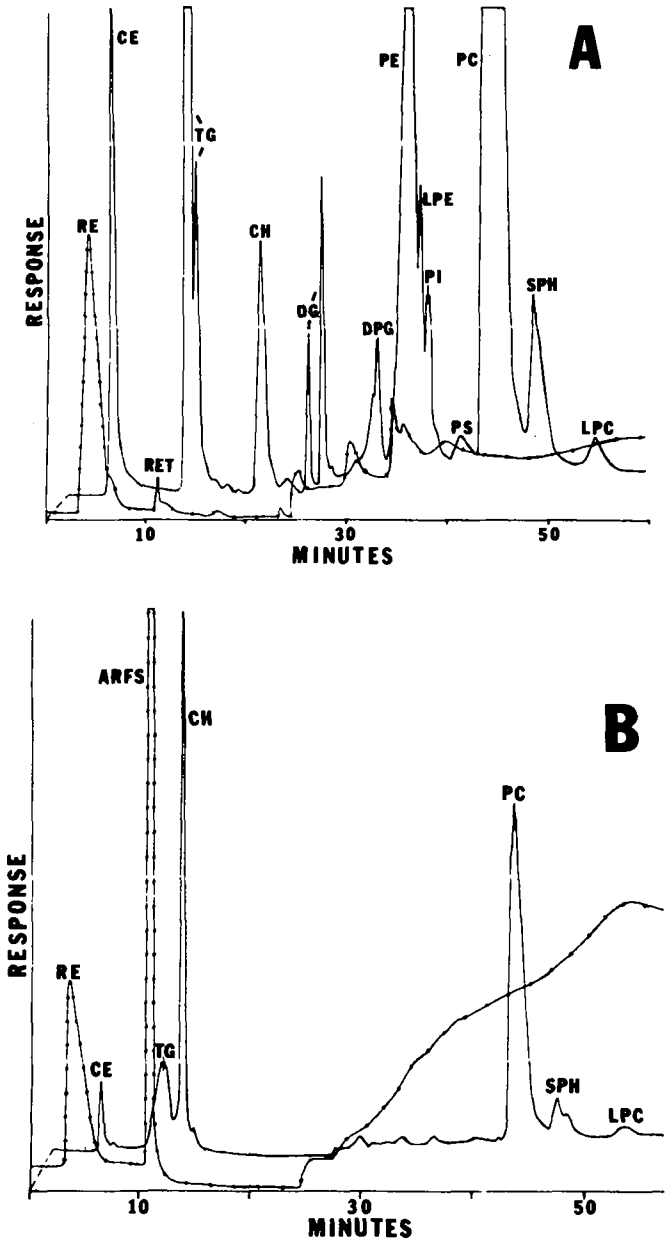

FIG. 5. FID and fluorescence chromatograms of individual C/M extracts of rat liver after acetic acid extraction: A = 1st chloroform/methanol (C/M), 1:1, v/v, extract; B = 2nd C/M, 1:2, v/v, extract. The chromatogram of the lipid classes is offset from that of the fluorescent compounds by 2 min. Major peaks in the fluorescent chromatogram and major peaks in the profile of the lipid classes are as abbreviated in Fig. 1 (LPE, lysophosphatidylethanolamine).

extracted with chloroform/methanol and that contaminate lipid extracts (19). Generally, these substances are removed from chloroform/methanol extracts by solvent partition as in the Folch procedure (20), by aqueous extraction (21), or Sephadex chromatography (19). However, these procedures are time-consuming and introduce errors in the quantitative analysis of lipids. On the other hand, tissues preextracted with acetic acid can be exhaustively extracted with chloroform and methanol to provide a quantitative recovery of the lipid classes devoid of nonlipid substances. Spectrophotofluorometric analysis showed that no lipofuscin substances were lost by extraction with dilute acetic acid inasmuch as the excitation and emission maxima of these extracts were 280 and 345 nm, respectively. The excitation and emission maxima of lipofuscin substances are generally

TABLE I

Quantitative Analysis of Kidney Phospholipids by HPLC[a]

	Phosphorus analysis (18)	HPLC (peak areas)	HPLC (peak areas corrected for % carbon)
PC	34.29	37.46	37.13
PE	27.13	29.31	29.51
PS	7.18	6.71	6.98
PI	5.87	6.69	7.12
DPG	6.51	6.81	6.80
SPH	12.13	12.43	12.19
LPC	0.94	0.13	0.15

Total phospholipids (%) by HPLC using flame ionization detector. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; DPG diphosphatidylglycerol; SPH, sphingomyelin; LPC, lysophosphatidylcholine.

reported as 340-370 nm and 420-470 nm, respectively (10,22).

Lipofuscin substances are believed to arise generally via in vivo oxidation; therefore, the peak designated as ARFS might serve as an index of in vivo oxidation. It should be particularly valuable for this purpose because it also appears in blood. Current methods for the detection of in vivo oxidation include the TBA test (23), fluorescence measurements on fractions obtained from tissue extracts (11,12, 24), or the analysis of hydrocarbon gases in respired air (25,26); these are general tests. Hence, measurement of ARFS should be not only highly sensitive, but also specific for in vivo oxidation.

Although TLC will continue to be used for the quantitative analysis of the lipid classes, this study demonstrates the potential value of HPLC for the quantitative analysis of these compounds. HPLC should have little, if any, advantage over other methods of analysis if it must be combined with other methodology such as determination of phosphorus or fatty acid composition for quantitative analysis. The fact that the lipid classes are converted to hydrocarbons via introduction of hydrogen in the reactor of the detector provides a uniformity in the ionization reaction for all compounds. Therefore, the response of the lipid classes should be equivalent to their carbon content in general. This seems to be the case with the compounds of similar structures, such as the phospholipids (Table I). Lipids with very different structures, such as cholesteryl esters and triglycerides, varied considerably in their responses, although the response was linear to mass in each case (17). It appears that, for a quantitative analysis of the entire profile of the lipid classes, response factors will be required at least for some compounds. However, these factors should be fairly simple and, with their development, should enable a direct quantitative analysis of these compounds by HPLC.

In this study, each analysis was done on ca. 1 mg of tissue extract to detect the fluorescent compounds, which is highly advantageous in studies designed to interrelate these substances with the lipid classes (as in aging studies). However, the FID detector is capable of detecting the lipid classes in the low nanogram range (17). Hence, if as we have indicated, HPLC can be used directly for quantitative analysis, it will be a superior method for the analysis of these compounds as well as of lipofuscin substances.

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Fatty Acids and Sterols in Oils from Canola Screenings[1]

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ABSTRACT

One sample of canola seed (variety Tower) and five samples of screenings were commercially processed to yield first an "expeller oil" and subsequently an "extractor oil" by the hexane extraction of the residue. The screening samples contained 25-50% intact or broken canola seed. The balance included 21-31% weed seeds (especially lambsquarter and stinkweed), hulls, fragments of the embryo, and chaff. All the oil samples were analyzed for sterol and fatty acid composition. The extractor screening samples had slightly higher sterol contents than the corresponding expeller samples, while the Tower samples gave the lowest values. The averages (in mg/g oil or extract) for the extractor screening samples were: brassicasterol, 1.0; campesterol, 4.1; and β-sitosterol, 7.3. For expeller screening samples the averages were: 0.9, 3.6 and 6.2, and for the Tower oils they were, respectively, 0.9, 3.8, 5.3 and 0.9, 3.5, 4.7. The fatty acid compositions of the screening samples for both extractor and expeller oils were similar to that of the Tower oil except for the higher proportions of docosenoic acid (22:1) and eicosenoic acid (20:1) and the more obvious presence of three C_{18} conjugated dienes totalling up to 0.6% of one screening oil sample. The docosenoic acid level (mainly erucic acid) ranged from 3.0 to 7.0% for the extractor oils and from 2.5 to 8.0% for the expeller samples, compared to 0.1% for the two Tower oils. The oil contents of the screenings ranged from 20 to 30%, and the fatty acids and sterols appear to be nutritionally useful and innocuous in all respects.

INTRODUCTION

Screenings are an inadvertent but economically significant factor in the canola (registered name for low-glucosinolate, low-erucic-acid varieties of *Brassica napus* or *Brassica campestris*) industry in western Canada. Farm deliveries of canola seed include damaged and immature canola seeds, and some genetically related seeds (e.g., mustard), but a variety of weed seeds are always included. The whole of this undesirable material may be termed dockage. A substantial portion can be removed, accompanied by some loss of sound canola seed, as screenings. This material is not normally processed in any way for oil or meal production. As part of a program to investigate the characteristics of screenings in animal nutrition, two types of oil were prepared from each of five sets of screenings, respectively denoted as extractor and expeller oils. These oils were examined for fatty acids and sterols. Following our earlier investigation of rapeseed oils for minor fatty acids (1), we have now applied the same examination technology, and our current results indicate generally unimportant differences between canola oil and screenings oils. The screenings oils, however, had appreciable erucic acid, whereas the Tower oils had only 0.1%. The screenings oils also had up to 0.6% total conjugated octadecadienoic acids, compared to only traces in the Tower oils.

EXPERIMENTAL PROCEDURES

The five lots of screenings, and one lot of canola seed, variety Tower (*Brassica napus*), from the 1977 crop were delivered to the P.O.S. Pilot Plant Corporation, Saskatoon. The lots amounted to ca. 900 kg each from five separate locations in three provinces. All were sequentially flaked, cooked, expelled and extracted (hexane) by conventional procedures (2). The oils were shipped to Halifax for analysis and were allowed to stand to settle out any fine solids present. Then the upper two-thirds to three-quarters of the oils was decanted into nigrogen-purged containers as the sample for analysis. A small lot of the original mixture of screenings (B) was obtained later. Seeds of sample B were crushed and extracted in the laboratory by boiling with hexane for 1 hr under nitrogen.

[1] Presented in part at the ISF/AOCS World Congress, New York, April-May 1980.