# APPLICATIONS OF SUPERCRITICAL FLUID TECHNOLOGIES TO THE ANALYSIS OF FOOD COMPONENTS

by

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This thesis is dedicated to

with love.

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TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (cont)

LIST OF FIGURES (cont)

LIST OF TABLES

LIST OF TABLES (cont)

# I. INTRODUCTION

Supercritical fluid (SF) technologies are being investigated extensively by the food industry for a variety of applications. An SF is any material that is at a temperature above its critical temperature and at a pressure above its critical pressure. SFs have good density dependent solvating characteristics permitting "selective" fractionation during extraction and gradient elution in a chromatographic separation. Their greater diffusivity relative to liquids provides greater facile penetration into a sample matrix for ease of extraction. Greater diffusivity also leads to faster chromatographic separations when compared to liquid chromatographic separations. Carbon dioxide in the supercritical state is of particular interest to the food industry due to its extremely low toxicity in comparison with organic solvents. Since many of the components in food matrices react or degrade at elevated temperatures, $CO_2$ is desirable because of its low critical temperature.

Considerable research efforts in food chemistry relate to designing a systematic approach to determine food composition using a variety of analytical tools. We are exposed through our diets to such a myriad of chemicals both toxic and salubrious, that it is often exceedingly difficult to separate, identify and quantify one particular component or group of components from foods. This difficulty is primarily a result of limited specificity in current extraction techniques and limited volatility/solubility/detection in typical chromatographic techniques. The technologies of supercritical fluid chromatography (SFC) and supercritical fluid extraction (SFE) have significant potential for successful application in these problem areas. These techniques have enormous potential for identification of food constituents by interfacing with

spectroscopic instrumentation. The goal of this research was to investigate potential applications of chromatography and extraction employing supercritical fluids in the analysis of food components and natural products. Three separate applications were investigated. The first application (Chapter III) related to a study of peracetylated nitrogen derivatives. This study employed SFC with Fourier transform infrared (FT-IR) and mass spectrometric detection. The second application (Chapter IV) related to the use of SFC/FT-IR in monitoring changes in chemical composition of soybean oil due to processing. Samples of refined soybean oil that were hydrogenated to different extents were analyzed. The third application (Chapter V) dealt with SFE of a model compound system based on the coumarin structure. The effect of several variables on the extraction of these compounds was studied. These variables included, temperature, matrix and restrictor type. The literature review (Chapter II) that introduces this work deals mainly with SFC and SFE of foods and natural products. This summary continues the extensive review published by Randall (1) in 1982.

## II. HISTORICAL

Supercritical fluid technologies have been studied since the late 1800s. Hannay and Hogarth (2) were the first to report on the solubility phenomena in supercritical fluids in 1879. In 1962 Klesper et al. (3) reported on the first use of supercritical fluids as mobile phases in chromatography. Interest in supercritical fluid technologies increased in the early 1980s due to readily available commercial equipment on the analytical scale. Randall (1) reviewed the status of dense (supercritical) gas chromatography and extraction essentially up to 1981. The following section will discuss the area of SFC and SFE as related to food components and natural products involving that work which has been published since 1981. This review is not intended to be all inclusive, but to be used as a means of evaluating the various applications of SFs to the analysis of food components. The discussion will deal only with the use of supercritical $CO_2$ (SC-$CO_2$) and modified SC-$CO_2$ since these are the fluids of most interest to the food industry.

### A. SFC of Food Components and Natural Products

Table I lists a variety of compounds and the SFC conditions for their separations found in the literature. The compounds investigated include alkaloids, amino acid derivatives, herbicides and pesticides, lipids, mycotoxins, steroids and carbohydrates. Packed and open tubular columns are employed with a variety of detection modes that include ultra violet (UV), flame ionization detection (FID), mass spectrometry (MS) and Fourier transform infrared (FT-IR) detection.

TABLE 1. Summary of Work in SFC Related to Food Components and Natural Products

| Commodity | Conditions | Column[a,b] | Detector | References |
|---|---|---|---|---|
| **Alkaloids:** | | | | |
| Caffeine | $CO_2$ with varying % of $CH_3OH$; 75°C; 2-6 mL/min; 230 -271 bar back pressure. | ODS or RP-8, 100 to 250 mm x 4.6 mm i.d., 3 to 10 μm $d_p$ | UV | 4 |
| Xanthines | $CO_2$ with varying % of 2-methoxy-ethanol, 2-propanol, chloroform or methylene chloride; 60°C; 5000 psi back pressure; 4 mL/min. | Hypersil Silica, 100 x 4.6 mm i.d., 5 μm $d_p$ | UV | 5 |
| Xanthines, ergot alkaloids | $CO_2$/12% $CH_3OH$; 70°C; 385 bar $CO_2$/15-20% $CH_3OH$; 75°C; 365 bar | Silica LiChrosorb and amino bonded Spherisorb, 100 x 4.6 mm i.d., 5 μm $d_p$ | UV,MS | 6 |
| pyrrolizidine alkaloids | $CO_2$; 130°C; 100 atm hold 20 min, 100 to 250 atm at 3 atm/min; splitless; integral restrictor | SB–Methyl–100 or SB-Biphenyl-30, 10 m x 50 μm i.d., 0.25 μm $d_f$ | FID | 7 |
| opium alkaloids | 5 to 25 wt% $CH_3OH$ in $CO_2$; 40.7°C; mean pressure 220 bar; 4 mL/min | LiChrosorb-$NH_2$, 230 x 4.6 mm i.d., 10 μm $d_p$ | UV | 8 |
| indole alkaloids | $CO_2$; 5-15% $CH_3OH$; 65°C; pressure not reported | Amino Spherisorb, 100 x 4.5 mm i.d., 5 μm $d_p$ | UV,MS | 9 |
| Nicotine | $CO_2$; 125°C; 120 atm for 3 min, 120 to 300 atm at 25 atm/min, 300 to 400 atm at 10 atm/min; integral restrictor | Deltabond™ methyl, 250 x 1 mm i.d., 5 μm $d_p$ | FT-IR flow-cell | 10 |
| Caffeine | $CO_2$; 60°C; 100 to 175 atm in 15 min, 175 to 400 atm in 5 min | SB-Cyanopropyl-25, 10 m x 100 μm i.d. | FT-IR flow-cell | 11 |
| Xanthine | $CO_2$/$CH_3OH$ 95:5; 70°C; inlet pressure of 4000 psi; 1.5 mL/min | S3CN Spherisorb, 100 x 4.6 mm i.d., 3 μm $d_p$ | EI-MS | 12 |
| **Amino Acid Derivatives:** | | | | |
| D- and L- amino acid tert-butyl esters | $CO_2$ gas flow at 2 L/min, $CH_3OH$ at 0.5 mL/min; 200 bar | chiral phase: (N-formyl-L-valyl-amino) propyl silica gel; 250 x 4 mm i.d.; 10 μm $d_p$ | UV | 13,14 |

[a]If the stationary phase is not specified as crosslinked then the original article did not specify it. SB– specified stationary phases are commercially available crosslinked stationary phases.

[b]Brand names were only given when the original reference listed them.

TABLE I. Summary of Work in SFC Related to Food Components and Natural Products (cont.)

| Commodity | Conditions | Column[a,b] | Detector | References |
|---|---|---|---|---|
| PTH-amino acids | mobile phase gradient SF-$CO_2$ and 0.001M tetramethyl ammonium hydroxide in methanol; 40°C | Cyanopropyl, 250 x 4.6 mm i.d., 5 µm $d_p$ | UV | 15,16 |
| **Carbohydrates:** | | | | |
| arabinose (underivatized) | $CO_2$ with 2.5 wt % $CH_3OH$; 60°C; 95 to 155 atm at 10 atm/min | 5% phenylmethyl siloxane, 2 m x 50 µm i.d. | MS | 17 |
| TMS glucose oligo-polysaccharides (DP2 to 18) | $CO_2$; 150 or 89°C; pressure programmed; integral restrictor | DB-1, 10 m x 65 µm i.d. or 10 m x 50 µm i.d., 0.2 µm $d_f$ | FID | 18 |
| permethylated glucose polymers, TMS glucose polymers (DP2 to 15) | $CO_2$; 90°C; 100 to 405 atm at 5 atm/min; 115 to 400 atm at 3 atm/min; Guthrie restrictor | DB-5, 10 m x 50 µm i.d., 0.2 µm $d_f$ | FID, MS | 19 |
| peracetylated aldononitrile derivatives of monosaccharides | $CO_2$; 100°C; 130 atm hold 8 min, 130 to 270 atm at 25 atm/min; direct injection; integral restrictor | Cyanopropyl Deltabond™, 250 x 1 mm i.d., 5 µm $d_p$ | FID, FT-IR flow-cell | 20 |
| permethylated maltodextrins | $CO_2$; 120°C; 110 atm for 5 min, 110 to 400 atm at 5 atm/min, hold at 400 atm; Guthrie restrictor | DB-5, 10 m x 50 µm i.d., 0.2 µm $d_f$ | FID, MS | 21 |
| **Herbicides/Pesticides:** | | | | |
| carbamate pesticides | $CO_2$; 75°C; programmed for 75 atm at 100 atm/min; split injection; linear restrictor | SE-54 (crosslinked), 0.9 m x 25 µm i.d. 0.2 µm $d_f$ | FID | 22 |
| carbamate and acid pesticides | $CO_2$; 75 or 100°C; 75 to 300 atm at 50 atm/min; split injection; linear restrictor | SE-54 (crosslinked), 2 m x 50 µm i.d. | MS | 23 |
| carbamates | $CO_2$/15% $CH_3OH$; 70°C; 179 bar | Silica Lichrosorb and amino bonded Spherisorb, 100 x 4.6 mm i.d., 5 µm dp | UV,MS | 6 |
| herbicides, pesticides | $CO_2$; 100°C; 0.45 g/mL hold for 5 min, to 0.6 g/mL at 0.1 g/mL/min, hold for 5 min; split injection; | SB-Phenyl-50, 3 m x 100 µm i.d. | FID | 24 |

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TABLE I. Summary of Work in SFC Related to Food Components and Natural Products (cont.)

| Commodity | Conditions | Column[a,b] | Detector | References |
|---|---|---|---|---|
| herbicides, pesticides | 5% $CH_3OH$ in $CO_2$; 45°C; 5 mL/min (Conditions varied depending on compound analyzed. Used conditions for Oust herbicide as an example) | Zorbax SIL, 250 x 4.6 mm i.d. | UV | 24 |
| sulfonylurea | 2% $CH_3OH$ in $CO_2$; 40 °C; 223 bar; 6.0 mL/min | packed unspecified | UV | 25 |
| Bendiocarb, carbaryl, alachlor, duiron, metalaxyl | $CO_2$; 77°C; 0.23 g/mL hold 1 min, 0.23 to 0.45 g/mL over 34 min; direct injection; linear restrictor | 5% phenylmethyl silicone, 12 m x 100 µm i.d., 0.25 µm $d_f$ | UV | 26 |
| pyrethrins | $CO_2$; 125°C; 0.18 g/mL hold 6 min, to 0.34 g/ml at 0.02 g/mL/min, to 0.38 g/mL at 0.004 g/mL/min, to 0.68 g/mL at 0.02 g/mL/min; split injection; frit restrictor | SB-Biphenyl-30, 2 m x 50 µm i.d. | FID/FT-IR flow-cell | 27 |
| pork fat spiked with DDT | $CO_2$; 100°C; 0.25 to 0.76 g/mL at 0.025 g/mL/min; fat sample was extracted with $CO_2$ at 5000 psi 60°C, 30 min | SB-Methyl-100, 3 m x 50 µm i.d. | ECD | 28 |
| **Lipids:** | | | | |
| fatty acid phenyl esters | $CO_2$; isothermal, 35-65°C; isobaric, 85-190 bar | Perisorb A, 258 x 4.5 mm i.d. Perisorb RP8, 256 x 4.5 mm i.d. | UV | 29 |
| free fatty acids, C10 to C18 | $CO_2$; 90°C; 130 atm hold 10 min, 130 to 190 atm in 30 min; split injection; integral restrictor | BP-10 (crosslinked), 9 m x 100 µm i.d., 0.1 µm $d_f$ | FID | 30 |
| mono-, di-, and triglycerides | $CO_2$; 90°C; 150 atm hold 20 min, 150 to 300 atm at 1 atm/min; split injection; integral restrictor | DB-5, 19 m x 100 µm i.d., 0.25 $d_f$ | FID | 31 |
| free carboxylic acids | $CO_2$; 50°C; 0.19 g/mL for 10 min, 0.19 to 0.75 g/mL at 0.007 g/mL/min (other conditions also reported) | SE-54 (crosslinked), 15 m x 50 µm i.d. 0.25 µm $d_f$ and 50% cyanopropyl (crosslinked), 12 m x 50 µm i.d., 0.25 µm $d_f$ | FID | 32 |

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TABLE I. Summary of Work in SFC Related to Food Components and Natural Products (cont.)

| Commodity | Conditions | Column[a,b] | Detector | References |
|---|---|---|---|---|
| Polyglycerol esters. free and silyl derivatives | $CO_2$; 140°C; pressure program depended on column used; split injection; integral restrictor | DB-1 (0.2 μm $d_f$) or DB-17 (0.1 μm $d_f$), 10 m x 50 μm i.d. | FID | 33 |
| free fatty acids | $CO_2$ or Freon; isothermal, 40 to 90°C; isobaric, 2600 to 4500 psi; 1.5 to 3.0 mL/min | PRP-1, 150 x 4.1 mm i.d., 5 μm or 10 μm $d_p$ | FT-IR flow-cell | 34 |
| triglycerides $C_{36}$ to $C_{54}$ | $CO_2$; isothermal, 90 to 250°C; 190 bar hold 10 min, 190 to 250 bar at 5 bar/min; split injection; integral restrictor | SE-54 (crosslinked), 10 m x 100 μm i.d., 0.2 mm $d_f$ | FID | 35 |
| archaebacterial glycerol tetraether lipids | $CO_2$; 120°C; 150 to 300 atm in 20 min; linear restrictor | 50% methylphenyl polysilioxane (crosslinked), 10 m x 75 μm i.d., 0.25 μm $d_f$ | FID | 36 |
| saturated and unsaturated $C_{18}$ free fatty acids | $CO_2$; temperature program: 160 to 210°C at 1°C/min; pressure program: 100 to 300 bar at 1 bar/min, hold 20 min; integral restrictor | Supelcowax 10, 14 m x 25 μm i.d. 0.25 μm $d_f$ | FID | 37 |
| permethylated glycophingolipids | $CO_2$; 120°C; 110 atm for 5 min, 110 to 400 atm at 5 atm/min, hold at 400 atm; Guthrie restrictor | DB-5, 10 m x 50 μm i.d. 0.2 μm $d_f$ | FID,MS | 21 |
| isoprenoids, glycerol esters | $CO_2$; 100°C; 150 atm for 30 min, to 410 atm at 6 atm/min; splitless injection; linear restrictor | SB-Biphenyl-30, 10 m x 50 μm i.d. 0.25 μm $d_f$ | FID | 38 |
| triacylglycerides | $CO_2$; 150°C; 13.8 MPa for 2 min, to 37.9 MPa in 38 min, hold for 5 min; split injection; Guthrie restrictor | DB-5, 5 or 10 m x 50 μm i.d., 0.20 μm $d_f$ | FID | 39 |
| free fatty acids, fatty acid esters, lipids | $CO_2$; 45°C; 100 to 160 atm in 20 min; integral restrictor (conditions for FFAs reported as an example) | ODS-Silica gel, 250 x 4.6 mm i.d., 5 μm $d_p$ | FID/UV | 40 |
| butter fat, triacylglycerides | $CO_2$; 150°C; 13.8 MPa hold 2 min, 13.8 to 27.6 MPa in 26 min, 27.6 to 34.5 MPa in 17 min; integral restrictor | DB-5, 5 m x 50 μm i.d., 0.20 μm $d_f$ | FID, EI-MS | 41 |

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TABLE I. Summary of Work in SFC Related to Food Components and Natural Products (cont.)

| Commodity | Conditions | Column[a,b] | Detector | References |
|---|---|---|---|---|
| unsaturated fatty acid methyl esters | $CO_2$; 49.5°C; 192 bar; 2 g/min | Hypersil Silica, 235 x 4.6 mm i.d., 5 µm $d_p$ | UV | 42 |
| **Mycotoxins:** | | | | |
| trichothecenes | $CO_2$; ≤100°C; density gradient for long column separation; pressure gradient for short column separations; split injection | SE-54 (crosslinked), 1.75 or 15 m x 50 µm i.d., 0.25 µm $d_f$; 0.8 m x 25 µm i.d., 0.2 µm $d_f$ | FID/MS | 43 |
| trichothecenes | $CO_2$; 100°C; 0.25 to 0.75 g/mL at 0.025 g/mL/min; split injection or retention gap; frit restrictor | methylsilicone (bonded phase), 10 m x 50 µm i.d. | MS | 44 |
| **Steriods:** | | | | |
| progesterone, methyltestosterone, estrone, Vitamin $D_2$ | $CO_2$ with varying % 2-methoxy-ethanol, 2-propanol, $CHCL_3$, or $CH_2CL_2$; 60°C; 5000 psi back pressure; 4 mL/min | Hypersil Silica, 100 x 4.6 mm i.d., 5 µm $d_p$ | UV | 5 |
| testosterone, progesterone, hydrocortisone and others | $CO_2$/20% methoxyethanol; 75°C; 195 bar | Silica LiChrosorb and amino bonded Spherisorb, 100 x 4.6 mm, 5 µm $d_p$ | UV, MS | 6 |
| progesterone, testosterone, 17-hydroxypro-gesterone, 11-deoxy-cortisol, corticosterone | $CO_2$; 60°C; 100 to 150 atm in 15 min, to 400 atm in 10 min, to 440 atm in 6 min; split injection | SB-Cyanopropyl-25, 10 m x 100 µm i.d., 25 µm $d_f$ | FID/FT-IR (flow-cell) | 45 |
| ecdysteriods | $CO_2$; 120°C; 0.4 to 0.71 g/mL at 0.015 g/mL/min after an initial 5 min hold; split injection; frit restrictor | SB-Cyanopropyl-50, 10 m x 50 µm i.d., 0.25 µm $d_f$ | FID | 46 |
| | 10% $CH_3OH$ in $CO_2$; 50°C; inlet pressure 300 atm; 10 µL loop injection | Spherisorb ODS-2 or cyanopropyl, 250 x 4.6 mm i.d., 5 µm $d_p$ | UV | 46 |
| | $CO_2$- $CH_3OH$ (4:1); 80°C; 300 bar; 4 mL/min | Hypersil, 100 x 4.6 mm i.d., 5 µm $d_p$ | UV/MS | 47 |

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[b]Brand names were only given when the original reference listed them.

TABLE I. Summary of Work in SFC Related to Food Components and Natural Products (cont.)

| Commodity | Conditions | Column[a,b] | Detector | References |
|---|---|---|---|---|
| **Other:** | | | | |
| azo compounds | $CO_2$; 40°C; 0.225 g/mL hold 15 min, 0.225 to 0.575 g/mL; split injection, linear restrictor | SE-54, 34 m x 50 μm i.d., 0.25 μm $d_f$ | FID | 48 |
| erythromycin A | $CO_2$; 40°C; 0.225 g/mL hold 24 min, 0.225 to 0.70 g/mL at 0.05 g/mL/min, hold 30 s, 0.70 to 0.83 g/mL at 0.005 g/mL/min | SE-54, 19 m x 80 μm i.d., 0.3 μm $d_f$ | FID | 49 |
| ferrocene, acetyl-ferrocene, 1,1-diacetyl ferrocene[c] | $CO_2/CH_3OH$ (98/2); isothermal 50 to 100°C; isobaric, 2000 to 5000 psi; 10 μL loop injection | Silica, ODS, PRP-1, phenyl, column size and diameter varied | UV | 50 |
| volatile citrus oil | $CO_2$; 50°C; isobaric, column head pressure 1750 psi, back pressure 1400 psi; 0.5 μL injection | PRP-1, 150 x 4.1 mm i.d., 5 μm $d_p$ | FT-IR flow-cell | 51 |
| triazole fungicide metabolite | $CO_2$; 100 atm hold 4 min, 100 to 300 atm at 40 atm/min; time-split injection; frit restrictor | SB-Methyl-100, 5 m x 50 μm i.d. | ECD | 52 |
| fat-soluble vitamins | $CO_2$; 150°C; 175 atm hold 25 min, 175 to 350 atm at 4.17 atm/min; split injection; integral restrictor | DB-Wax, 10 m x 100 μm i.d., 0.1 μm $d_f$ | FID | 53 |
| ouabin | $CO_2$; 80°C; 200 atm for 8 min, 200 to 400 atm at 0.02 g/mL/min; SFE sample introduction; frit restrictor | SB-methyl-100, 3 m x 50 μm i.d. 0.25 μm $d_f$ | FID | 54 |

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[c]While these compounds are not food related, they potentially could be used as models for those naturally occuring metal containing compounds.

Separation of alkaloids on traditional packed columns requires the addition of a polar modifier. Since polar modifiers are used, detection is limited to UV or MS. The modifier used most often was methanol (4, 6-9, 12). A study by Randall (5) compared the effectiveness of four different modifiers that included 2-methoxyethanol, 2-propanol, chloroform and methylene chloride. For the separation of xanthines, 2-methoxyethanol mixed with SC-$CO_2$ provided the best chromatography. 2-Propanol eluted caffeine, theophylline and theobromine but not xanthine even at the maximum modifier concentration employed (9.5%). The recent availability of more highly deactivated silica based packed columns permits the analysis of some alkaloids without the addition of modifier. The separation of nicotine on a Deltabond™ methyl column (7) demonstrated the effectiveness of this column. Since no polar modifier was employed, FT-IR flow cell detection was used. Alkaloids are also separated readily on open tubular columns (6,10) with 100% $CO_2$.

Amino acid derivatives have been separated under SFC conditions using $CO_2$ modified with polar solvent (13-16). The addition of a small amount of ion pair reagent (tetramethyl ammonium hydroxide) to the organic modifier greatly enhanced the separation thereby reducing the amount of organic solvents needed (15-16). This combination of solvents has the potential of being extended to ionic species although it limits the available detection modes to LC type detectors.

Carbohydrates are generally derivatized for analysis under SFC conditions with 100% $CO_2$. The types of derivatives analyzed include trimethylsilated oligo- and polysaccharides (18,19), permethylated glucose polymers (19,21) and peracetylated aldonitrile derivatives of monosaccharides (20). Wright et al. (17) detected underivatized arabinose using methanol modified $CO_2$ with a capillary column and a mass

spectrometer. The advantage of SFC over GC in the analysis of carbohydrate derivatives is that detection of higher molecular weight species is possible.

As with the analysis of alkaloids, herbicides and pesticides generally require the addition of modifier when using packed column SFC (6,24-25). Silica or amino packed columns were employed with UV and/or MS detectors. The columns employed with capillary SFC generally were methyl or phenylmethyl polysiloxane columns (22-23, 24, 26-28). Since 100% $CO_2$ was used a variety of detection modes were employed including FID, MS, FT-IR and electron capture detection (ECD). Richter et al. (28) demonstrated the selectivity of the ECD detector by detecting DDT from SC-$CO_2$ extracted pork fat. By using a 50 μm i.d. open tubular column, the baseline shift due to the background contribution of the $CO_2$ during density programming can be subtracted. When a 1 mm i.d. packed column or a 100 μm i.d. open tubular column is used the baseline rise cannot be adequately corrected because the $CO_2$ flow rates are too large.

Both packed and open tubular columns have been used in the analysis of lipids as fatty acid esters (29, 40, 42), free fatty acids (FFAs) (30, 32, 34, 37) and triacylglycerols (31, 35, 39, 41). Hellgeth et al. (34) analyzed FFAs using a PRP-1 column with FT-IR detection. Severe distortion occurred for the C18 carboxylic acids found in soybean oil. Inspection of the FT-IR spectra showed that this peak distortion resulted because chromatographic resolution was not achieved between the saturated and unsaturated C18 fatty acids. Markides et al. (32) could resolve the saturated and unsaturated FFAs using a 50% cyanopropyl polysiloxane capillary column. They employed a FID detector and indicated that C18 positional as well as cis and trans isomers were separated although, without baseline resolution. Gorner and Perrut (42) could separate unsaturated fatty acid methyl esters using a packed silica column with

100% $CO_2$. They did not indicate in their report whether positional or cis and trans isomers were separated under the chromatographic conditions employed. Lipids were separated by carbon number with non-polar columns (31, 35, 39, 41). With polar columns separation by degree of unsaturation is also accomplished (37,40). Other lipids that have been chromatographed successfully under SFC conditions include: polyglycerol esters (33, 36, 38), permethylated glycophingolipids (21) and isoprenoids (38).

Mycotoxins are secondary mold metabolites. Within the SFC literature a specific class of mycotoxins, trichothecenes, has received attention (43-44). These compounds can be chromatographed using open tubular columns with non-polar stationary phases and 100% $CO_2$. The two laboratories working with the trichothecenes coupled SFC to MS detection. The attraction of SFC in trichothecene analysis resulted from the ability to analyze the compounds without derivatization. Generally these compounds were derivatized for GC analysis.

Separation of steroids has been achieved using packed and capillary column SFC (5-6, 45-47). As with many other polar compounds, $CO_2$ modified with polar solvents was used when employing packed columns. Randall (5) reported that when 2-methoxyethanol and 2-propanol were used as modifiers similar chromatographic behavior was observed. Ecdysteroids are polyhydroxylated steroids related to ecdysone. SFC separation is achieved with both packed and open tubular columns (45-47). Raynor et al. (46) found that only those polar steroids containing up to four hydroxyl groups could be separated on open tubular columns. While on a packed column employing $CO_2$ modified with 10% methanol steroids containing 7 hydroxyl groups are chromatographed.

The literature shows that a wide variety of compounds related to foods and natural products can be chromatographed using SFC. Alkaloids, amino acid derivatives, carbohydrate derivatives, herbicides, pesticides, lipids, mycotoxins and steroids have been successfully separated using SFC with both packed and open tubular columns. Many of these compounds have multiple polar functional groups. Generally, when using packed column SFC the addition of modifier to SC-$CO_2$ is required. The highly deactivated stationary phases used in open tubular columns and recently available packed columns usually require no modifier when separating moderately polar compounds. The availability of a variety of detection modes, UV, FID, FT-IR and MS, increases the versatility of SFC. But, the addition of polar modifiers limit the detection modes. Derivatization is still required when analyzing highly polar compounds such as carbohydrates. The low capacity of open tubular columns may stimulate the development of highly deactivated stationary phases for packed columns.

B. SFE of Food Components and Natural Products

Supercritical fluid extraction (SFE) techniques are being investigated extensively in the food industry as well as other industries (55-61). The interest in SFE is evident by the extensive patent activity related to foods since the early 1970s. Over 50 patents have been issued or applied for nationally or internationally. Research and process development using SFE technologies are of interest due to the unique properties of SFs. With increasing cost and awareness of the safety hazards relating to the use and disposal of conventional organic solvents (62), investigation of SFs that are inexpensive, nontoxic, nonflammable and highly pure is attractive. Of particular

interest to the food industry is SC-$CO_2$. The potential advantages to the food industry include higher yields, better quality products and the use of a nonflammable, nontoxic solvent. Currently as regulated by Food and Drug Administration (Code of Federal Regulation 21.184.1240(c)) $CO_2$ is generally recognized as safe as a direct human food ingredient. $CO_2$ can also be used in food with no limitations other than current good manufacturing practice.

The use of traditional organic solvents requires the removal of residual solvent to permitted levels. Usually this removal requires some distillation which can cause off-flavors due to decomposition of components at the elevated temperatures used in these distillation techniques. The chances of off-flavors resulting from residual solvents is eliminated when extracting with SC-$CO_2$ because of the ease of removing $CO_2$ from the food matrix. Extensive investigations of food processing applications using SC-$CO_2$ began in the early 1970s.

Due to the low critical temperature and pressure of $CO_2$, less mechanically complex equipment is required in the separation of complex sample matrices. Other motivations to investigate SFs in the food industry include the potential for new product development and more stringent pollution controls that increase the cost of waste disposal for traditional solvents (61). Although the solubility range of SC-$CO_2$ is broad, the use of entrainers such as ethanol can enhance further the solvating properties of the fluid. Furthermore, while a neat compound may be soluble in SC-$CO_2$, it may not be extractable without the addition of an entrainer. This phenomenon is demonstrated in the decaffeination of coffee (63); neat caffeine is soluble in dry SC-$CO_2$, but moist SC-$CO_2$ or moist coffee is necessary for the extraction of caffeine from coffee beans. This same phenomenon occurs with decaffeination by traditional organic solvents. It is

hypothesized that water frees the chemically bound caffeine in the coffee matrix.

Brunner and Peter (64) demonstrated that the advantages of an entrainer in SC-$CO_2$ extractions include a) enhanced solubility; b) thermal regeneration of the extraction fluid gas; and c) depending on the entrainer, enhancement of the separation factor. In studies of the solubility of palm oil in SC-$CO_2$ with ethanol as the entrainer, they found that with 10 wt% ethanol at 200 bar and 70°C, more than 5 wt% glycerides dissolved in the SF phase. At the same conditions with neat SC-$CO_2$ only 0.25 wt% was dissolved. It was also noted that at 130 bar the solubility of the palm oil decreased from 2 wt% at 70°C to a negligible amount at 110°C. Thus, it was possible to completely regenerate the extracting fluid by simply increasing the temperature.

An SFE system contains five basic components: pump, extraction vessel, temperature controls, pressure controls and separator. For processing, three possible recovery strategies are viable: a) change temperature; b) change pressure; and c) use a suitable absorbent material. The complexity of the processing SFE system depends on the desired application and the mode of recovery. Rizvi et al. (60) divided SFE applications related to foods into essentially three categories: a) total extraction, b) deodorization, and c) fractionation. Total extraction is the removal of a component or group of related compounds from an insoluble matrix. This type of process is exemplified by the extraction of vegetable oils with SC-$CO_2$. Deodorization relates to operating the extraction system at less than the maximum solubility. The extraction conditions are usually held constant while the components of interest, generally the more soluble ones, are preferentially removed from the matrix. This type of application is appropriate for the removable of objectionable aromatics or the extraction of desirable odor components such as with spices. While all extraction applications

fractionate the original matrix somewhat, Riviz et al. (60) use the term fractionation to describe the separation of coextracted components from each other. They also use the term to describe the concentration of components either as the extractant or in the residual material.

Table II lists the extraction conditions using SF-$CO_2$ for a variety of compounds found in the literature. These compounds have either been intentionally added or are naturally present in foods and include essential oils, herbicides or pesticides, and lipids.

Generally, the best spice extracts have all the organoleptic factors of the spice even after dilution of the extract and are similar to the commercially obtained extracts. In 1981 Caragay (101) reviewed the extraction condition of spices including cloves, cinnamon and vanilla pods. Stahl and Gerard (65) studied the solubility and fractionation of essential oils in SC-$CO_2$. They were able to obtain quantitative recovery of volatile oils free of undesirable substances without fractionation of the essential oils themselves. Once the essential oil components were extracted, further fractionation into certain substance groups was possible. Several laboratories have employed off-line extraction techniques in the analysis of flavors from ginger, pimento berries, apple essence (66) and lemon peel (68). Using GC as the means of analysis these laboratories were able to show some fractionation of the flavor components as a function of the extraction density. Other laboratories have investigated the use of SFE in flavor analysis by direct coupling of the extractor to a chromatographic technique such as GC (67,70) and SFC (69).

Herbicides and pesticides have been extracted using SC-$CO_2$ with (25, 73-74) and without (71-72) the addition of polar modifiers. Schafer and Baumann (71) determined the solubility limit of several pesticides in 100% $CO_2$ at 40°C and 200 bar.

TABLE II. Summary of Work in SFE Using SC-$CO_2$ Related to Food Components and Natural Products

| Commodity | Conditions | Reference |
|---|---|---|
| **Flavors/Spices:** | | |
| essential oil components (limonene, carvone, anethole, eugenol, caryophyllene, valeranone) | 40-120°C; 40-120 bar | 65 |
| ginger, pimento berries | 50°C; 1500-5000 psi | 66 |
| rosemary | 45°C; 300 atm; 10 min; cryotrapping -50 to 25°C | 67 |
| lemon peel oil | 30-58°C; 90-250 $kg/cm^2$ | 68 |
| cold-pressed grapefruit oil | 70°C; 0.1767-0.8579 g/mL; 12 min; cryotrapping -65 or -10°C | 69 |
| eucalyptus leaves, lime peel, lemon peel, basil | 45°C; 300 atm; 10 min.; cryogenic trapping -50 to 30°C | 70 |
| **Herbicides/Pesticides:** | | |
| sulfonylurea herbicides and metabolites | 2% methanol in $CO_2$; 40°C; 6 mL/min; 223 bar; 1.5-8 min | 25 |
| Linuron, methoxychlor, Diclofopmethyl, Diclofop and 2,4 D | 40°C; 200 bar; 6-540 min | 71 |
| Lindane, aldrine, pp'-DDT | 138 bar; 15 min; no temperature reported | 72 |
| diuron, linuron | 0-20% methanol or 10% acetonitrile in $CO_2$; 75-100°C; 110-338 bar | 73 |
| diuron, linuron in sassafras soil and wheat grain | methanol or ethanol in $CO_2$ or sample matrix; 120°C; 0.4-0.7 g/mL; 35-105 min. | 74 |

TABLE II. Summary of Work in SFE Using SC-$CO_2$ Related to Food Components and Natural Products (cont.)

| Commodity | Conditions | Reference |
|---|---|---|
| **Lipids:** | | |
| palm oil | 10 wt% ethanol in $CO_2$; 50-110°C; 50-200 bar | 64 |
| soybeans | 50-60°C; 2000-10,000 psi | 75,76 |
| triacylglycerols from ground copra | 40 or 60°C; 300 to 900 bar | 77 |
| rapeseed, sunflower seed, soybeans | 40 or 50°C; 300 bar<br>40°C; 300-700 bar | 78 |
| cottonseed oil | 50-80°C; 8000-15,000 psi | 79 |
| soybean, soya flakes, lupineseed, cottonseed, jojoba | 40°C; 350 bar | 80 |
| soybeans, peanuts, cottonseed | 50°C; 8000 psi | 81 |
| wet and dry milled corn | 50-90°C; 5000-8000 psi | 82,83 |
| butter oil | 40°C; 300-350 kg/cm$^2$ | 84 |
| oils from mackerel powder | 40°C; 4.9-24.5 MPa | 85 |
| soybeans | 80-100°C; 10,600-12,400 psi; 20 min | 86 |
| oils from Antarctic krill | 40-80°C; 250 or 400 kg/cm$^2$ | 87 |
| cheese, butter, coffee, tobacco, camomile | 40°C; 100 bar | 37 |
| canola seed | 55°C; 36 MPa | 88 |
| menhaden oil fatty acid ethyl esters | 40-100°C; 2200 or 2500 psi | 89 |
| soybean | 40.6°C; 8.9-18.4 MPa | 90 |

TABLE II. Summary of Work in SFE Using SC-$CO_2$ Related to Food Components and Natural Products (cont.)

| Commodity | Conditions | Reference |
|---|---|---|
| **Other:** | | |
| steroids | 40°C; 80-200 bar | 91 |
| coffee | 20-80°C; 100-250 bar; 0-20% moisture, 20-80 min | 92 |
| Shiitake mushrooms | 40°C, 3000 psi; 4 L/min | 93 |
| trichothecenes from wheat | 61-98°C; 100-300 bar | 94 |
| Vitamin $K_1$ in powdered infant formulas | 60°C; 8000 psi; 15 min | 95 |
| carotene and lutein from leaf | 40°C; 10-70 MPa; 5-6 L/min | 96 |
| tocopherols in wheat germ | 40°C; 250 bar | 97 |
| ouabain | 80°C; 400 atm; 30 min | 54 |
| furocoumarins | $H_2O$ or ethanol in $CO_2$ or sample; 6L/min; 30-60°C; 100-400 bar | 98 |
| coumarins, lignans, phenylflavonoids | 3 wt % $H_2O$ or ethanol in $CO_2$; 6 L/min; 40°C; 400 bar | 99 |
| pigments | 3 wt % $H_2O$ or ethanol in $CO_2$; 6 L/min; 35 or 40°C;130-400 kg/cm$^2$ | 100 |

Two acid pesticides, 2-[4-(2,4-dichlorophenoxy)-phenoxy]propanoic acid and (2,4-dichlorophenoxy)acetic acid had low solubilities in the $CO_2$. Engelhardt and Grob (72) could extract non-polar pesticides at the 10 ppm level from a soil matrix with 100% $CO_2$. McNally and Wheeler (25, 73-74) have studied the effect of modifier, temperature, density and matrix on the extractability of a variety of herbicides and pesticides. The use of polar modifiers increases extraction efficiencies. Enhancement of the extractions depended on the modifier employed, the solute of interest and the sample matrix. The matrices investigated include sassafras soil (73-74), reagent grade sand, whole wheat kernels, wheat flour, wheat straw and cell culture medium (25). Reagent grade sand provided a non-interactive matrix for the urea herbicides extracted.

A variety of lipid containing products including vegetable, animal and fish products have been extracted under SFE conditions. The characteristic properties of extracted seed oils are dependent on the extraction conditions. The properties that are readily affected include: color, turbidity, odor, and solubility (75-76, 79, 80-83, 85-86). Crude oils obtained from SC-$CO_2$ extraction processes investigated by the United States Department of Agriculture (USDA) (75-76, 79-83) had lower refining losses. These lower refining losses were attributed to the lower phosphorus levels in the SC-$CO_2$ extracted crude oils because of the virtual insolubility of phospholipids in SC-$CO_2$. Comparable phosphorus levels are obtained in hexane extracted oils only after degumming. Thus, an advantage to SC-$CO_2$ extraction is the elimination of this processing step. A consequence of initial low phosphorus content, however, is that the SC-$CO_2$-extracted crude oils are less stable to oxidation.

By using SFE methodology fractionation of lipids can be done to improve the final product. Shishikura et al. (84) could lower the cholesterol level and improve the

spreadability of butter oil. Generally the triacylglycerols are readily extracted but after approximately 80% of the oils are extracted, a decrease in extractability is reached. The spreadability of the extracted butter oil was improved because the triacylglycerols larger than C46 were concentrated in the latter fractions and the residual oil. The cholesterol content of the butter oil was only lowered after passing the SC-$CO_2$ extract through a silicic acid column. Fattori and co-workers (88) demonstrated the fractionation of lipids in canola seed extracts. They found that later fractions of the canola seed extracts were richer in C22 and C24 fatty acids. The concentration of the C24:0 fatty acid (erucic acid) in the final fractions makes it possible to produce low erucic acid canola oil. This apparent fractionation does not occur with a hexane extraction process.

Studies have also dealt with the effect of SFE on the residual proteins of the oilseed meals. (76, 80, 86) Generally these studies investigated protein solubility, flavor, and amino acid content and showed that SC-$CO_2$ extractions had negligible influence on the quality of the extracted meals. Friedrich and Pryde (76) noted that the germ flour obtained from SC-$CO_2$-extracted corn germ had a nearly 10-fold reduction in residual peroxidase activity. This reduction was attributed to the denaturization of the enzyme and indicated a favorable control of oxidative rancidity during storage.

Other compounds that have been investigated using SFE techniques include steroids (91), trichothecenes (94) and ouabin (54). Stahl and Glatz (91) extracted steroids with 100% $CO_2$ at 40°C and various pressures. While they were able to extract steroids with three hydroxyl groups below 300 bar, they were unable to extract those steroids containing four hydroxyl groups, three hydroxyl and one acid group, one phenolic hydroxyl with two other hydroxyl groups and any of the glycosides. Kalinoski

et al. (94) could extract deoxynivalenol (DON), a trichothecene with three hydroxyl groups, from a wheat matrix. Diacetoxyscirpenol and T-2 toxin were also extracted. These compounds have only one hydroxyl group and are less polar than DON. Xie et al. (54) were recently able to extract ouabin, a steroid-derived glycoside with eight hydroxyl groups using 100% $CO_2$.

In summary, while many compounds have been extracted using SC-$CO_2$, the majority of the work related to food products can be divided into three broad catagories: flavor/spices, herbicides/pesticides and lipids. SFE techniques have been shown to improve the final food product. The loss of the volatile components desired in spice extracts is reduced, since $CO_2$ is a gas at room temperature and the higher temperatures required in distillation are not needed. Apparent fractionation of higher MW lipids can reduce the erucic acid, a toxic substance, content of canola oil. A processing step in the preparation of refined vegetable oil is eliminated when using SFE. SFE is becoming a desirable sample preparation method due to the ease of interfacing SFE instrumentation to a variety of chromatography systems. Also, recovery of extracted material is easily achieved since $CO_2$ is a gas at room temperature. While many compounds are soluble in SC-$CO_2$ the addition of small quantities of polar organic solvents or $H_2O$ can enhance extraction recoveries from complex matrices. As more polar modifiers are added, the selectivity of the extraction is reduced requiring additional purification steps. SFE is generally rapid when dealing with mg sample sizes. As the sample size is increased the extractions take longer. Statistical studies using real sample matrices are highly desirable to ensure sample homogeneity and to determine sample sizes required for trace analysis ($\leq$ ppb).

The research presented here deals with three applications of supercritical fluid

technologies. The first application relates to the study of peracetylated nitrogen derivatives. The literature indicated that a higher MW range of certain carbohydrate derivatives was separated under SFC conditions than that achieved using GC conditions. The study was initiated to increase the detectable MW range of the peracetylated aldonitrile (PAAN) derivatives by employing SFC. The PAAN derivatives were not previously separated under SFC conditions. During the study more than one reaction product per sugar was detected when employing SFC. Some of these additional reaction products were identified using SFC with on-line FT-IR and MS detection.

The second application relates to the use of SFC/FT-IR in monitoring changes in chemical composition of soybean oil due to processing. Processing of soybean oil can readily change the fatty acid content of the original oil. Analytical methods that permit the identification of individual isomers caused by partial hydrogenation is desirable. IR detection is used to determine the amount of conversion of cis isomers to trans isomers. SFC is easily interfaced to flow-cell FT-IR detection. The literature has shown that FAMEs, FFA and triacylglycerols can be separated under SFC conditions. A packed column SFC/FT-IR report (34) indicated that the saturated and unsaturated C18 fatty acids were partially resolved spectroscopically even though chromatographic resolution was not achieved. Another study using SFC/FID only, indicated that by using an open tubular column with a polar stationary phase the unsaturated C18 fatty acids were resolved (32). Triacylglycerols also can be separated under relatively mild conditions using SFC. A report from 1985 (31) indicated that the C54 triacylglycerols, tristearin, triolein, trilinolein and trilinolenin, were separated using a 50% cyanopropylphenyl polysiloxane open tubular column in 130 min. These reports prompted the investigation of SFC/FT-IR with open tubular columns as a potential analytical method in the

determination of isomers in partially hydrogenated vegetable oil.

The third application deals with SFE of a model compound system based on the coumarin structure. This study was initiated because of preliminary SFC work with aflatoxins and coumestrol. The aflatoxins required high density to elute from an open tubular column even though there are no hydroxyl or carboxyl groups on their backbone. Coumestrol was not detected employing SFC. The reasons these compounds were difficult to separate using SFC were not readily explained by the empirical rules relating to solubility in SC-$CO_2$. Examination of their structures showed that they contained the coumarin structure within their backbones. The SFE literature indicated that two laboratories (98-99,102) did limited work with the coumarins. Due to the availability of coumarins with a variety of functional groups, a study was initiated to determine if the empirical rules within the literature could be enhanced by studying this structural system.

## III. PERACETYLATED NITROGEN DERIVATIVES OF MONOSACCHARIDES

### A. INTRODUCTION

Chromatographic methods for the analysis of carbohydrates encompass a broad range of techniques including HPLC, GC and SFC. Many HPLC methods have been published in the literature (103), but a basic limitation using HPLC methods is that generally only two detection modes are available, namely, ultraviolet (UV) and refractive index (RI). The lack of convenient chromophores on underivatized carbohydrates requires the use of UV detection at very low wavelengths (190-210 nm), thus limiting the usable solvents to high purity acetonitrile and water. Impurities in the sample also can lead to interferences if monitoring at these low wavelengths. Mobile phase gradients cannot be used with IR detection, thus limiting its usefulness in complex sample analysis. A recent report (104) describing the use of pellicular ion exchange resins and pulsed amperometric detection shows some promise in addressing these problems.

GC methods for carbohydrate analysis require derivatization. Most of the sugar derivatization methods (105) yield more than one chromatographic peak per sugar thus complicating the separation. An advantage of multiple peaks is that a fingerprint is provided for more confident sugar assignment. Preliminary modification of the sugar to its alditol or oxime prior to the formation of volatile derivatives has been shown to produce single peaks for each saccharide under GC conditions. A serious disadvantage of many of the derivatization methods is that anhydrous conditions are required. However, Brobst and Lott (106) formed the trimethylsilyl derivatives using

trimethylsilylimidazole as a reagent in the presence of water. Reduction of carbohydrates to their corresponding alditols usually yields single compounds. The procedure can cause quantitation problems if fructose and glucose are present in the mixture being analyzed. This problem arises because fructose is reduced to a mixture of mannitol and glucitol; while, glucose is reduced to glucitol alone. The time required to form alditols negates their usefulness in the routine analysis of many samples. For example, a derivatization method previously reported by Englyst et al. (107) and considered simple and rapid in a recent review (105), requires four hours. Guerrant and Moss (107) could form the alditol acetate derivatives in 2 hr.

The acetylation of sugar oximes leads to the formation of peracetylated aldonitrile (PAAN) derivatives. The formation of the acetylated nitrile from the oxime is the first step in the well-known Wohl sugar degradation. The preparation of these derivatives using acetic anhydride in pyridine (109) or N-methylimidazole (110) is rapid and simple. A later modification (111) of the procedure using N-methylimidazole showed that the PAAN derivative could be formed in the presence of water or aqueous acid, thus eliminating the need to neutralize and dry hydrolysates of complex carbohydrates. The formation of the oxime precursor involves both aldoses and ketoses, but subsequent nitrile formation is limited to the aldoses (Figure 1). Thus the method is not useful in the analysis of fructose (i.e., a ketose). It is generally assumed that the PAAN derivatization procedure yields only one product per sugar, but a review of the literature indicates that this is not necessarily the case. Furneaux (112) studied the reaction products arising from D-galactose and D-glucose preparatory-scale derivatization under anhydrous conditions via NMR analysis. The study revealed that several oxime hexaacetates (Figure 2) were formed as additional reaction products of

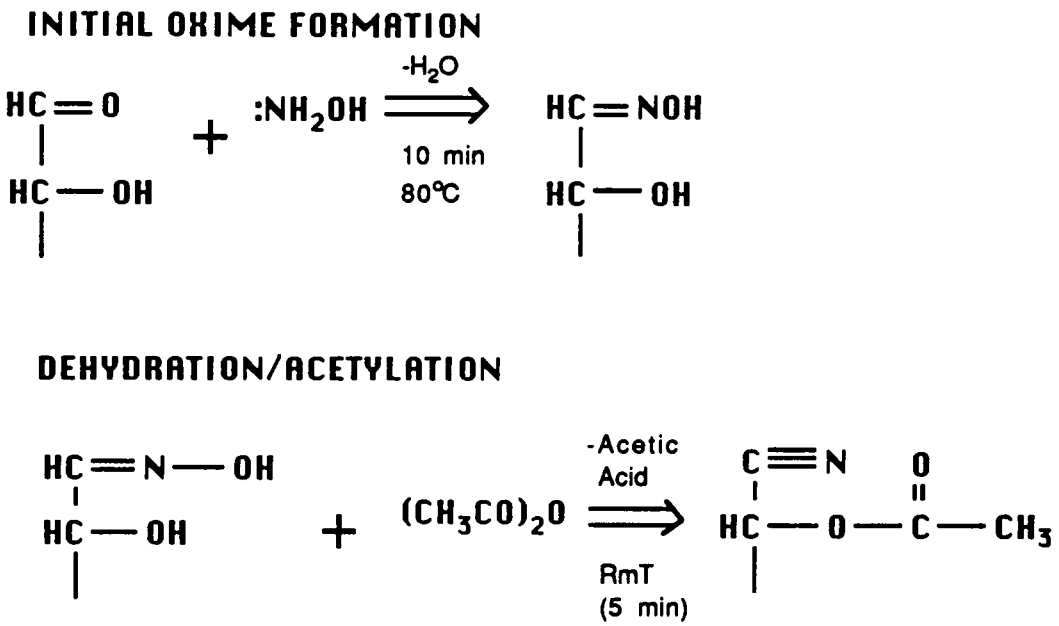



**Figure 1.** Derivatization scheme for the monosaccharides.

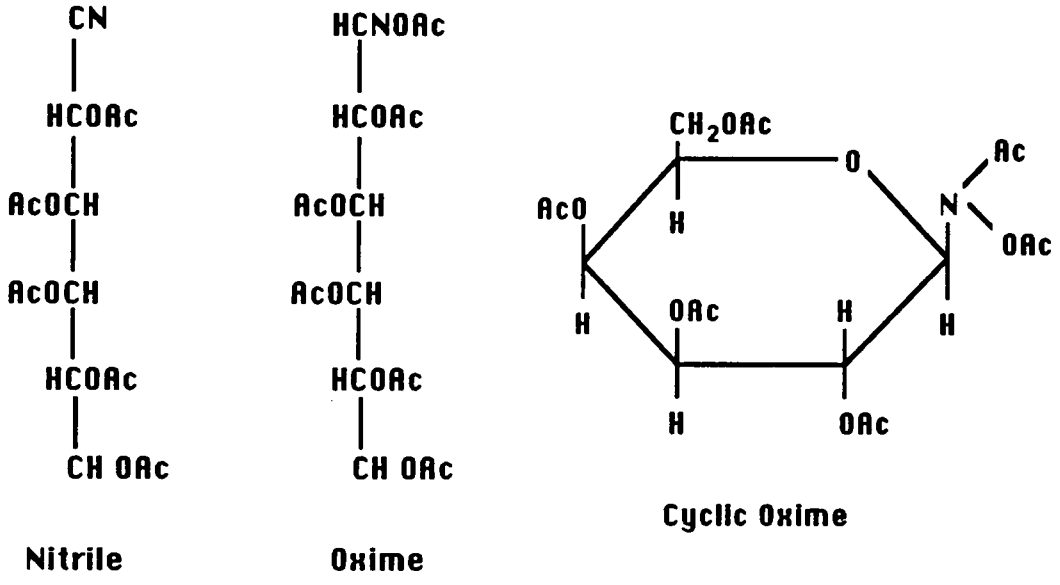



**Figure 2.** Molecular structures of possible products from the dehydration and acetylation of galactose oximes.

D–galactose and D–glucose. Early work of Deulofeu and coworkers (113) indicated that under anhydrous derivatization conditions, D-arabinose, D-xylose and L-rhamnose gave the nitrile exclusively. Several studies indicated that the peracetylated oximes (114) and the o-methyloxime acetates of amino sugars (115) are more difficult to analyze by GC on polar packed columns than the peracetylated nitrile derivatives. The o-methyloxime acetates of amino sugars only chromatographed after the oven temperature reached 240°C using 1% diethylene glycol adipate on 100-240 mesh Chromosorb W (I/8" o.d. x 6') as the stationary phase. The paper did not indicate the temperature of the injector, but the initial oven temperature was I70°C. Guerrant and Moss (108) separated acetylated o-methyloximes and alditol derivatives of neutral, alcohol and amine sugars employing a 50 m OV-1 capillary column. With this column, the o-methyloxime acetate derivatives of the neutral sugars generally eluted below 250°C. The o-methyloxime acetate derivatives of the amino sugars analyzed eluted between 250 to 260°C. Peracetylated nitrile sugar derivative procedures via GC are limited to the analysis of mono and disaccharides due to the low volatility of derivatives of higher oligosaccharides. PAAN derivatives of the monosaccharides can be completely resolved under isothermal conditions (195°C) using 1% diethylene glycol adipate on 100-120 mesh Chromosorb WHP (3.05 m x 3.2 mm) as the stationary phase (111).

Chester et al. (18) used capillary SFC for extending the separation range of oligo- and polysaccharide TMS derivatives beyond that achieved with GC. This work prompted the investigation of SFC for the analysis of peracetylated aldonitrile derivatives. Packed column and capillary SFC with FID were employed. Since multiple peaks were obtained for the monosaccharides studied, FT-IR and MS detection were used to identify the additional reaction products (20,116).

## B. EXPERIMENTAL

### Packed column SFC

A Suprex (Pittsburgh, PA) Model 200A supercritical fluid chromatograph equipped with a FID was used. SFC/FT-IR data were collected with a Nicolet (Madison, WI) 5SXC spectrometer equipped with a prototype 0.6 mm i.d. x 5 mm pathlength (1.4 μL) high pressure flow-through cell. All spectra were obtained in real time at 8 $cm^{-1}$ resolution. Spectra were acquired by collecting one file/sec, four scans/file in real time. The flow cell was maintained at 33°C.

A cyanopropyl Deltabond™ column (Keystone Scientific, Inc., Bellefonte, PA) of 250 mm x 1 mm i.d. with 5 μm particle diameter was used. Chromatographic separation was achieved with SFC grade $CO_2$ (Scott Specialty Gases, Plumsteadville, PA) as the mobile phase. The chromatographic conditions are reported in the Figure captions.

### Capillary column SFC

A Lee Scientific, Inc. (Salt Lake City, UT) Model 501 SFC with a FID was used. SFC/FT-IR data were collected with a Nicolet (Madison, WI) 740SX spectrometer equipped with a 0.6 mm i.d. x 5 mm pathlength (1.4 μL) high pressure flow-through cell. All spectra were obtained in real time at 8 $cm^{-1}$ resolution. Spectra were acquired by collecting 1.1 files/s, 8 scans/file in real time. The flow cell was maintained at 35°C and the transfer line was 100°C. A SB-cyanopropyl-25 bonded fused silica capillary column (Lee Scientific, Inc., Salt Lake City, UT) of 7 m length and 100 μm i.d. with 0.25 μm film thickness was used for the SFC/FT-IR work. In most experiments

FID (350°C) was employed subsequent to FT-IR wherein a frit restrictor of 100 μm i.d. was employed. Chromatographic separation was achieved with Coleman grade $CO_2$ (Matheson, Dorsey, MD.) which was used as the mobile phase. A 100 nL direct injection or a 200 nL timed-split injection of 1 second duration was employed for the analysis of the derivatization mixtures. The chromatographic conditions for each separation are reported in the Figure captions.

SFC/MS data were collected by J. A. G. Roach (FDA, Division of Contaminants Chemistry, Washington, D.C.) with a Finnigan 3300 CI Mass Spectrometer interfaced to a Lee Scientific, Inc., Model 501 SFC. The interface employed has been previously described (44). A SB-cyanopropyl-25 bonded fused silica capillary column of 3 m length by 100 μm i.d. with 0.25 μm film thickness was used for the MS work. Chromatographic separation was achieved with Coleman grade $CO_2$ (Matheson, Dorsey, Md.) which was used as the mobile phase. Reagent gases were methane and ammonia. The frit restrictor tip was maintained at 200°C. For analysis of the model saccharide derivatization mixture a 200 nL spilt injection was performed. The data obtained for the xylose derivatization mixture utilized 2 μL injections with a retention gap.

Derivatization

The PAAN derivatives were synthesized by J. Rose (Department of Food Science and Technology, VPI&SU, Blacksburg, VA) using a modification of the method described by McGinnis (111). A stock solution of the derivatizing reagent was prepared by dissolving 0.5 g hydroxylamine hydrochloride in 19.9 mL N-methylimidazole and then adding 0.1 mL internal standard stock solution (i.e. 10 mg methylglucopyranoside/mL methylimidazole). A mixed sugar stock solution was prepared to contain 1 mg/mL of

each of the following sugars in $H_2O$: rhamnose, fucose, arabinose, xylose, mannose, glucose and galactose (Figure 3). Stock solutions of some of the individual sugars were also prepared at a concentration of 2.5 mg/mL. Derivatizations were accomplished by mixing 0.4 mL of the derivatizing stock reagent and 0.2 mL of the sugar stock solution in a screw-cap vial with a Teflon-lined septum. The vial was heated with stirring for 10 minutes at 80°C, allowed to cool, and then 1 mL of acetic anhydride was added. After 5 minutes at room temperature, 1 mL of chloroform was added after which the solution was extracted twice with 1 mL of water. The aqueous extracts were discarded and the chloroform fraction after drying over approximately 0.4 g anhydrous sodium sulfate was used for the chromatography study. The PAAN derivatives were initially analyzed by GC to ensure that the derivatization reaction had proceeded per the literature reference. The GC conditions employed were as follows: 1% diethylene glycol adipate packed column (3 m x 0.32 cm) (Supelco, Inc., Bellefonte, PA); oven temperature = 200°C; injection port = 225°C; detector = 225°C; $N_2$ carrier gas (35 mL/min); detector gases: $H_2$ (35 mL/min) and air (300 mL/min).

## C. RESULTS AND DISCUSSION

The GC trace (Figure 4) obtained for the derivatized reaction mixture shows as expected seven peaks via flame ionization detection for the seven model monosaccharide derivatives chosen for study here and a peak for the internal standard. When this apparent eight component mixture was chromatographed under SFC conditions with a CN-Deltabond™ packed column, pressure programming and flame ionization detection 13 major peaks with retention times between 16 and 22 minutes were observed in addition

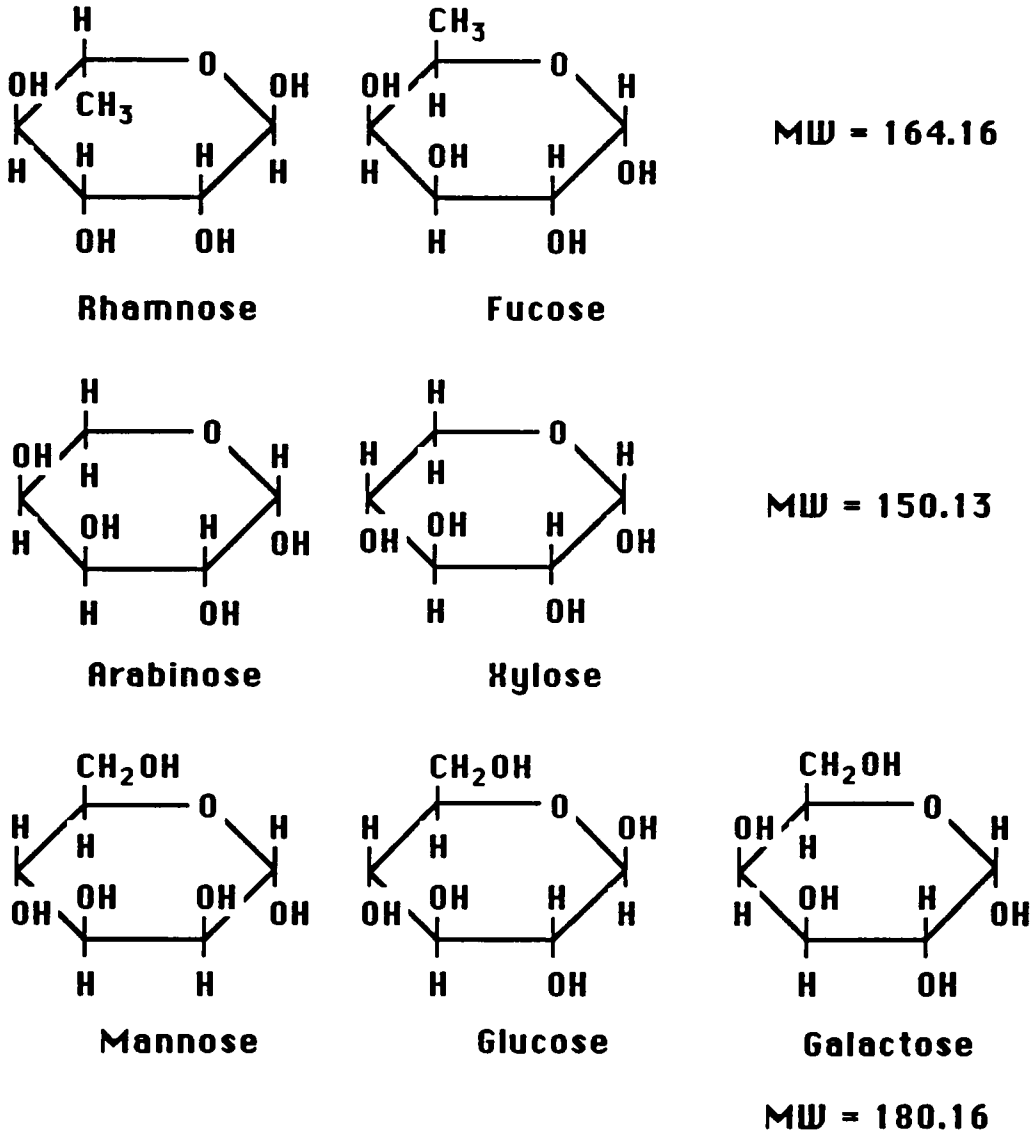



**Figure 3.** Structures of the seven model monosaccharides employed in this study.

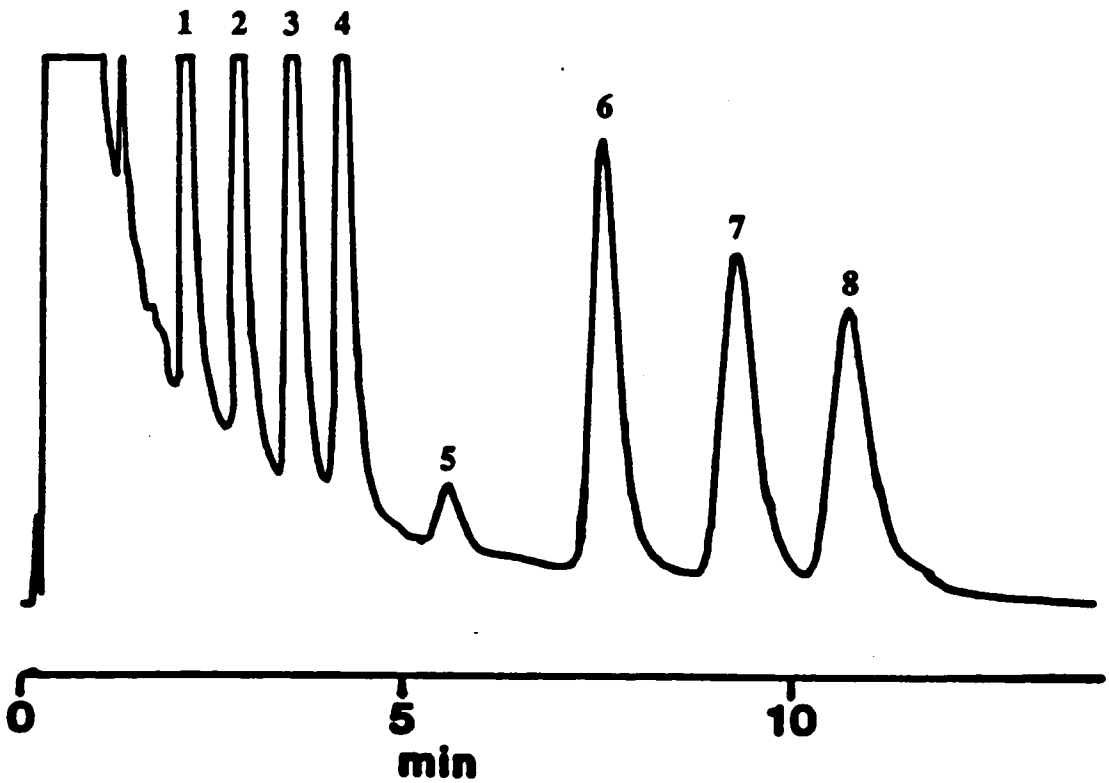



**Figure 4.** Gas chromatogram of seven component peracetylated aldonitrile monosaccharide derivatives. 1% diethylene glycol adipate packed column (3m x 0.32 cm): oven temperature (isothermal) = 200°C; injection port = 225°C; flame ionization detector = 225°C; $N_2$ carrier gas (35 mL/min); detector gases: $H_2$ (35 mL/min) and air (300 mL/min). 1. Rhamnose; 2. Fucose; 3. Arabinose; 4. Xylose; 5. Internal standard; 6. Mannose; 7. Glucose; 8. Galactose.

to the earlier eluting solvent/reagent peaks (Figure 5a).

When the derivatization reaction mixture was chromatographed on a CN-propyl polysiloxane open tubular column the best separation for SFC/FID with a fixed restrictor (the approximate initial linear velocity was 2.3 cm/s measured at 100°C and a $CO_2$ density equal to 0.2 g/mL) was achieved at 140°C, with a $CO_2$ density program. Complete resolution of all the components was not achieved. A greater number of peaks with shoulders was evident beyond the solvent/reagent peaks than observed with the packed column separation (Figure 5b). Work by Furneaux (112) showed that the nitrile, the cyclic oxime and acyclic oximes were formed with galactose and glucose. Deulofeu and coworkers (113) reported that D-mannose, D-arabinose, D-xylose, and L-rhamnose gave exclusively the nitrile under anhydrous derivatization conditions. Quantitation of the individual sugars can be accomplished by combining the area counts of each peak attributed to a specific sugar. The presence of a complex mixture places extreme importance on complete resolution and accurate peak assignment.

In an effort to identify the additional derivatization products observed via SFC, FT-IR detection was performed on the eluate using a flow SFC/FT-IR interface. An equivalent of 50-100 ng based upon the underivatized sugar was chromatographed. Figure 6 is the Gram-Schmidt Reconstruction (GSR) of the packed column separation of the mixed sugar mixture. The on-line FT-IR spectral data show that the components responsible for the first six peaks in the cluster (16.4 to 19.2 min) are similar; while, the components responsible for the last seven peaks (19.4 to 22.0 min) have similar spectra to each other but the spectra are different from the first set. The separated sugar derivatives by the capillary SFC run, were not clustered by compound type as observed with the packed column separation. The major difference observed

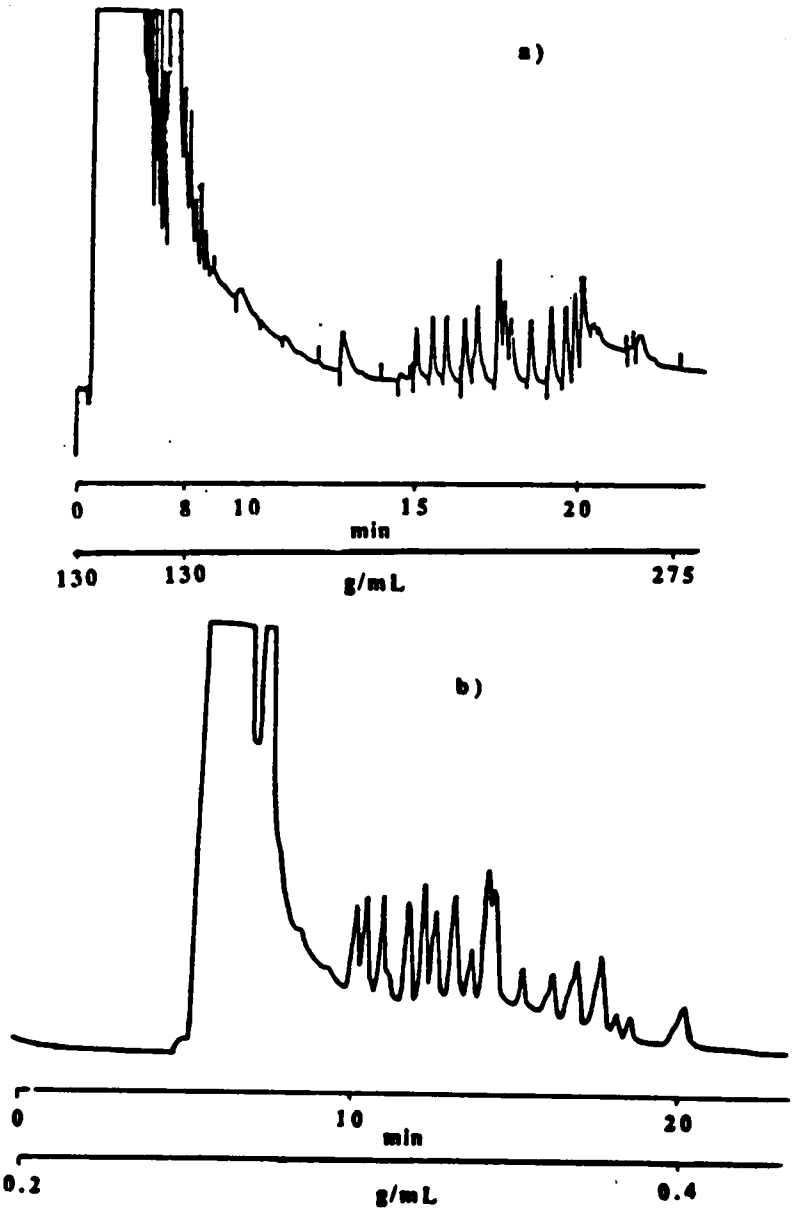



**Figure 5.** SFC separation of derivatized monosaccharide mixture with flame ionization detection. (a) Cyanopropyl Deltabond™ (250 x 1.0 mm i.d., 5 µm), $CO_2$, 100°C, Pressure program: 130 atm hold 8 min, 130 to 275 atm in 15 min. (b) SB-cyanopropyl-25 (10 m x 100 µm, i.d.), $CO_2$, 140°C, Density program: 0.2 to 0.5 g/mL at 0.01 g/mL/min.

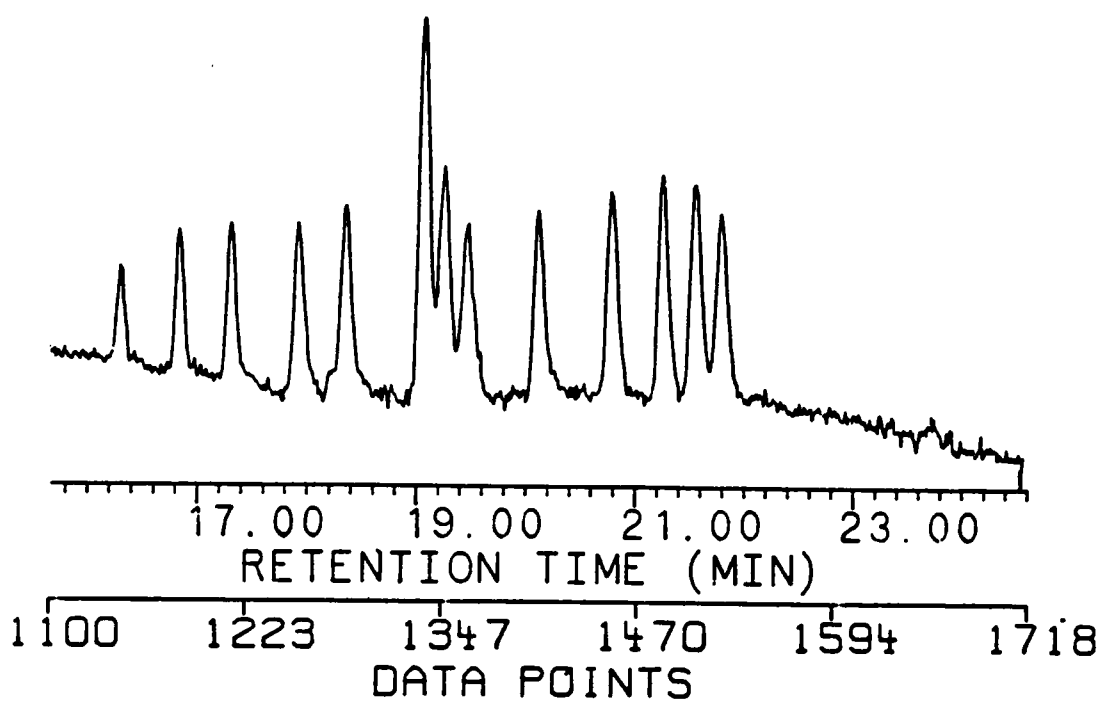



**Figure 6.** Partial Gram Schmidt Reconstruction of packed column separation of derivatized monosaccharides mixture. Cyanopropyl Deltabond™ column (250 x 1.0 mm i.d., 5 μm), $CO_2$, 100°C, Pressure program: 130 atm hold 8 min, 130 to 275 atm in 15 min.

between the components appears in the carbonyl stretching region (1750 to 1800 $cm^{-1}$). The first compound type gives rise to spectra (Figure 7a) where only one carbonyl stretch is observed at approximately 1760 $cm^{-1}$ consistent with the aldonitrile acetate derivative. The second compound type yields FT-IR spectra (Figure 7b) that exhibit two carbonyl stretching modes (i.e., 1760 $cm^{-1}$ and 1790 $cm^{-1}$). The higher frequency stretch can be attributed to the acetate moiety which is attached to the nitrogen of the oxime. Due to the incomplete resolution and the complex chromatographic profile obtained, xylose and glucose were chosen for further study using capillary SFC. The identification of the reaction products observed under SFC conditions was achieved by employing both FT-IR and MS detection. The FT-IR data obtained from the xylose and glucose derivatives were similar. MS data suggested similar molecular adduct ions and fragment ions although, their absolute values were different due to the difference in MW between the two sugars (Figure 3). Only the data for the xylose derivatives are reported.

Figure 8 is a partial GSR of the xylose derivatization mixture. The on-line FT-IR data show that the spectra of the components responsible for the first two peaks in Figure 8 are similar. The first peak is the response from the peracetylated methyl-D-glucose internal standard, while the second peak is the response from the peracetylated nitrile of xylose. These two components have spectra (Figure 9a,b) where only one carbonyl stretch is observed at approximately 1760 $cm^{-1}$ consistent with a single type of acetate moiety. A major difference between these two structures is in the C-O stretching vibration region. The C-O band for the internal standard (Figure 9a) absorbs near 1230 $cm^{-1}$ while the C-O stretching vibration for the nitrile (Figure 9b) absorbs near 1215 $cm^{-1}$. The shift to higher frequency of the C-O stretch is perhaps indicative

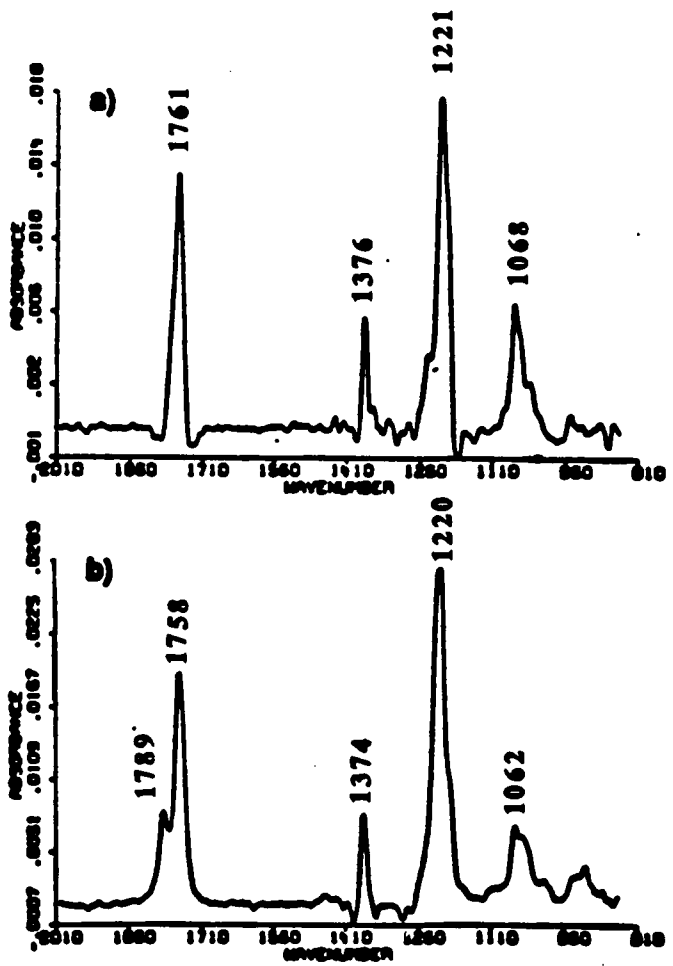



**Figure 7.** On-line FT-IR chromatographic file spectra of (a) early eluting rhamnose peak (i.e. peracetylated nitrile) and (b) late eluting rhamnose peak (i.e. peracetylated oxime). See Figure 5b for chromatographic conditions.

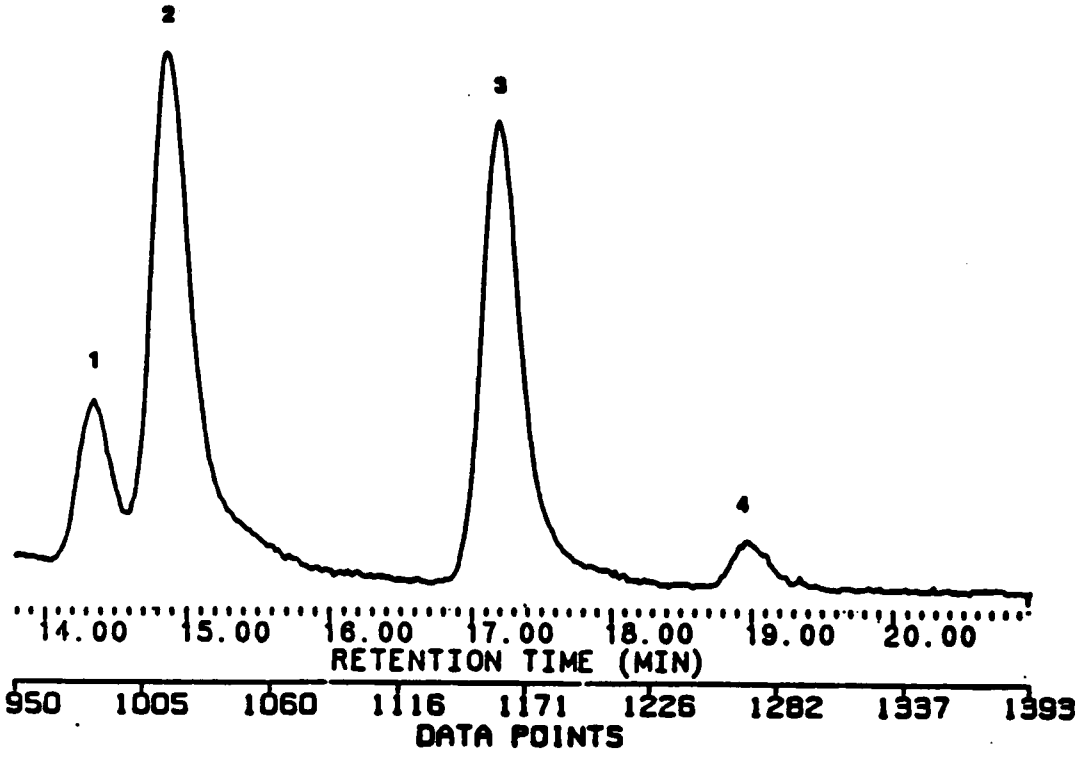



**Figure 8.** Partial Gram-Schmidt Reconstruction plot of a mixture of peracetylated nitrogen derivatives of xylose. Separation was performed on an SB-cyanopropyl-25 (7 m x 100 µm, i.d.) column at 100°C, $CO_2$. Density program: 0.20 to 0.28 g/mL at 0.01 g/mL/min, 0.28 to 0.40 g/mL at 0.025 g/mL/min, 0.40 to 0.60 g/mL at 0.01 g/mL/min, 0.60 to 0.70 at 0.025 g/mL/min hold 10 min. (1) internal standard, (2) nitrile, (3) acyclic oxime, (4) cyclic oxime.

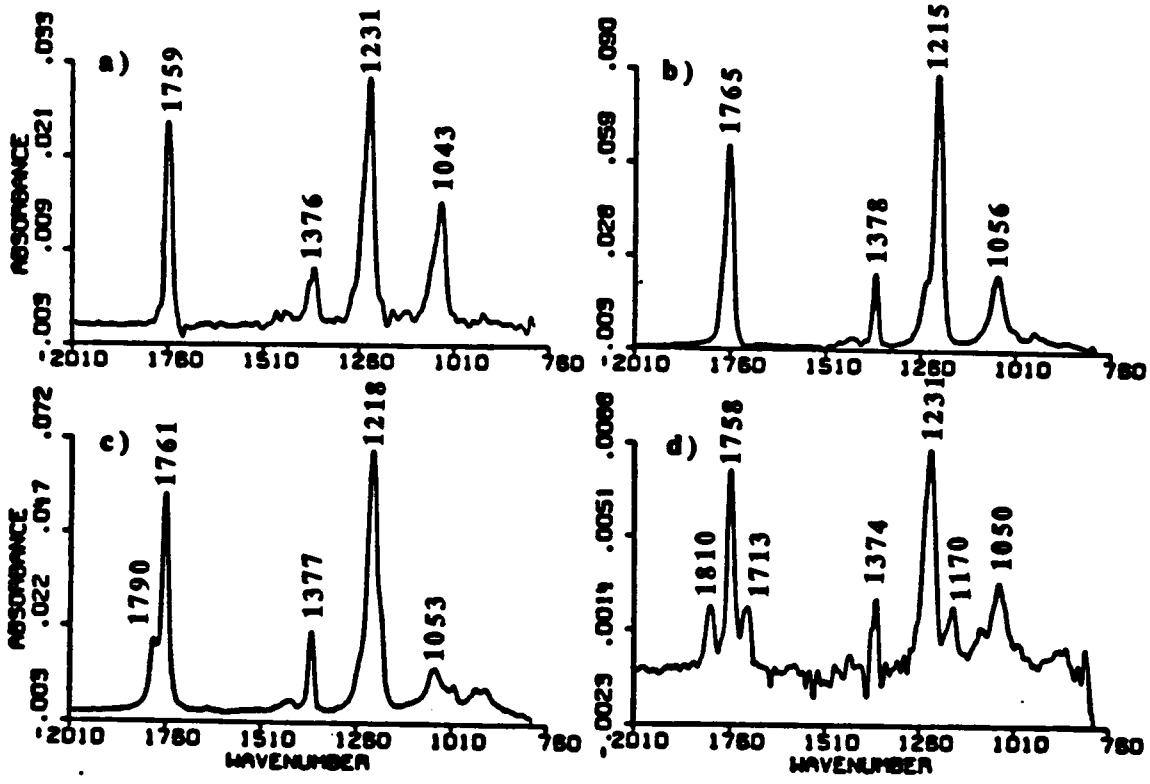



**Figure 9.** On-line FT-IR chromatographic file spectra of peracetylated nitrogen derivatives of xylose. (a) internal standard, 12 file coadded; (b) nitrile, 14 file coadded; (c) acyclic oxime, 16 files coadded; (d) cyclic oxime, 17 files coadded (8 scans/file).

of the increased constraint caused by the cyclic structure of the acetylated internal standard. The second xylose component (peak 3, Figure 8) yields a FT-IR spectrum (Figure 9c) that exhibits two carbonyl stretching modes (i.e. 1760 $cm^{-1}$ and 1790 $cm^{-1}$). The higher frequency C=O stretch as previously mentioned is attributed to the acetate moiety that is attached to the nitrogen of the oxime. The other major bands observed in the FT-IR spectra of the nitrile and oxime components include a strong band due to the C-O stretching vibration of the acetate moieties at 1220-1210 $cm^{-1}$, the C-H bending vibration of the $CH_3$ groups at 1380-1375 $cm^{-1}$ and the O-C-C ("alcohol" carbon-oxygen stretch) band corresponding to esters of primary and secondary alcohols between 1100-1000 $cm^{-1}$. The FT-IR spectrum of the third minor component (peak 4, Figure 8) resulting from the derivatization of xylose shows the presence of three C=O groups. A cyclic oxime structure such as is shown in Figure 2 should have three types of carbonyls present. The absorption of the N-acetyl group can be assigned to 1713 $cm^{-1}$. The carbonyl frequency of a tertiary amide (N-C=O) occurs in the range of 1680-1630 $cm^{-1}$ as reported by Tipson (117). However, electron attracting groups attached to the N should increase the frequency of absorption since such groups effectively compete with the carbonyl oxygen for the electrons of the N, thus increasing the force constant of the C=O band. Also, dissolution in supercritical $CO_2$ has been observed to shift the carbonyl as well as C-O stretching vibrations (118). The absorption of the carbonyl attached to the N-O group could be assigned to 1809 $cm^{-1}$. The O-C-C band also became broader with potentially two distinct bands, although due to the S/N in this region it is difficult to interpret.

Since the nitrile stretching region (2400-2100 $cm^{-1}$) is masked by the absorbance of supercritical $CO_2$, the presence of the nitrile functionality could not be

confirmed by on-line SFC/FT-IR. The weak C=N band (1660-1610 $cm^{-1}$) also is not assignable in the supercritical $CO_2$ matrix. SFC/FT-IR experiments on other individual derivatized sugars corroborated the hypothesis that each sugar produced the nitrile as well as the acyclic oxime. SFC/FID data indicate that a third component was also present.

Comparable experiments on the derivatized sugar mixture and individual sugars were carried-out via SFC/MS to determine if the multiple components per derivatized sugar exhibited different masses. When a split injection was employed for the MS experiments the third minor peak observed via the FID was not detected. The positive ion chemical ionization (PICI) MS data using methane as the reagent gas showed that both the first and second peak of each sugar had the same base peak: m/z 270 for rhamnose and fucose derivatives; m/z 256 for arabinose and xylose derivatives; and m/z 328 for mannose, galactose and glucose. These ions correspond to the loss of acetic acid from the protonated molecular ions of fully acetylated nitriles. A report (114) on the fragmentation pattern of the PAAN derivatives for 6-deoxy-L-mannose using GLC-PICI-MS (methane) also showed a base peak ion at m/z 270. The 6-deoxy-L-mannose derivative corresponds to the PAAN derivatives of rhamnose and fucose in our model mixture. For the other types of sugars in the model mixture, the most intense ion observed by previous workers (114) was $[(MH-60)-42]^+$. This ion was attributed to the loss of acetic acid and ketene ($CH_2=C=O$). This loss was not observed in our study.

In order to obtain molecular weight information ammonia ($NH_3$) was used as the reagent gas for both the derivatized sugar mixture and xylose. In CIMS with ammonia protonated molecular ions ($MH^+$) as well as, $[M+NH_4]^+$ adduct ions may be formed. For the derivatized mixture, the internal standard and two major peaks per sugar were observed. Figure 10 shows the reconstructed ion chromatogram (RIC) and the mass

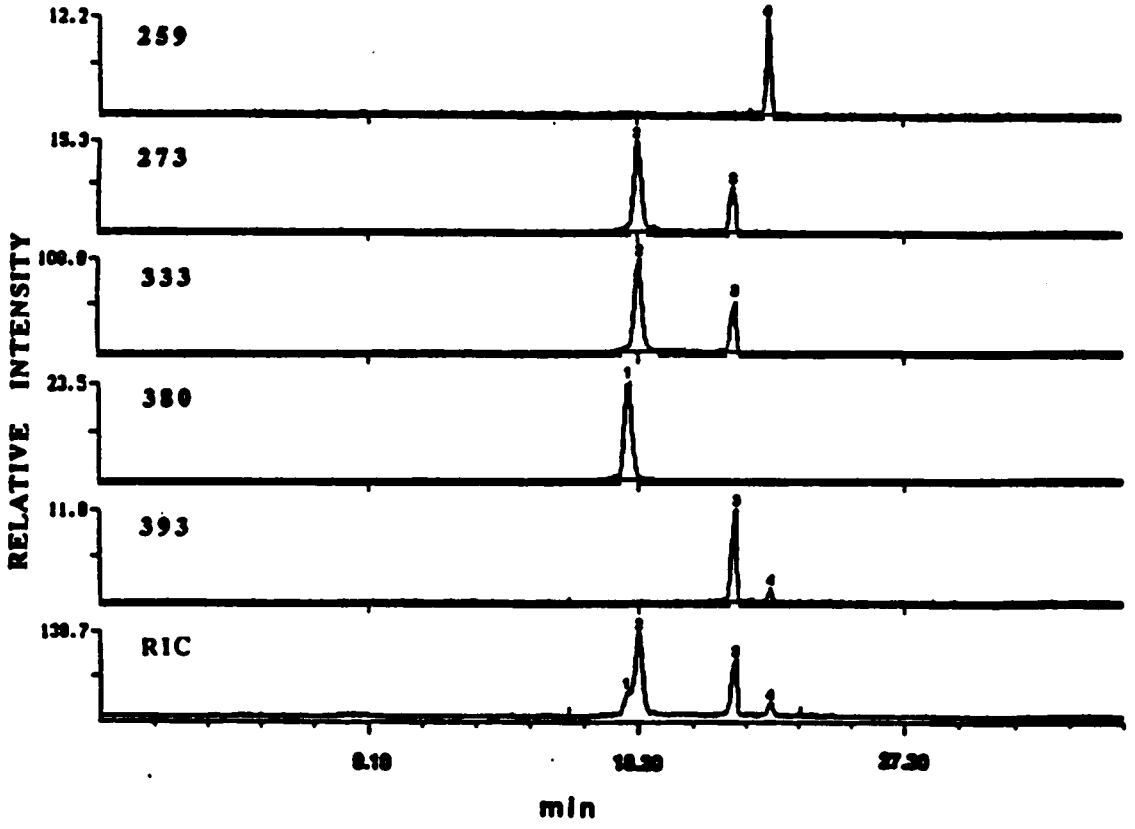



Figure 10. Selected ion profiles of the peracetylated nitrogen derivatives of xylose. SB-cyanopropyl-25 (3 m x 100 µm, i.d.) column, $CO_2$, 100°C, density program as in Figure 8. (1) internal standard {380}, (2) nitrile {333}, (3) acyclic oxime {393}, (4) cyclic oxime {393}. (Note: number in {} is the mass of the $NH_4^+$ adduct ion formed. RIC means reconstructed ion chromatogram)

chromatograms for the xylose derivatives. A third component was detected for xylose beyond that previously observed for the derivatized sugar mixture. The PICI ($NH_3$) mass spectrum of the peracetylated methyl-D-glucose, the internal standard, gave an ammonium adduct ion at m/z 380 (Figure 11a). The mass spectra of the first two peaks associated with xylose, the acetylated nitrile and the acetylated acyclic oxime, gave ammonium adduct ions m/z 333 and 393 respectively. The mass spectrum of the acetylated nitrile (Figure 11b) also gave fragment ions, m/z 273 and 256, corresponding to the loss of acetic acid from the ammonium adduct and the $MH^+$ ion respectively. The characterization of m/z 333 and 256 of the acetylated nitrile have been reported previously by Seymour and co-workers. (114) The mass spectrum of the acetylated acyclic oxime (Figure 11c) gave ions at m/z 333 (base peak), 273 and 256.

The FT-IR spectrum of the third minor peak (Figure 9d) and its corresponding CIMS spectrum (Figure 11d) obtained from derivatized xylose appears to correlate with one of the cyclic oximes that Furneaux (112) described having either a pyranose orfuranose structure. The current data do not permit an exact assignment. The mass spectrum of the third component (Figure 11d) is more complicated than the first two peaks associated with the peracetylated derivatives of xylose. In addition to the $NH_4^+$ adduct ion at m/z 393 and the $MH^+$ ion at 376, the base peak ion observed at m/z 259 corresponds to the loss of 117 Daltons from the protonated molecular ion possibly resulting from cleavage of the ring nitrogen-carbon bond of the cyclic oxime (see Figure 2). The mass spectrum of the internal standard, also a cyclic structure, yields no major fragmentation corresponding to the loss of acetic acid. Thus, since the third component is postulated to have a cyclic structure it would follow that the primary fragmentation observed would occur at the side chain. The additional ions observed, m/z 318 and 276

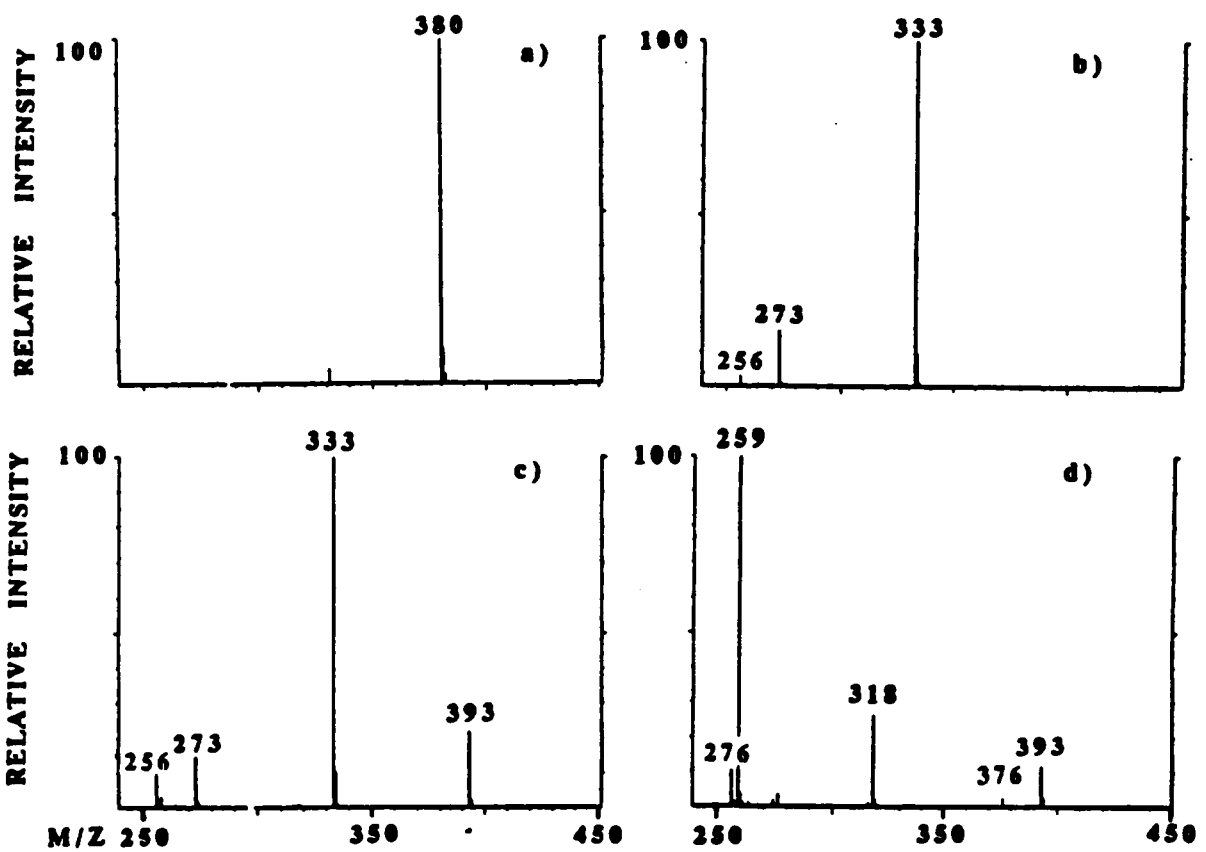



**Figure 11.** On-line MS chromatographic file spectra of peracetylated nitrogen derivatives of xylose. (a) internal standard, (b) nitrile, (c) acyclic oxime, (d) cyclic oxime.

could correspond to fragmentation of the protonated molecular ion via loss of acetone in the former and loss of acetone and ketene in the latter case.

## D. CONCLUSIONS

The FT-IR and MS data indicate that with the derivatization procedure using N–methylimidazole and acetic anhydride in the presence of $H_2O$, all the monosaccharides investigated produced not only the nitrile but at least one oxime acetate as reaction products. SFC provided a means of studying those derivatization products that were not observable under conventional GC conditions either because the high temperatures employed lead to decomposition of the products or because some of the derivatization products were not volatile. With the lower temperatures and solvating capabilities inherent to SFC, some of these reaction products were easily observed with a variety of detectors. While derivatization of polar compounds makes them amenable to analysis by GC as well as SFC, GC derivatization methods are not necessarily applicable to SFC. The attraction for using PAAN derivatives is that the GC analysis is simplified (109-111). The SFC separation is not simplified due to the presence of multiple peaks for each sugar. An advantage of multiple peaks is that a fingerprint is provided for more confident sugar assignment. Furthermore, the effect of modifications to derivatization procedures which are designed to eliminate additional reaction products can be quickly investigated employing SFC.

## IV. A Study on the Use of SFC/FT-IR in Investigating the Changes in the Chemical Composition of Soybean Oil Due to Processing

### A. INTRODUCTION

Soybean oil is one of the most important edible oils in the US. It is a valuable commodity because it is an inexpensive edible oil that has many of the characteristics of premium vegetable oils (corn, safflower and sunflower oils). Soybean oil has a high linoleic acid content (51%) and a low saturated fatty acid content (15%). The oil remains liquid over a wide temperature range. Considerable efforts have been made in the modification of soybean oil for food technology applications. It can be selectively hydrogenated for blending with semisolid or liquid oils. Although the phosphatides are present in large amounts (about 2%) they can be removed by processing. If these are not removed off-flavors occur upon heating. The refined soybean oil contains 7-8% linolenic acid that could cause off-flavors from oxidation. Selective hydrogenation can reduce the linolenic acid content to 3% which improves the stability of the product (119). Refined soybean oil contains palmitic, stearic, oleic, linoleic and linolenic fatty acids distributed in a series of triacylglycerols (TGs) (120). Table III lists the triacylglycerols found in soybean oil. The order of the fatty acids within each triacylglycerol listed in Table III does not indicate a specific positional distribution (i.e. LnLL vs LLLn) but indicates that the three fatty acids are present within the molecule. Christie (121) reported on the positional distribution of fatty acids in triacylglycerols. For soybean oil, palmitic acid (C16:0) and stearic acid (C18:0) were generally found on the first and third carbon, while 70 mol % of the fatty acid found on the second carbon

Table III: Distribution of Triacylglycerols (TGs) Found in Refined Soybean Oil (120).

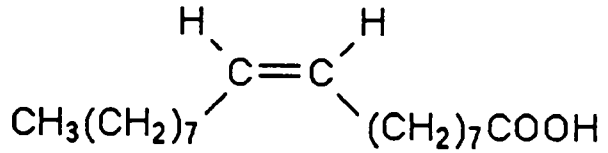
| TG[a] | C# | #Double Bonds | %Area |
|---|---|---|---|
| LnLL | 54 | 7 | 7.2 |
| LLL | 54 | 6 | 22.0 |
| LLO | 54 | 5 | 16.9 |
| LOO | 54 | 4 | 9.0 |
| OOO | 54 | 3 | 4.2 |
| SOO | 54 | 2 | 0.6 |
| LLP | 52 | 4 | 13.8 |
| LOP | 52 | 3 | 12.1 |
| POO | 52 | 2 | 6.2 |
| POP | 50 | 1 | 1.7 |
| Other | | | 6.3 |
| | | Total | 100.0 |

[a] Ln=linolenic acid (C18:3); L=linoleic acid (C18:2); O=oleic acid (C18:1); S=stearic acid (C18:0); P=palmitic acid (C16:0).

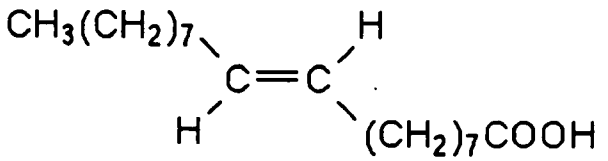
was linoleic acid (C18:2). Processing of soybean oil can readily alter the fatty acid content of the original oil. Partial hydrogenation of vegetable oils leads to an increase in the level of saturation and isomerization of the unsaturated fatty acids. That is, double bonds normally found in the cis configuration are converted to the trans configuration or vice versa. (Figure 12). The C18:2 Δ9,Δ12 isomer (linoelaidic acid) is found in soybean oil only after hydrogenation and has been shown to cause adverse physiological effects (122). Therefore, analytical methods that permit the identification of individual isomers from the partial hydrogenation of soybean oil is desirable.

Wojtusik et al. (123) recently reviewed the status of the separation and detection of triacylglycerols by HPLC. The modes of separation involve argentation chromatography, normal phase, micellar and reverse phase HPLC. Argentation chromatography uses silica gel impregnated with silver nitrate and separates triacylglycerols by degree of unsaturation and differences in geometric configuration. Normal phase HPLC generally separates TGs by chain length with some separation achieved due to degree of unsaturation. Reverse phase HPLC resolves TGs by carbon number and degree of unsaturation. Micellar HPLC can reduce the retention times of TGs with either normal or reverse phase HPLC separations. Wojtusik et al. (123) believe that the best chromatographic resolution can be obtained by using reverse phase HPLC. Complete characterization of naturally occurring TGs although, requires the use of several analytical techniques.

Separations of triacylglycerols have also been achieved using GC (124-132). TGs are readily separated by carbon number (124-127). Due to the high temperature of the separations very stable polar stationary phases are required to separate TGs by degree of unsaturation (128-132). Grob et al. (125) listed a variety of requirements when analyzing TGs by GC. These include highly deactivated columns to prevent degradation of the TGs




cis-9-Octadecenoic Acid (18:1,c9)




trans-9-Octadecenoic Acid (18:1,t9)

**Figure 12.** Position of the double bonds in cis– and trans–9–octadecenoic acid.

during the analysis and cold on-column injections to suppress discrimination of the higher molecular weight components. Hinshaw and Ettre (132) recently investigated cold split/splitless injection and cold on-column injection. They used saturated TGs in the study to suppress possible bond migration in unsaturated compounds due to the high temperatures required for separation.

A variety of detectors are used in the HPLC analyses of lipids (123). These include refractive index (RI), ultraviolet absorption (UV), infrared (IR), flame ionization detection (FID) and mass detection. RI detection has been used widely because it is a universal detector, easy to use and inexpensive. Due to the presence of very weak chromophores UV detectors are operated in the range of 190-230 nm. While this limits the available solvents for chromatographic separations, gradient elution is possible with UV detection thus permitting the analysis of more complex triacylglycerol mixtures. A moving-wire FID can be used in the analysis of triacylglycerol mixtures. While the detector is compatible with gradient elution it is not commercially available. Mass detection is achieved using a light scattering detector. Gradient elution is permitted since the solvent is nebulized. The solvents used with a light scattering detector are limited to those that are more volatile than triacylglycerols. Although the response is nonlinear it is reproducible and related to sample mass.

IR detection in lipid analysis is generally used for two reasons. The first reason for using IR detection is that it is a selective detector for HPLC when monitoring the carbonyl stretching region (5.75 μm) (133-135). Baseline drift occurs with gradient elution although it can be minimized with the proper choice of solvents and gradient conditions. The second reason for using IR detection is to determine the amount of conversion of the fatty acid isomers from the cis to the trans configuration by hydrogenation (136-140).

Generally IR methods are used to determine the total trans content without separation by analyzing the methyl ester derivatives of the fatty acids (136-139). A recent GC analysis of fatty acid methyl esters (FAMEs) coupled with matrix isolation (MI) FT-IR permitted the identification of low levels of trans isomers resulting from hydrogenation of edible oils (140).

SFC has recently been employed in the analysis of lipids. Separation of lipids as fatty acid esters (29, 40, 42), free fatty acids (FFAs) (30, 32, 34, 37) and TGs (31, 35, 39, 41) has been achieved using both packed and open tubular columns. Hellgeth et al. (34) analyzed FFAs using a PRP-1 column with FT-IR detection. Severe distortion occurred for the C18 carboxylic acid found in soybean oil. Inspection of the FT-IR spectra showed that the peak distortion resulted because chromatographic resolution was not achieved between the saturated and unsaturated C18 fatty acids. Markides et al. (32) could resolve the saturated and unsaturated FFAs using a 50% cyanopropyl polysiloxane capillary column. They employed a FID detector and indicated that C18 cis and trans isomers as well as isomers differing in the position of the double bonds were separated although, without baseline resolution. Gorner and Perrut (42) could separate unsaturated FAMEs using a packed silica column with 100% $CO_2$. Their report did not indicate whether cis and trans isomers were separated under the chromatographic conditions employed. TGs are separated by carbon number with non-polar columns (31, 35, 39, 41). Separation of TGs by degree of unsaturation is accomplished with polar columns (37,40).

An advantage of SFC over GC analysis is that triacylglycerols can be chromatographed at temperatures below 200°C. An advantage of SFC over HPLC is that it is readily interfaced to FID and IR detection. On-line FT-IR detection using SFC is not limited to monitoring only the carbonyl region (34). Therefore, an opportunity exists to

determine the extent of unsaturation and isomerization in a single analysis. TGs and FFAs can be chromatographed under similar SFC conditions (141), making the analysis of complex mixtures containing both types of compounds relatively easy.

The purpose of this study was to evaluate the ability of SFC with on-line FT-IR detection to investigate the changes in the chemical composition of vegetable oil due to processing. Refined soybean oil and soybean oil that was partially hydrogenated were chosen for the study due to their dietary importance. Supporting SFC/FID and SFC/FT-IR data were also obtained with FFA, FAME and TG standard solutions.

## B. EXPERIMENTAL

A Model 501 supercritical fluid chromatograph (Lee Scientific, Inc., Salt Lake City, UT) with a flame ionization detector (FID) set at 350 or 375°C was used. The SFC pump and the injector were cooled to <5°C using a recirculating bath. Chromatographic separation was achieved with Coleman grade or SFC grade $CO_2$ (Matheson, Dorsey, MD) as the mobile phase. Restriction was achieved employing a 100 μm i.d. frit restrictor. The chromatographic column was directly attached to the injector employing a polyimide ferrule previously described (44). The chromatographic conditions varied with the components analyzed and are given in the Figure captions. A 1 s timed-split injection employing a 200 nL rotor was used.

FT-IR spectra were obtained using a 740SX FT-IR spectrometer (Nicolet Instrument Corps., Madison, WI) equipped with a 0.6 mm i.d. x 5 mm pathlength (1.4 μL) high pressure flow cell in-line with an FID. All spectra were obtained in real time at 8 $cm^{-1}$ resolution. Spectra were acquired by collecting 1.1 files/s, 8 scans/file in real time.

FT-IR data acquisition was started 20 minutes into the chromatographic run for the FFAs and the TGs separations. The transfer line was maintained at or near the oven temperature. The flow-cell was maintained at 35°C.

Refined soybean oil and soybean oil that was partially hydrogenated with a Ni or Ni–S catalyst were analyzed. Soybean oil consists primarily of triacylglycerols. The soybean oil was hydrogenated with a Ni and Ni-S catalyst in a one-gallon pressure vessel reactor (Autoclave Engineers, Erie, PA) at 1000 rpm and 138 kPa hydrogen pressure. Aliquots of these samples were hydrolyzed and then esterified to form the fatty acid methyl esters (140). The FAMEs and unhydrolyzed soybean oil samples (Table IV) were obtained from R.M. McDonald (FDA, Division of Food Chemistry and Technology, Chicago, IL). Working solutions were prepared using iso-octane. The FFAs from the unhydrolyzed soybean oil samples were obtained by hydrolyzing an aliquot of the oil with 0.5 N aqueous NaOH with stirring and addition of heat overnight. The non-hydrolyzed TGs were removed from the NaOH solution by washing 3 times with 4 mL aliquots of $CH_2Cl_2$. The aqueous layer was acidified with 1N $H_2SO_4$ and the FFAs were extracted from the aqueous layer with three 4 mL hexane washes. Triacylglycerol standard mixtures containing tristearin, triolein, trilinolein and trilinolenin at 25 mg each were obtained from Sigma (St. Louis, MO). Two TG standard solutions were prepared. Solution I - standard mixture dissolved in 100 mL volume of approximately 30% toluene and 70% isooctane. Solution II - standard mixture dissolved in 100 mL toluene. Other lipid standard solutions were prepared in the following concentrations: Methyl palmitate - 5 mg/mL MeOH; C17:0, C18:1 t9, C18:2 t9,t12 methyl esters - 44 µg/mL isooctane; Linoleic acid (C18:2 c9,c12) - 2 mg/mL $CH_2CL_2$; Elaidic acid (C18:1 t9)- 6.5 mg/mL hexane.

Table IV: Fatty Acid Methyl Esters (FAMEs) from Soybean Oil and Unhydrolyzed (TG) Soybean Oil Samples obtained from McDonald.

| ID# | Iodine Value[a] | Catalyst | Isomer Conversion[b] |
|---|---|---|---|
| 7-155-2 (FAME) | 131 | – | – |
| 7-168-2 (FAME) | 95 | Ni | Low |
| 8-13-7A (TG) | 131 | – | – |
| 8-13-7B (TG) | 100 | Ni | Low |
| 8-13-7C (TG) | 83 | Ni | Low |
| 8-13-7D (TG) | 98 | Ni-S | High |
| 8-13-7E (TG) | 85 | Ni-S | High |

a. The Iodine Value (IV) is a measure of unsaturation; the lower the value, the less the level of unsaturation. The IV for refined soybean oil averages 131-133.
b. The isomeric conversion is a qualitative indication of how much conversion from cis to trans isomers there was during hydrogenation.

## C. RESULTS AND DISCUSSION

### Lipid Standard Analysis:

With non-polar stationary phases lipids are separated according to carbon number. As the polarity of the column increases separation based on the degree of unsaturation is also obtained. Figure 13 is the SFC separation of the TG standard mixture (Solution I) on a 30% biphenyl polysiloxane open tubular column. The chromatogram is the FID trace with the FT-IR flow cell in–line. The analysis time was over 50 min without baseline separation of the four standards with tristearin and triolein coeluting. The separation of the same TG standards is achieved in under 42 minutes with a 25% cyanopropyl polysiloxane capillary (Figure 14). While the FID trace does not show baseline resolution, the GSR (Figure 15) indicates that baseline separation was achieved. Therefore, the loss in resolution can be attributed to the SFC/FT-IR flow cell interface. This effect on resolution is further demonstrated by the separation of methyl esters of refined soybean oil without (Figure 16a) and with (Figure 16b) the FT-IR flow cell on line. Wieboldt et al. (142) recently described the requirements for an optimized flow cell design for open tubular column SFC. The flow cell design used in the Nicolet SFC interface was a compromise between the conflicting requirements of an absorbance detector (longer pathlength) and a chromatographic detector (small cell volume). The flow cell design resulted in a loss of chromatographic resolution greater than 1%. To date, the best separation for the triacylglycerol standard mixture has been obtained using a 50% cyanopropyl polysiloxane capillary column (Figure 17). Sharper chromatographic peaks were obtained because the separation was done without the FT-IR flow cell in-line.

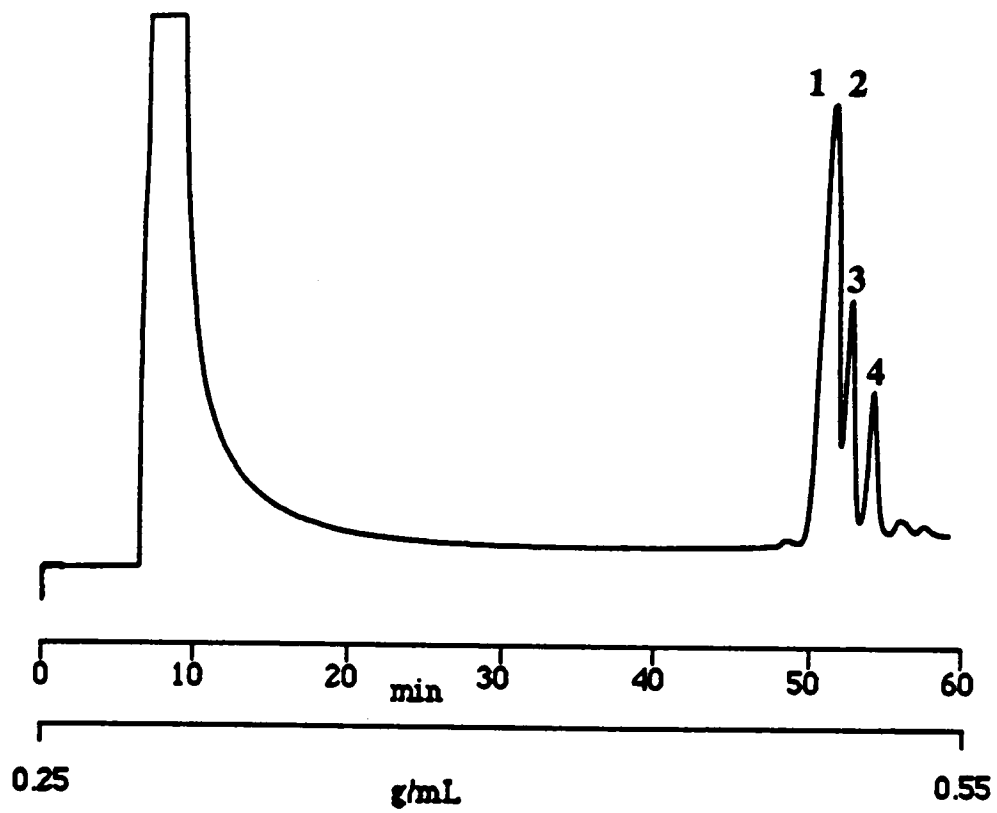



**Figure 13.** SFC chromatogram of triacylglycerol standard mixture on a 30% biphenyl-70% methyl polysiloxane open tubular column (10 m x 100 µm i.d., 0.25 µm film thickness) 1) tristearin; 2) triolein; 3) trilinolein; 4) trilinolenin. Oven temperature: 100°C. Density program: 0.25 to 0.60 g/mL at 0.005 g/mL/min, hold at 0.60 g/mL for 10 min. FID detector post FT-IR flow cell.

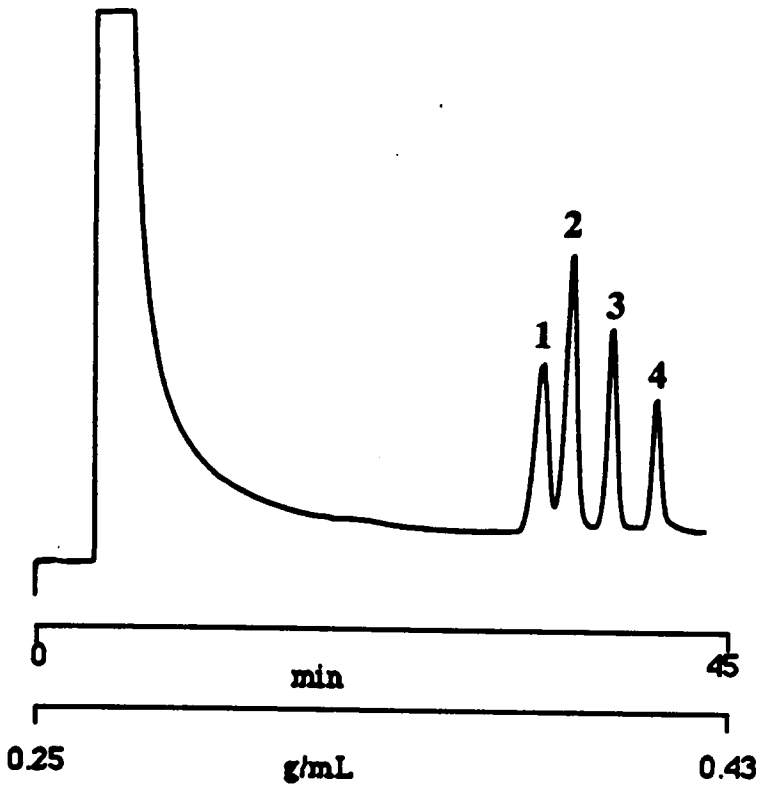



**Figure 14.** SFC chromatogram of triacylglycerol standard mixture on a 25% cyanopropyl – 25% phenyl – 50% methyl polysiloxane open tubular column (7 m x 100 µm i.d., 0.25 µm film thickness) 1) tristearin; 2) triolein; 3) trilinolein; 4) trilinolenin. Oven temperature: 140°C. Density program: 0.25 to 0.60 g/mL at 0.005 g/mL/min, hold at 0.60 g/mL for 10 min. FID detector post FT-IR flow cell.

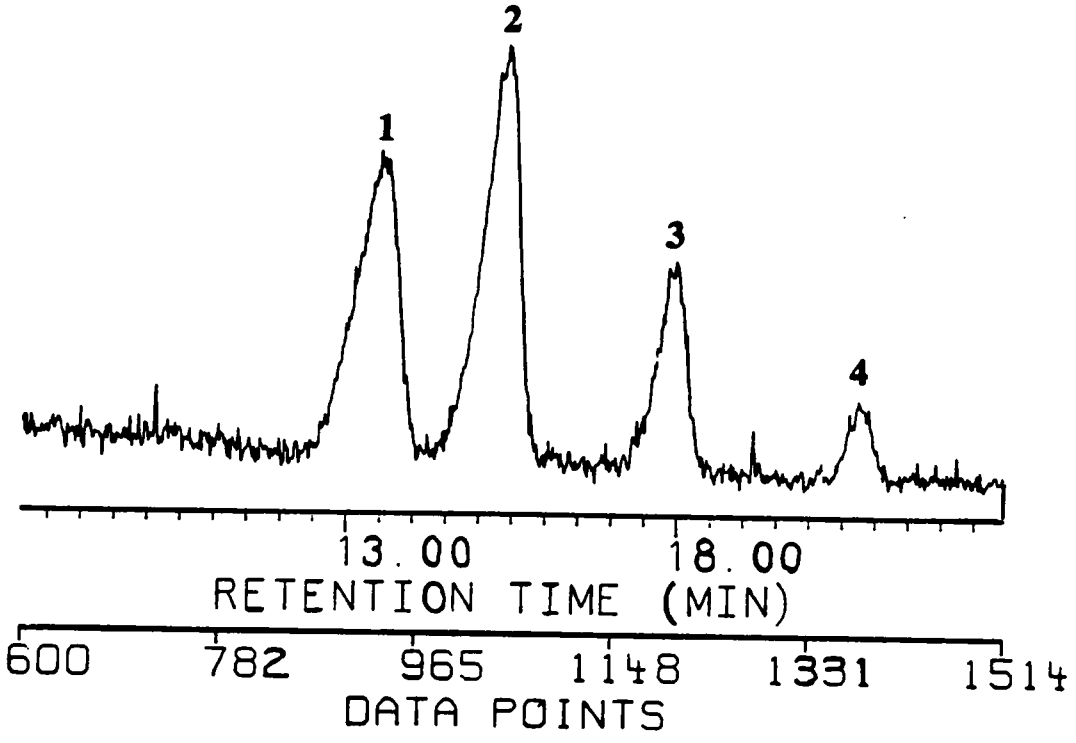



**Figure 15.** Gram-Schmidt Reconstruction of triacylglycerol standard mixture on a 25% cyanopropyl – 25% phenyl – 50% methyl polysiloxane open tubular column (7 m x 100 µm i.d., 0.25 µm film thickness) 1) tristearin; 2) triolein; 3) trilinolein; 4) trilinolenin. See Figure 13 for chromatographic conditions.

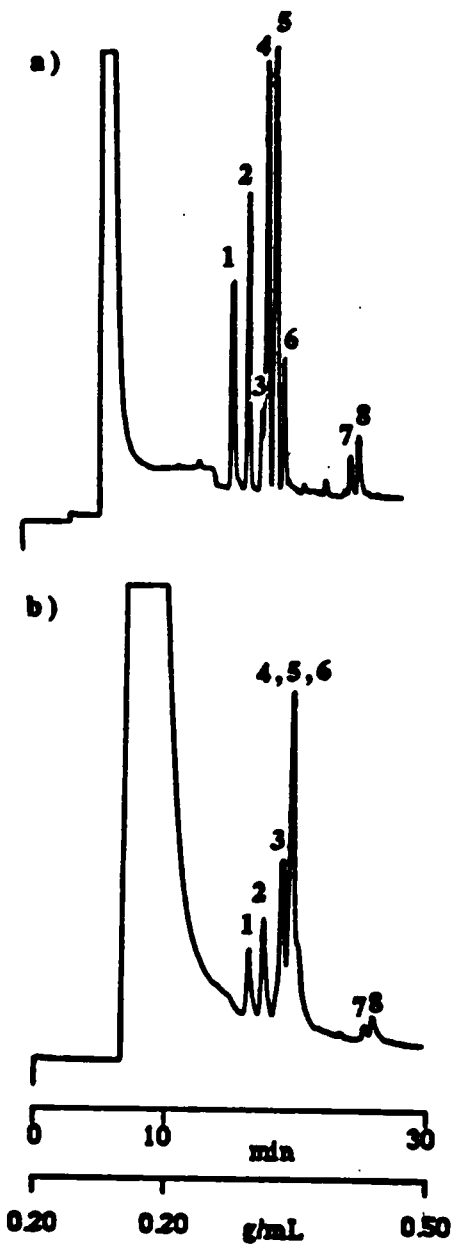



**Figure 16.** SFC chromatograms of FAMEs of refined soybean oil on a 25% cyanopropyl – 25% phenyl – 50% methyl polysiloxane ( 7 m x 100 µm i.d., 0.25 µm film thickness) a) FID detector without FT-IR flow cell on-line, b) FID detector with FT-IR flow cell on-line. Chromatographic oven; 100°C. FT-IR flow cell: 35°C. Transfer line: 100°C. FID: 350°C. Density program: 0.2 g/mL hold 10 min., 0.2-0.6 g/mL at 0.015 g/mL/min, hold 15 min. 1) C16:0; 2) C17:0; 3) C18:0; 4) C18:1; 5) C18:2; 6) C18:3; 7 & 8) Unidentified FFAs.

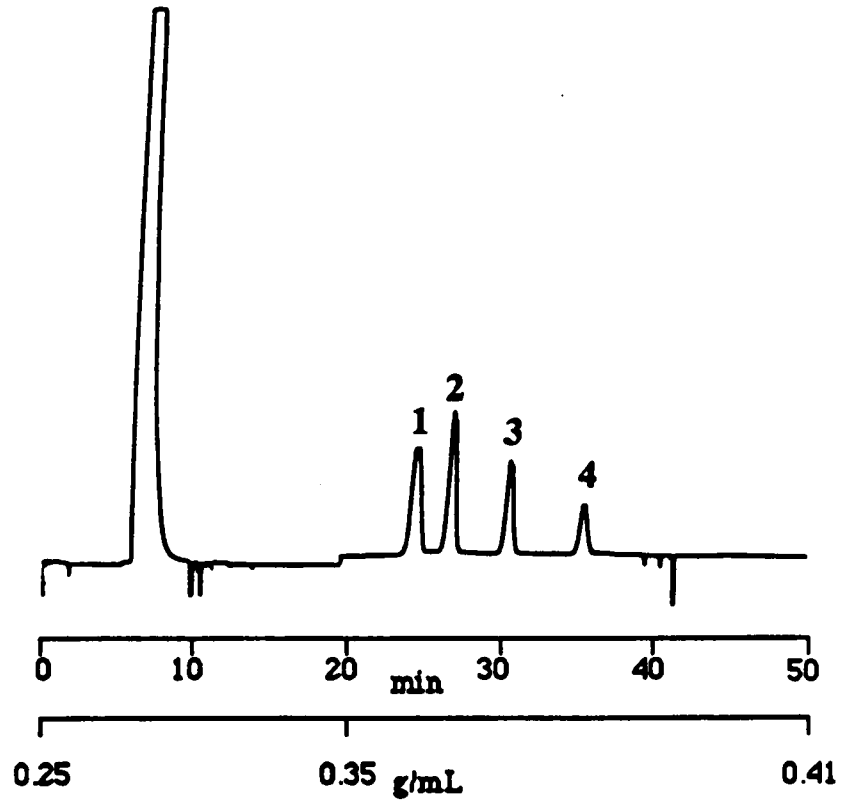



**Figure 17.** SFC chromatogram of triacylglycerol standard mixture on a 50% cyanopropyl-50% methyl polysiloxane open tubular column (10 m x 100 µm i.d., 0.25 µm film thickness). 1) tristearin; 2) triolein; 3) trilinolein; 4) trilinolenin. Oven temperature: 140°C. Density program: 0.25 to 0.35 g/mL at 0.005 g/mL/min, 0.35 to 0.45 g/mL at 0.002 g/mL/min, 0.45 to 0.60 g/mL at 0.01 g/mL/min, hold at 0.60 g/mL for 10 min. FID directly connected.

The difference in retention time between tristearin and triolein increased 0.45 min from the retention times on the 25% cyanopropyl polysiloxane column with a corresponding decrease in total analysis time of 5 min. Although the FT–IR flow cell was in–line when the 25% cyanopropyl polysiloxane column was used, the difference in retention times should not be significantly affected.

Due to the low FT-IR absorbance of triacylglycerols, the amount needed for detection could lead to column overload. The high hydrocarbon nature of the lipids causes column overload to become more prevalent as more polar columns are employed. Polar columns are needed for separation of unsaturated lipids. The use of a thicker film can compensate somewhat for this column overload problem. The lipid standard solution II was concentrated 5 and 10 times. The resulting solutions were chromatographed on two 50% cyanopropyl polysiloxane columns with film thicknesses of 0.25 and 0.50 μm. The separation of the solution concentrated 5 times on both columns is shown in Figure 18. As expected the solution concentrated 5 times resulted in severe peak distortion on the 0.25 μm film. Separation of the C18:0 TG resulted in column overload before the unsaturated lipids, as is evident with the solution concentrated 5 times on the 0.5 μm film (Figure 18b). Column overload is first observed with the C18:0 TG because this is the least polar of the TGs investigated. As the TG becomes more unsaturated its polarity increases slightly, thus severe column overload on polar columns is not observed at similar concentrations.

The FT-IR spectra of FAMEs, TGs and FFAs are similar (Figures 19,20). The absorbances between 2800-3000 $cm^{-1}$ are due to methylene and methyl symmetric and asymmetric stretching. Absorbances above 3000 $cm^{-1}$ are due to unsaturation in the

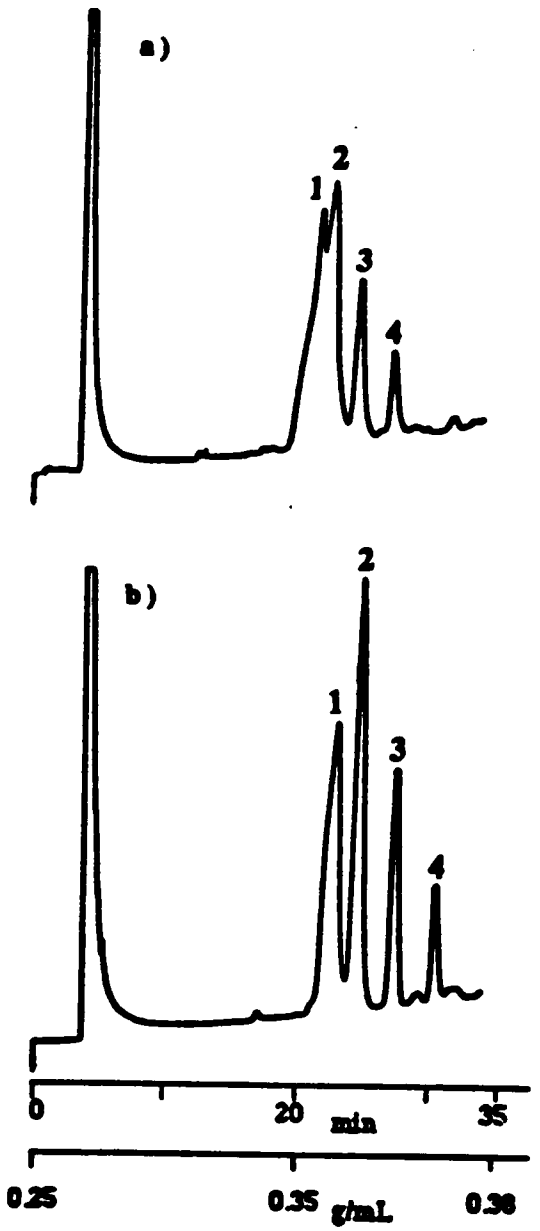



**Figure 18.** SFC chromatogram of triacylglycerol standard mixture concentrated five times employing a 50% cyanopropyl – 50% methyl polysiloxane column. A) 0.25 μm film thickness; B) 0.50 μm film thickness. See Figure 17 for chromatographic conditions. FID directly connected. 1) tristearin; 2) triolein; 3) trilinolein; 4) trilinolenin.

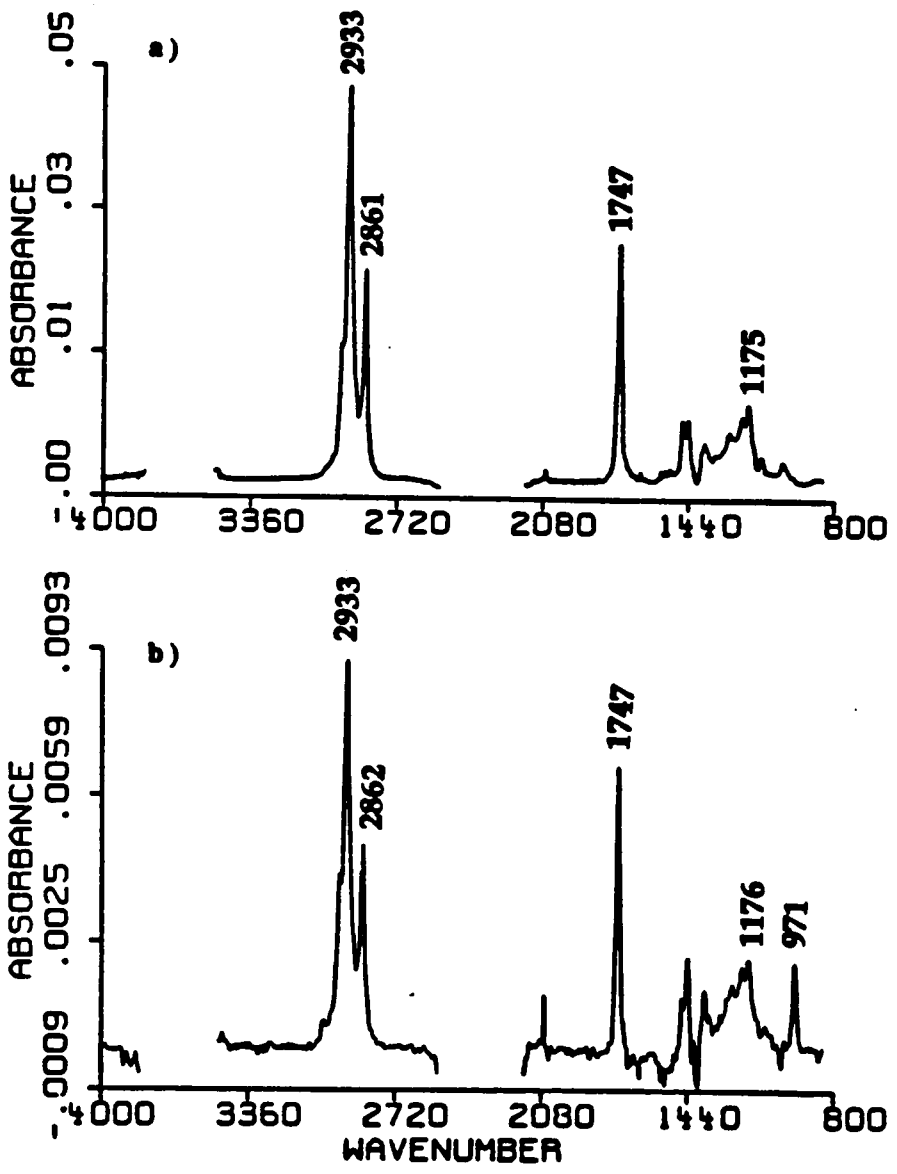



**Figure 19.** On-line FT-IR chromatographic file spectra of FAME standards. a) methyl palmitate,14 files coadded; b) C18:2 $\Delta$9,$\Delta$12 methyl ester,17 files coadded (8 scans/file).

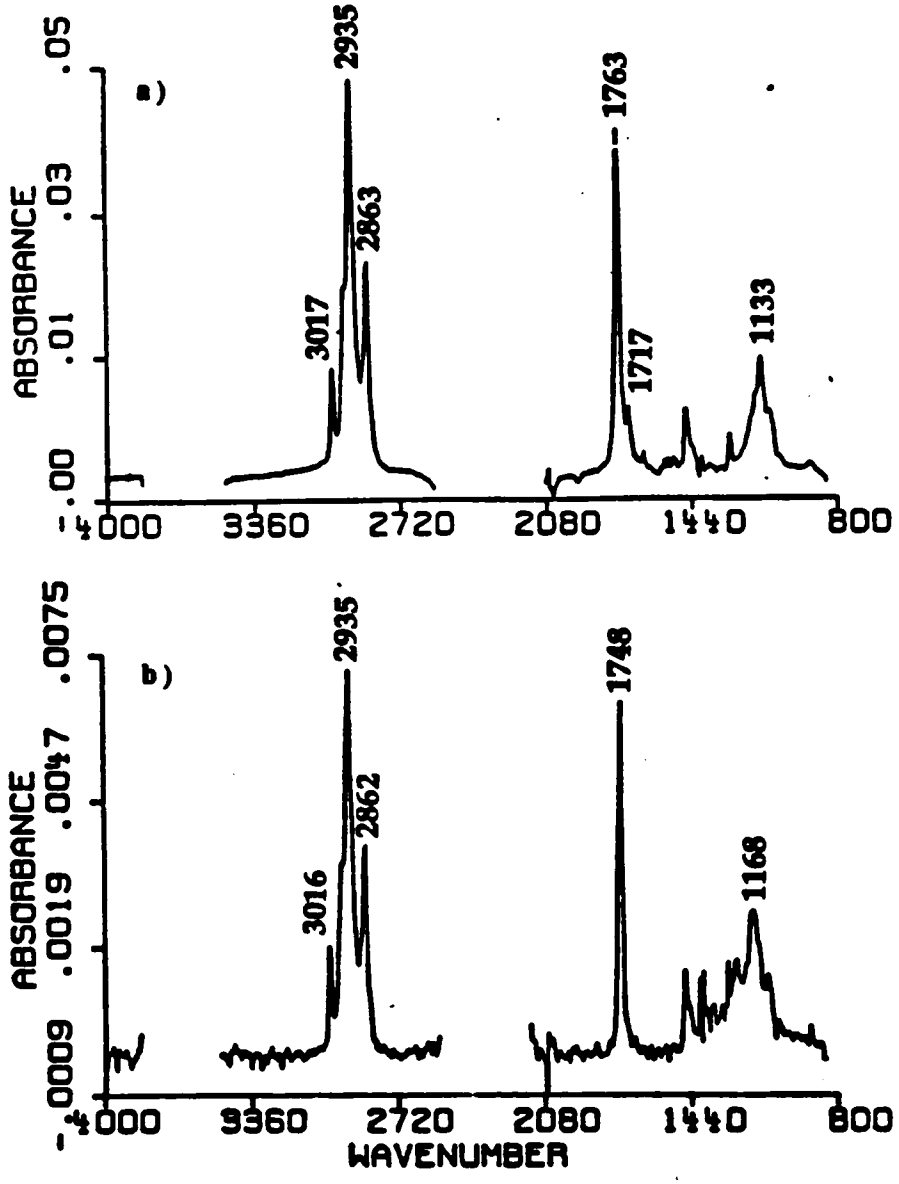



**Figure 20.** On-line FT-IR chromatographic file spectra of FFA and TG standards. a) linoleic acid, 20 files coadded; b) trilinolein, 23 files coadded (8 scans/file).

hydrocarbon chain. An absorbance at 3018-3016 $cm^{-1}$ indicates the presence of at least two double bonds in the cis, cis configuration. A single double bond in the cis configuration has an absorbance at 3012-3010 $cm^{-1}$. The C=O stretching vibration shifts from 1748 $cm^{-1}$ in the methyl ester and triacylglycerol to 1762 $cm^{-1}$ in the free acid. The C–O stretching vibration also shifts from 1160-1175 $cm^{-1}$ in the methyl ester and triacylglycerol to 1132 $cm^{-1}$ in the free acid. In Figure 19b the FT-IR spectrum of C18:2 Δ9,Δ12 methyl ester shows absorbance at 972 $cm^{-1}$ (CH deformation) indicating that there is a double bond(s) in the trans configuration. If one examines the carbonyl region of a FFA standard (Figure 20a) in SC-$CO_2$, two peaks (1762 and 1716 $cm^{-1}$) are readily observed. The absorbance at 1762 $cm^{-1}$ is caused by the FFA in the monomer state while the absorbance at 1716 $cm^{-1}$ is caused by the presence of the FFA in the dimer state (32). A difference that is observed between the methyl ester and the triacylglycerol is the bandwidth of the C-O stretching vibration. In the methyl ester the absorbance in the C–O stretching region is partially resolved into multiple bands. The broader unresolved band in the triacylglycerol may reflect the average of the environments encountered by the three hydrocarbon chains. The intensity of the $CH_2$ asymmetric stretching band decreased relative to that of the carbonyl stretch as the degree of unsaturation in the TGs increased (Table V). Also the intensity of the cis olefinic CH stretching band increased with increasing unsaturation. The intensity of the CH deformation band (972 $cm^{-1}$) would need to be used when the trans isomers are present. These changes in intensity could potentially be used as an aid in identifying the level of unsaturation in the triacylglycerols in vegetable oils.

Table V: Normalized Intensity of the cis =C–H and Aliphatic C–H Stretching Bands for $C_{54}$ Triacylglycerols Relative to the Ester Carbonyl Stretching Vibration.

| Triacylglycerol | =C–H stretch (cis)[a] | aliphatic C–H stretch[b] | C=O stretch[c] |
|---|---|---|---|
| 18:0 | – | 2.06 | 1.0 |
| 18:1 | 0.15 | 1.59 | 1.0 |
| 18:2 | 0.28 | 1.08 | 1.0 |
| 18:3 | 0.38 | 0.75 | 1.0 |

a. 3012 - 3018 $cm^{-1}$
b. 2932-2936 $cm^{-1}$
c. 1748 $cm^{-1}$

Soybean Oil Analysis:

The following interpretation of the SFC/FID and SFC/FT-IR data is partially based on the work of Mossoba et al. (140). Their work dealt with the identification and quantitation of t-9, t-12 octadecadienoic acid methyl ester and other fatty acid methyl esters. These esters were obtained by the transesterification of partially hydrogenated soybean oil. They employed GC/MI/FT-IR for the analyses.

Two samples of fatty acid methyl esters of refined soybean oil and Ni hydrogenated soybean oil containing C17:0 methyl ester as an internal standard were chromatographed under SFC conditions (Figure 21). The amount of internal standard was different in the two samples. Separation of the FAMEs in refined soybean oil was achieved on a 25% cyanopropyl polysiloxane column. In both FAME samples the C16:0 methyl ester was readily resolved from the C18 methyl esters. Comparison of retention times between C16:0 and the C18 methyl esters indicated that the hydrogenated soybean oil lost a majority of the C18:3 fatty acid as expected. Assuming that the amount of C16:0 was unaffected by hydrogenation, peak height data also indicate that some of the C18:2 was lost. The increase in the C18:1 relative peak height accounts for the loss of the other two fatty acids. Hydrogenation of the fatty acids does not necessarily result in complete loss of unsaturation. While some of the C18:1 may have converted to the C18:0 fatty acid more of the polyunsaturated fatty acids were converted to the C18:1 hence the increased concentration of C18:1. Due to incomplete resolution between the C18:0 and the C18:1, an increase in the C18:0 fatty acid cannot be determined.

Although the standard C54 triacylglycerols were readily resolved on the 50% cyanopropyl polysiloxane column (Figure 17), the TGs from refined soybean oil were

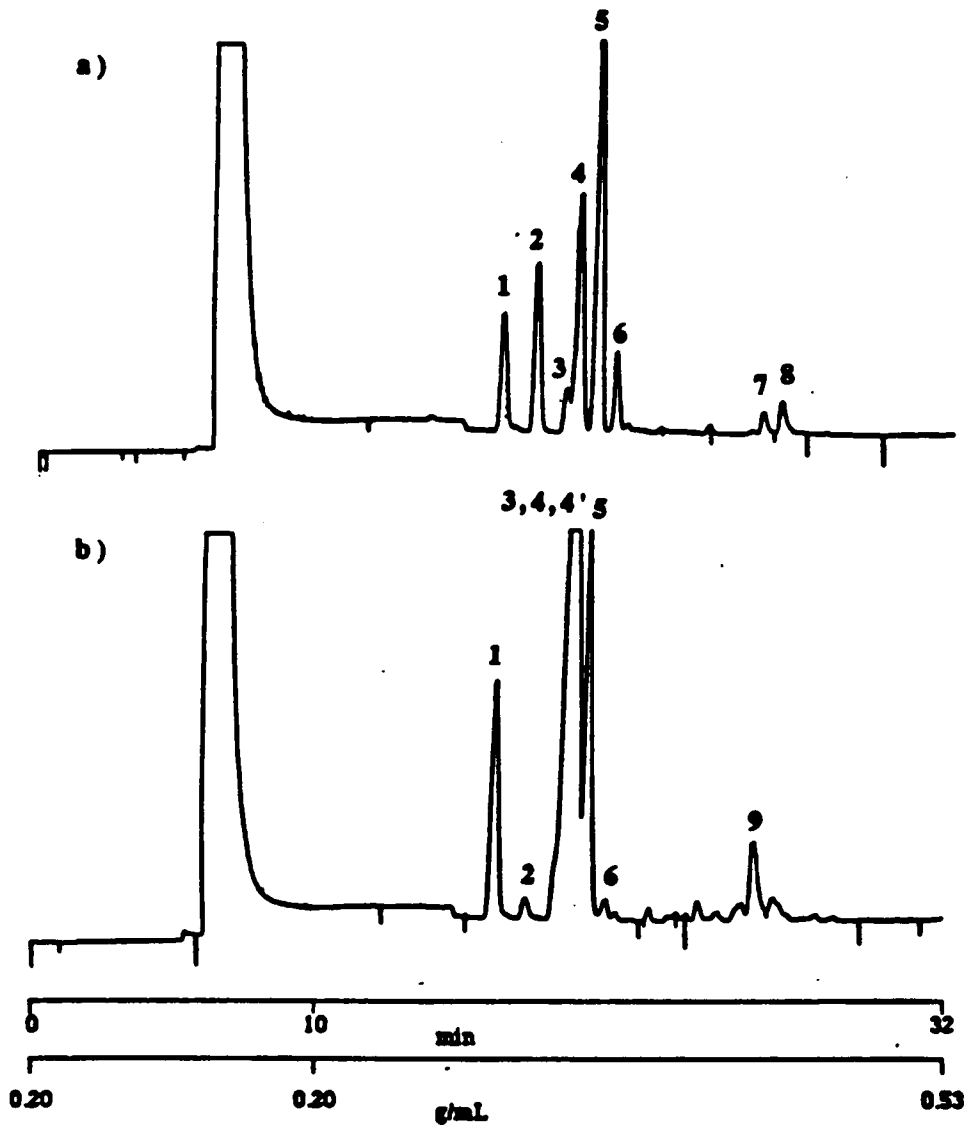



**Figure 21.** SFC chromatogram of FAMEs from a) refined soybean oil (ID# 7-155-2); b) Ni hydrogenated soybean oil (ID# 7-168-2). Column: 25% cyanopropyl – 25% phenyl – 50% methyl polysiloxane, (Lee Scientific, Inc.) 7 m x 100 µm i.d.; Chromatographic oven; 100°C. FT-IR flow cell: 35°C. Transfer line: 100°C. FID: 350°C. Density program: 0.2 g/mL hold 10 min., 0.20-0.53 g/mL at 0.015 g/mL/min, hold 15 min. FID detector directly connected. 1) C16:0; 2) C17:0; 3) C18:0; 4) C18:1c; 4') C18:1t; 5) C18:2; 6) C18:3; 7, 8 & 9) Unidentified FFAs.

not completely resolved (Figure 22). This can be explained in part by the distribution of TGs in soybean oil. As shown in Table III there are at least 6 TGs containing 54 carbons (excluding the glycerol carbons) with the number of double bonds ranging from 2 to 7. Also while separation of lipids is first by carbon number and then by degree of unsaturation, a C54 triacylglycerol could possibly elute before a C52 triacylglycerol depending on the level of unsaturation in both glycerols. As with the FAMEs, the FID profiles change significantly upon hydrogenation (Figure 22). The increase in saturation of the Ni and Ni-S samples were similar. The increased saturation is measured by the iodine value (Table IV). The lower the value, the greater the level of saturation. The chromatograms shown in Figure 21 are from samples 8–13–7A, 8–13–7B and 8–13–7D. Samples 8-13-7B and 8-13-7D were partially hydrogenated to similar levels of saturation. Longer times are required when using the Ni-S catalyst than the Ni catalyst during hydrogenation to obtain similar levels of saturation (140). Loss, in the partially hydrogenated samples (Figures 22b,c), of the later eluting components found in the refined soybean oil (Figure 22a) is indicative of increased saturation of the fatty acids. Although the iodine values (Table IV) show similar levels of total saturation, the FID profiles were significantly different. The nature of the Ni-S catalyst surface appears to favor more complete hydrogenation of the highly unsaturated TGs found in soybean oil. This is shown in the FID profile (Figure 22c) by the loss of the later eluting components found in the refined soybean oil and the Ni hydrogenated soybean oil (Figures 22a,b). Also, the Ni-S catalyst converts a higher percentage of cis isomers to trans isomers. This greater conversion to trans isomers can account for the broad peak in the SFC/FID chromatogram.

An oven temperature of 60°C was used for the elution of the FFAs based on a

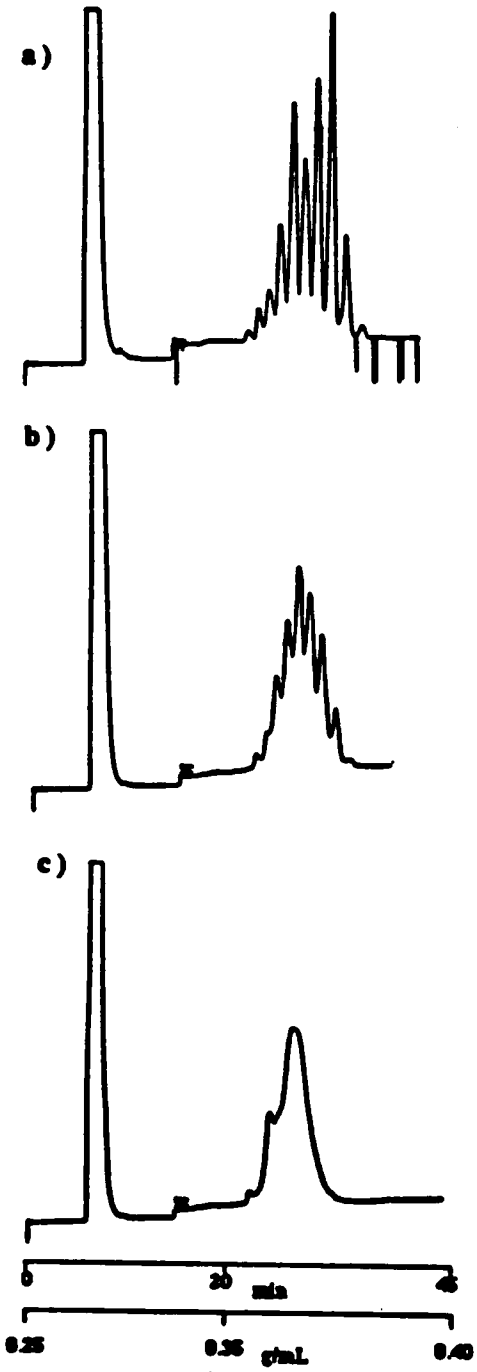



**Figure 22.** SFC chromatograms of triacylglycerols. a) refined soybean oil (ID# 8-13-7A); b) Ni hydrogenated soybean oil (ID# 8-13-7B); c) Ni-S hydrogenated soybean oil (ID# 8-13-7D). See Figure 17 for chromatographic conditions. FID detector directly connected.

report of Markides et al. (32). While slight improvement in resolution was obtained with a slower gradient rate, this improvement came with an analysis time over one hour. The density gradient program chosen for the FFAs separation resulted in an analysis time of approximately 40 min. The density program used resulted in the elution of trans C18:1 between C18:0 and cis C18:1. Trans isomers also elute before the corresponding cis isomers of the FAMEs. The FID profiles (Figure 23) of the FFAs separation shows more clearly than the triacylglycerol chromatograms the loss of unsaturated components as well as the isomerization of the fatty acids. In the Ni hydrogenated soybean oil profile the presence of the trans isomers is shown by the broader C18:1 peak and the front shoulder on the C18:2 fatty acid peak. A more concentrated solution of the Ni hydrogenated sample more readily shows that some C18:3 is still present. In the Ni-S hydrogenated soybean oil, almost complete conversion to trans C18:1 occurs as determined by the shift in retention times. Comparison of peak height ratios between the hydrogenated samples is difficult because FID response factors for cis and trans isomers were not determined. A comparison of the peak height ratios of the C16:0 and C18:0 fatty acids is possible. These ratios indicate that the Ni hydrogenated soybean oil (Figure 23b) had a significantly greater amount of C18:0 than the refined soybean oil (Figure 23a) or the Ni-S hydrogenated soybean oil (Figure 23c). The changes due to hydrogenation in the FID profiles were confirmed with on-line FT-IR analysis of the samples of refined and hydrogenated soybean oil. Figure 24 shows representative on-line FT-IR chromatographic file spectra of triacylglycerols from refined soybean oil and hydrogenated soybean oil. The spectra represent chromatographic peaks with similar retention times observed in the three samples. The

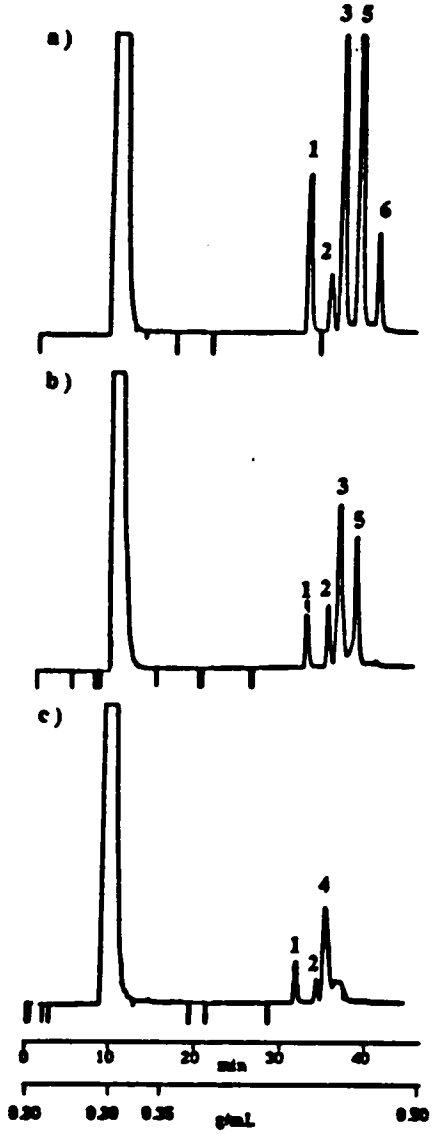



**Figure 23.** SFC chromatograms of FFAs from a) refined soybean oil; b) Ni hydrogenated soybean oil; c) Ni-S hydrogenated soybean oil. Column: 50% cyanopropyl-50% methyl polysiloxane (10 m x 100 µm i.d., 0.25 µm df). Oven temperature: 60°C. Density program: 0.20 g/mL, hold 10 min, 0.20 to 0.35 g/mL at 0.025 g/mL/min, 0.35 to 0.50 g/mL at 0.005 g/mL/min. FID detector directly connected. 1) C16:0; 2) C18:0; 3) C18:1c; 4) C18:1t; 5) C18:2c; 6) C18:3c.

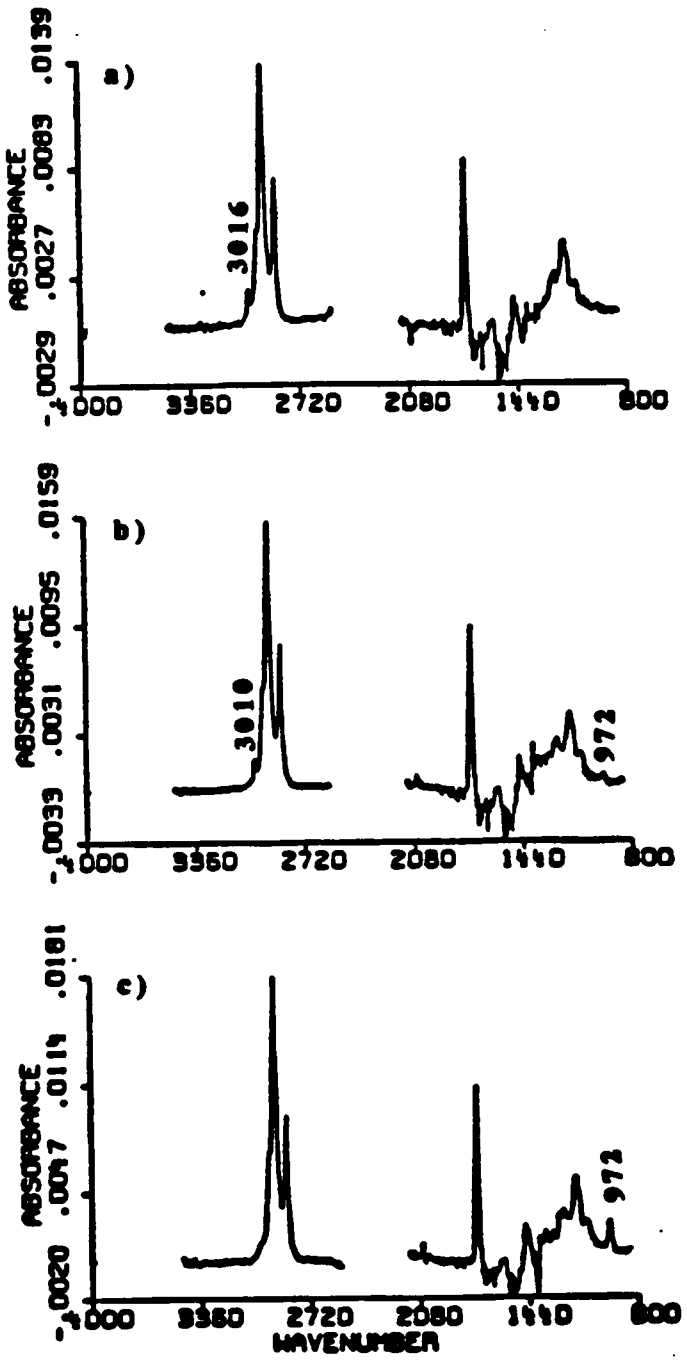



**Figure 24.** On-line FT-IR chromatographic file spectra of triacylglycerols found in a) refined soybean oil, 23 files coadded; b) Ni hydrogenated soybean oil, 27 files coadded; c) Ni-S hydrogenated soybean oil, 21 files coadded (8 scans/file).

spectra indicate a change in the configuration of some double bonds from cis to trans. This isomerization was demonstrated by the loss of absorbance at 3016 $cm^{-1}$ and the presence of absorbance at 972 $cm^{-1}$. The FT-IR spectra of the triacylglycerols of the Ni–S hydrogenated soybean oil indicated that there was greater conversion to trans isomers than with the Ni hydrogenated soybean oil. The greater conversion to trans isomers was determined by the intensity of the absorbance at 972 $cm^{-1}$. This conversion to trans isomers can account for the broad peak in the FID trace. Due to the low isomer content of the Ni hydrogenated soybean oil, the presence of the absorbance at 972 $cm^{-1}$ was difficult to observe in the intact triacylglycerols but became more evident in the spectra of the FFAs. Table VI lists the S/N of the trans absorbance at 972 $cm^{-1}$ from the Ni hydrogenated soybean oil. The noise was measured as the background peak–to–peak absorbance between approximately 950 and 850 $cm^{-1}$. The intensity of the 972 $cm^{-1}$ is less in the triacylglycerols because the trans fatty acid is distributed among several of the triacylglycerols. The absorbance at 972 $cm^{-1}$ in the FFAs spectra of the Ni-S hydrogenated soybean oil also increases from that observed in the TGs spectra. Examination of the FID traces of the FAMEs separations (Figure 21) reveal several late eluting components. FT-IR spectra of these components indicate that they are free fatty acids.

As previously mentioned, the intensity of the $CH_2$ asymmetric stretching absorbance decreased relative to that of the carbonyl stretch as the degree of unsaturation in triacylglycerols increased. The ratios determined from FFAs and TGs separation of refined soybean oil are listed in Table VII and VIII. As with the triacylglycerol standards, the ratio of the $CH_2$ asymmetrical stretch to the carbonyl

Table VI: S/N of the trans Absorbance at 972 $cm^{-1}$ from Ni Hydrogenated Soybean Oil.

| Triacylglycerols Retention Time[a] (minutes) | S/N | Free Fatty Acids Retention Time[b] (minutes) | S/N |
|---|---|---|---|
| 13.87 | 0.67 | 13.35 | 0.43 |
| 14.70 | 2.0 | 14.08 | 3.5 |
| 15.65 | 1.8 | 14.35 | 1.8 |
| 16.53 | 1.3 | 14.71 | 1.9 |
| 17.39 | 1.8 | 15.86 | 1.0 |

a. See Figure 33 in Appendix B.
b. See Figure 34 in Appendix B.

Table VII: Normalized Intensity of the cis =C–H and Aliphatic C–H Stretching Bands for FFAs in Refined Soybean Oil Relative to the Ester Carbonyl Stretching Vibration.

| Retention Time[a] (min.) | =C–H stretch (cis)[b] | aliphatic C–H stretch[c] | C=O stretch[d] |
|---|---|---|---|
| 10.52 (16:0)[e] | – | 1.82 | 1.0 |
| 13.11 (18:0) | – | 2.16 | 1.0 |
| 14.27 (18:1) | 0.19 | 1.62 | 1.0 |
| 16.18 (18:2) | 0.30 | 1.17 | 1.0 |
| 18.50 (18:3) | 0.41 | 0.79 | 1.0 |

a. See Figure 35 in Appendix B.
b. 3012-3018 $cm^{-1}$
c. 2932-2936 $cm^{-1}$
d. 1762 $cm^{-1}$
e. Designates which free fatty acid elutes at the given retention time.

Table VIII: Normalized Intensity of the cis =C–H and Aliphatic C–H Stretching Bands for Triacylglycerols in Refined Soybean Oil Relative to the Ester Carbonyl Stretching Vibration.

| Retention Time[a] (min.) | =C–H stretch (cis)[b] | aliphatic C–H stretch[c] | C=O stretch[d] |
|---|---|---|---|
| 12.33 | 0.14 | 1.41 | 1.0 |
| 13.40 | 0.16 | 1.42 | 1.0 |
| 14.28 | 0.19 | 1.36 | 1.0 |
| 15.28 | 0.20 | 1.37 | 1.0 |
| 16.27 | 0.22 | 1.33 | 1.0 |
| 17.10 | 0.24 | 1.25 | 1.0 |
| 17.88 | 0.27 | 1.14 | 1.0 |
| 18.71 | 0.36 | 1.06 | 1.0 |

a. See Figure 36 in Appendix B.
b. 3012-3018 $cm^{-1}$
c. 2932-2936 $cm^{-1}$
d. 1748 $cm^{-1}$

stretch in the FFAs decreased with increasing unsaturation. Also the ratio decreased as the carbon number decreased (C16:0 vs C18:0). Slight increases in the ratio values of $CH_2$ stretch to C=O stretch in the FFAs seems to indicate that the intensity of the C=O in the FFAs is less than in the ester. When the ratios were determined for the TGs in refined soybean oil, small changes in the ratio values occurred. There was a trend to lower values at longer retention times indicating increased unsaturation in the compounds. Further evidence of unsaturation can be seen with the increased value for the ratio of the cis =C–H stretch to the carbonyl stretch. Less dramatic changes in these ratio values compared with the values obtained for the TG standards (Table V) may result from incomplete resolution of the TGs in refined soybean under the SFC conditions employed in the study. The distribution of the different fatty acids (C16 as well as C18 fatty acids) among the TGs also may average the absorbances measured so that identifiable ratios are not possible with mixed fatty acid moieties.

## D.CONCLUSION

SFC with on-line FT-IR detection shows promise in the ability to monitor changes in the chemical composition of vegetable oils due to processing. A major advantage of SFC over LC is that on-line detection using SFC permits the monitoring of the C-H deformation region (1000-900 $cm^{-1}$) in trans $R_1HC{=}CHR_2$ groups, the C–H stretching region (3020- 2800 $cm^{-1}$) and the carbonyl region (1800-1700 $cm^{-1}$). Therefore an opportunity to determine the extent of unsaturation and isomerization in a single analysis exists. The on–line FT–IR spectra of the TGs and FFAs from the partially

hydrogenated soybean oil showed that although similar levels of unsaturation were achieved with both catalysts during hydrogenation, the Ni-S catalyst produced higher levels of trans isomers. This higher trans isomer content was confirmed by the increased absorbance at 972 $cm^{-1}$ and loss of absorbance at 3010–3016 $cm^{-1}$ in the spectra obtained from the Ni-S samples as opposed to the Ni samples. One area of the IR spectra that is lost due to $CO_2$ absorbance is the C–H out–of–plane deformation vibrations of the cis $R_1HC{=}CHR_2$ groups. This absorbance occurs at 730 $cm^{-1}$. Use of SFC/FT–IR with solvent elimination would provide this information. A major advantage of SFC over GC is that FFAs and TGs can be analyzed using the same chromatographic column. Although refined soybean oil consists primarily of triacylglycerols, the analysis of complex mixtures containing both types of compounds would be relatively easy. A major disadvantage of the study is that complete resolution of the triacylglycerols and of the geometric (cis and trans) isomers was not obtained. This problem may be addressed by using a series of columns with different stationary phases such as a 50% cyanopropyl polysiloxane in series with a liquid crystalline phase that can separate isomers. As stationary phase technology improves additional phases may become available that will provide improved resolution of lipids.

# V. Supercritical Fluid Extraction Study of a Model Compound System

## A. INTRODUCTION

The empirical rules in the literature used to estimate solubility in SC-$CO_2$ did not provide sufficient information to predict the SFC behavior of such compounds as aflatoxins and coumestrol. Therefore, a supercritical fluid extraction (SFE) study dealing with a model compound system based on the coumarin structure was initiated.

McHugh and Krukonis (63) described the use of static and dynamic methods for measuring solubilities in supercritical fluids. In a static extraction the solute and solvent are loaded into a cell. In a dynamic extraction the solute is continuously swept with the SF. Static methods are generally used to determine phase behavior and solubilities of solutes. Dynamic techniques are used to determine solute solubilities in an SF and fractionation studies. Apparati for static methods are designed with high-pressure view cells containing a magnetic stirring bar to mix the content of the cell. Pressure of the system is adjusted by varying the cell volume. Dynamic methods use a high-pressure pump to deliver the fluid through the sample cell(s). The saturated fluid phase then exits the cell(s) through a heated metering valve and the solute falls from solution and is collected in a cold trap. By metering the amount of $CO_2$ that passes through the system and determining the amount of solute collected, solubility data can be obtained. SFE apparati for analytical scale extractions are generally designed for continuous or dynamic extraction, where the substance being extracted is continuously flushed with fresh fluid. The extracted material is generally trapped cryogenically on the head of a chromatographic column or precolumn, or into an accumulator containing

conventional organic solvents. In some instances the extracted material can be directly introduced to a detector without a chromatographic separation.

Estimates of solubility and/or extractability of a compound in SC-$CO_2$ can be based on previous solubility data of similar compounds found in the literature (143), chromatographic retention (144-145) or threshold pressure values (91,102,145-146). An extensive report on solubilities of substances in liquid $CO_2$ at 25°C and 955 psi was published by Francis (147). Dange et al. (143) developed structure-solubility correlations from the solubility data obtained by Francis (147) and additional data obtained in his laboratory at 25 or 35°C and 2500 psi. Dange's data were obtained using an extraction cell that was isolated for an equilibration period followed by flow of a known amount of $CO_2$ into an assay chamber. In this manner they determined the solubility of a variety of compounds including hydrocarbons, alcohols, phenols and acids. Some of the generalizations he developed were: 1) A decrease in solubility was observed for primary alcohols greater than C6. 2) Multiple hydroxyl groups decreased solubility. 3) Etherification or esterification enhanced solubility. 4) Branching increases solubility. 5) The nature of a substituent group and its position to a phenolic hydroxyl can affect solubility. 6) A decrease in solubility occurs for aliphatic carboxylic acids whose hydrocarbon chain length is greater than 9. 7) Aromatic acids are generally less soluble than aliphatic acids. 8) The acid chlorides of aromatic acids are soluble in $CO_2$.

Barker et al. (144) measured solubilities of naphthalene and 1–methylnaphthalene in SFs at pressures around and below the critical pressure using chromatographic retention. Solubilities can be obtained from chromatographic retention volumes if either vapor pressure or one previously measured solubility value is known.

The experimental apparatus consisted of a syringe pump that delivered $CO_2$ to an extraction cell attached to an UV detector via a 6-port sampling valve. Two valves before the extraction cell and sampling valve controlled the direction of the flow. With one valve opened the $CO_2$ was delivered to the extraction cell through a 20 μL loop into the UV detector to determine when a solution close to saturation was obtained. When a saturated solution was obtained the 1st valve was closed and the 2nd valve opened such that the $CO_2$ flowed through an open stainless steel tube to the UV detector. The sample was injected into the flowing stream and the migration time through the system observed. In this manner retention times were determined and converted to retention volumes. Capacity ratios were calculated and solubilities determined.

Smith et al. (145) studied solubilities in SFs by direct solubility measurements and by applying chromatographic measurements as well. They demonstrated the methods by using aromatic hydrocarbons and trichothecenes. In the direct solubility measurements the solute dissolved in $CH_2Cl_2$ was coated onto the wall of deactivated fused silica and the solvent pressure was raised stepwise with a 5 min equilibration time before CI-MS data were acquired. The system was calibrated for an absolute response factor. The solvent flow rate was calibrated by monitoring the EI signal vs the absolute flow through the restrictor with a flow meter. In the chromatographic measurement retention factors were obtained under isothermal conditions at various pressures using a 5% phenyl polymethylsilioxane stationary phase. They developed an equation that gives the relationship between retention, solubility and pressure at constant temperature for infinitely dilute solutions. With this equation they compared experimental and calculated retention factors for naphthalene in $CO_2$. The agreement between the data suggested that since available solubility data can predict retention in SFC, then

solubilities can be predicted from SFC retention data of infinitely dilute solutions.

Giddings et al. (148) studied the relative migration of various solutes in ammonia and carbon dioxide. The solutes investigated included squalene, cholesterol and sugars. Pressures up to 2000 atm for $CO_2$ and up to 200 atm for $NH_3$ were used. The $CO_2$ studies were conducted at 40°C, while the $NH_3$ studies were conducted at 140°C. Giddings reported a threshold pressure for some of the solutes in $CO_2$. He defined threshold pressure as that pressure at which detectable migration commences. He reported that threshold pressures for waxes and oils were generally below 200 atm, although silicon gum rubber did not migrate until 770 atm. Terpenes migrated at 170 atm, while sterols migrated at 510 atm. Purines, nucleosides, cortical steroids and underivatized monosaccharides did not migrate at all or not until the pressure was above 1300 atm.

King (146) reported a method to predict the pressure at which maximum solute solubility is attained in the supercritical fluid. The method is based on knowledge of solvent and solute solubility parameters. He indicated that extraction conditions for maximum solubility are desirable for extracting large amounts of a solute from a matrix. Lower pressures and hence lower solvent solubility parameters are generally sufficient for extracting trace quantities of an analyte. King described four basic parameters of SFE that are helpful in understanding solute behavior in supercritical fluids. These parameters are: 1) miscibility pressure that has been defined by Giddings as "threshold pressure"; 2) the pressure at which maximum solute solubility is attained; 3) the fractionation pressure range which is the range between the miscibility pressure and the solubility maximum pressure; and 4) knowledge of the solute's physical properties such as the melting point. By having this information development

of extraction methodology is more easily pursued.

Smith et al. (145) made threshold pressure measurements for some trichothecenes, pyrene and benzopyrene. The measurements were obtained using a FID. A 6 m x 100 μm i.d. length of deactivated fused silica was attached to a HPLC valve via a splitter. A linear restrictor was used to maintain system pressure. Solutions of the individual solutes in $CH_2Cl_2$ were injected. The pressure was maintained at 75 bar until the solvent eluted and then the pressure was ramped 2 or 4 bar/min. Threshold pressure measurements were taken at several temperature and corrected for transient time through the column.

Stahl et al. (91,102) extracted a variety of natural products in the pressure range up to 400 bar. They determined threshold pressures of such products as phenols, coumarins, lipids (102) and steroids (91) using a coupled SFC/TLC system. Using these data some structure-extractability correlations were established. The findings were summarized into "rules of thumb". 1) Hydrocarbons, esters, ethers, lactones and epoxides are easily extracted in lower pressure ranges 70-100 bar. 2) Introduction of –OH, –COOH groups makes the extraction more difficult. 3) Strongly polar substances are not extracted in the pressure range up to 400 bar. While he reported that benzene derivatives with three phenolic hydroxyl groups are somewhat extractable, the coumarin derivative, 6,7–dihydroxycoumarin, with two phenolic hydroxyl groups is not extractable (102). A steroid with one phenolic hydroxyl and two alcoholic hydroxyl groups is not extractable in the range studied (91).

In the study reported here, a series of compounds based on the coumarin structure have been evaluated for extractability. These compounds were chosen because of their widespread natural occurrence; their toxicological importance, e.g., aflatoxins

and coumestrol; the availability of coumarins with a variety of functional groups including hydroxyl, amines and phenyls; and to extend the previous work of Stahl et al. (102) where they only investigated three coumarins. The extractability of the model compounds was evaluated by using an empty liquid chromatographic (LC) guard column as the extraction cell attached directly to a supercritical fluid chromatograph equipped with a flame ionization detector. Apparent threshold densities of the model compounds were obtained using this dynamic extraction system. The effects of extraction temperature, detector temperature, restrictor type and matrix interaction on threshold densities were evaluated.

## B. EXPERIMENTAL

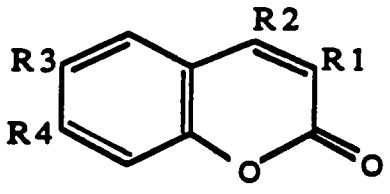
### Threshold Density Determinations:

A Model 501 supercritical fluid chromatograph (Lee Scientific, Inc., Salt Lake City, UT) with a flame ionization detector was employed. The extractions were performed using an LC stainless steel precolumn (2 cm x 2 mm i.d., 0.6 mL volume). Restriction was achieved employing a frit restrictor (61 cm x 100 μm i.d.) attached to the extraction cell with a Valco reducer (1/16" to 1/32"). Oven temperature, detector temperature and density gradient rate were evaluated to determine their effect on the threshold density value. Restrictor type (integral, frit and linear) was also evaluated. Each experiment was performed in triplicate except for the evaluation of the density gradient rate where duplicate runs were performed. Density gradients were established at the different temperatures to ensure that the pressure range was between 73 and 400 atm. The densities were determined using the Utility program for P/D calculations

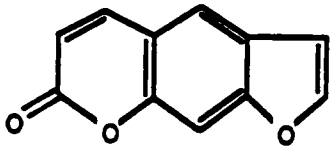
found in the LSI 600 series software. Supercritical fluid chromatography (SFC) grade $CO_2$ (Matheson, Dorsey, MD) was used as the extraction fluid. Carbon black and alumina traps were placed in line between the SFC pump and the gas cylinder for additional purification. Standards were obtained from Aldrich Chemical, vacuum dried and stored over desiccant. Figure 25 shows the structures of the model compounds used in this study.

Evaluation of Matrix Effects:

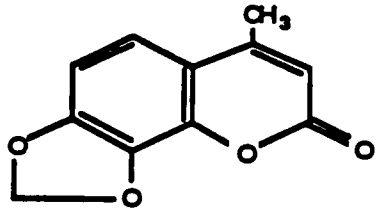
Four matrices were evaluated for their effect on threshold density as well as on extraction time. These matrices included carbon black (Fisher), acidic aluminum oxide (Baker), silica gel (Baker; 60-200 mesh) and corn starch. Extraction conditions were: oven temperature - 80°C, detector temperature - 375°C; density program: 0.14 to 0.82 g/mL at 0.05 g/mL/min, hold at 0.82 g/mL. Restrictor - 20.6 cm x 100 μm i.d. frit restrictor. Each experiment was performed in duplicate. The extraction vessel was filled with each matrix and the gradient program run to precondition the matrices. Two integrators were used. The HP integrator was used to record extraction profiles. When this integrator was used the detector values were recorded manually as the extraction progressed. After the initial extractions of 7–methoxycoumarin and 3–phenylcoumarin were completed, the integrator was changed to a Spectra Physic integrator that permitted basic programming and the series repeated. The Spectra Physics integrator was used during the duplicate runs because it digitized the detector output unattended. A basic program was written (Appendix C) to enable the recording of detector values at 0.5 min intervals beginning at 13 min into the run. The level values recorded are related to mV with 1000 = 1mV. The detector readings manually recorded



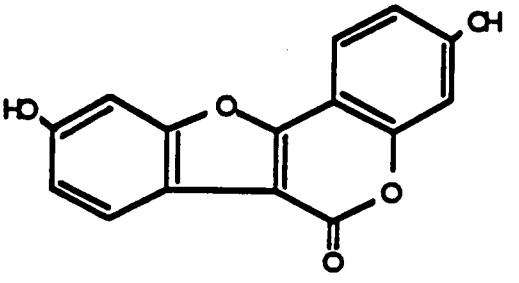

| | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| 1 | H | H | H | H |
| 2 | H | H | H | $OCH_3$ |
| 3 | $C_6H_5$ | H | H | H |
| 4 | $C(O)CH_3$ | $NH_2$ | H | H |
| 5 | H | H | $OCH_3$ | OH |
| 6 | H | H | H | OH |
| 7 | H | $CH_3$ | H | $NH_2$ |
| 8 | H | OH | H | H |
| 9 | H | $C_6H_5$ | H | OH |
| 10 | H | H | OH | OH |



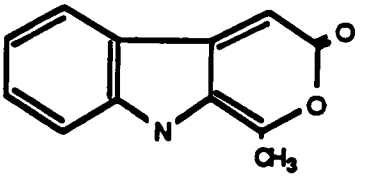

(11)



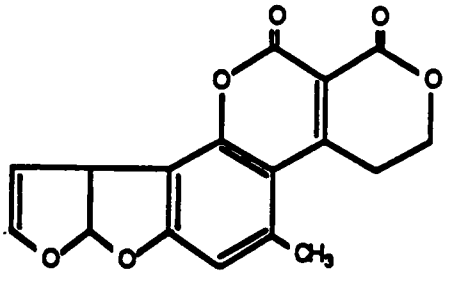

(12)




(13)




(14)




(15)

**Figure 25.** Structures of compounds employed in study. See Table IX for identification of structures 1-14. (15) Aflatoxin G2.

were in pA units. The detector readings for each integrator were converted to mV and compared.

## C. RESULTS AND DISCUSSION

The threshold densities obtained during this study should be considered "apparent threshold densities" since such factors as the surface effect of the extraction cell wall has been assumed negligible and FID response factors for all the model compounds were not determined. The use of a carbon black trap was intended to improve the solvent purity. At a recent meeting (ASTM Committee E19 Symposium on the Practice of Chromatography, October 1989) participants in a discussion related to $CO_2$ purity indicated that carbon black could actually increase the amount of impurities in the solvent. This is especially evident when extracting with $CO_2$ and concentrating the extract at the head of an analytical column or into an accumulator. During the experiments reported here the compounds were continuously extracted without accumulation, therefore any impurities present in the mobile phase were manifested in a baseline rise during the density gradient. The average difference between the initial detector reading at low density and the final detector reading at high density for 33 blank runs was 5.3±3.2 pA.

Figure 26 shows a representative extraction profile of 7-methoxycoumarin. For the purposes of evaluating the effect of extraction temperature, detector temperature, restrictor type and matrix interactions on threshold densities the apparent threshold density value was chosen to be that density that resulted in a detector response 10% full scale. All threshold densities were determined in this manner. Table IX lists the

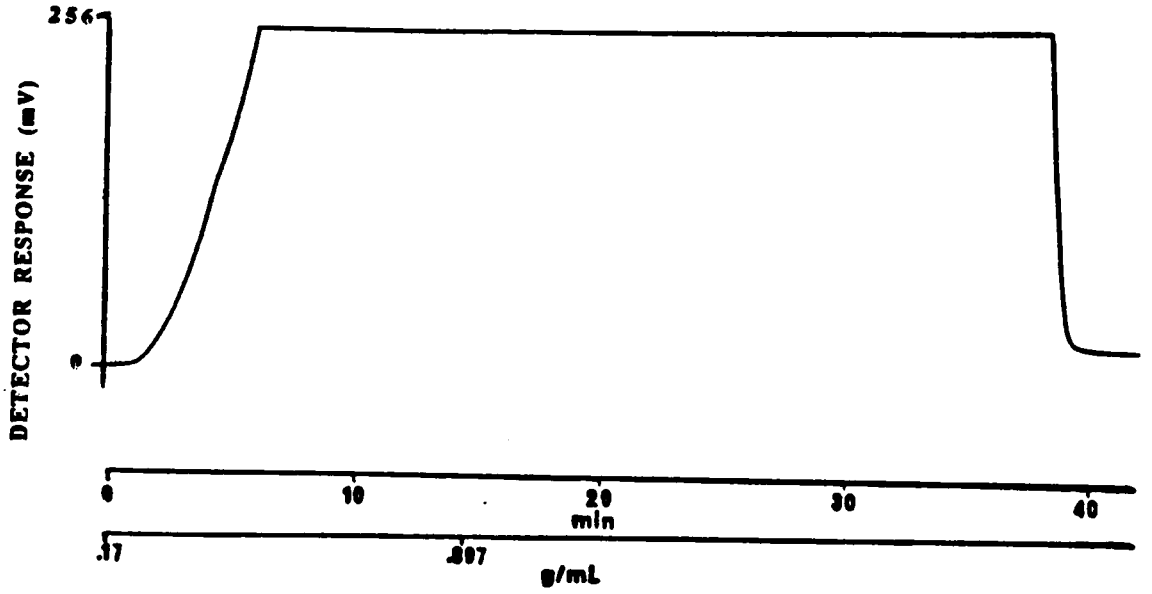



**Figure 26.** Extraction profile for 7-methoxycoumarin at 60°C, 0.17 to 0.90 g/mL at 0.05 g/mL/min.

Table IX: Apparent Threshold Densities of Model Compound Series Based on the Coumarin Structure.

| Compound | MW | M.P. | Threshold Density[a] |
|---|---|---|---|
| (1)[b] coumarin | 146.15 | 68.5-69.5°C | <0.17 g/mL |
| (2) 7-methoxycoumarin | 176.17 | 118-120 | 0.29±0.02 |
| (3) 3-phenylcoumarin | 222.00 | 140-141 | 0.41±0.01 |
| (11) psoralen | 186.17 | 158-161 | 0.47±0.01 |
| (12) "6-methyl-2H-1,3-dioxolo (4,5-H)(1)benzopyran" | 204.00 | 220-228(dec)[c] | 0.64±0.03 |
| (4) 3-acetyl-4-aminocoumarin | 203.00 | 120-130(dec)[c,d] | 0.67±0.05 |
| (5) 7-hydroxy-6-methoxycoumarin | 192.16 | 204 | 0.73±0.01 |
| (6) 7-hydroxycoumarin | 162.14 | 225-228(dec) | 0.76±0.01 |
| (7) 7-amino-4-methylcoumarin | 175.19 | 223-226 | 0.79±0.01 |
| (8) 4-hydroxycoumarin | 162.14 | 213-214(dec) | 0.83±0.03 |
| (14) "1-methylpyrano[3,4b]indol 3(9H)-one" | 199.21 | 246(dec) | not extractable |
| (9) 7-hydroxy-4-phenylcoumarin | 238.00 | 240-244(dec)[c] | not extractable |
| (10) 6,7 dihydroxycoumarin | 178.15 | 271-273 | not extractable |
| (13) coumestrol | 268.21 | 385 (sub.325) | not extractable |

a. Extraction conditions: 60°C, 0.17 to 0.90g/mL at 0.05 g/mL/min.
b. Numbers refer to structures in Figure 25.
c. Not reported in the literature., obtained in laboratory.
d. Appears to start melting at 120°C by 130°C bubble formation, apparent decomposition.

apparent threshold densities determined at 60°C with a density gradient of 0.05 g/mL/min from 0.17 to 0.90 g/mL. These values were the average of triplicate runs except for coumarin. Coumarin was so soluble that it started to extract before the shutoff valve was opened between the pump and the injector. The shutoff valve in principle isolates the SFC pump from the rest of the instrument to permit exchange of columns easily. In practice the shutoff valve still permits a very small flow of $CO_2$ through even when it is closed. Also reported in Table IX are the molecular weight and melting points of the model compounds investigated.

The first four compounds listed in Table IX were readily extracted with $CO_2$. That is, 0.5 to 1.5 mg of these compounds were completely extracted within an hour. With these four compounds the apparent threshold densities increase with increasing melting point but not necessarily with increasing molecular weight as evidenced with 3–phenylcoumarin and psoralen. The melting or decomposition points of those compounds that were partially extracted ranged between 200 and 140°C. For those compounds that were not extracted, under the constraints of the experiment, the melting or decomposition points were greater than 240°C. The threshold densities of the other compounds appear to be affected by the presence of functional groups as well as their position on the coumarin backbone. The data suggest that 7–hydroxycoumarin is more soluble in SC–$CO_2$ than 4–hydroxycoumarin although both compounds have the same MW and decompose at temperatures above 210°C. The increased solubility is evidenced by the decreased threshold density for 7–hydroxycoumarin. Stahl and Glatz (91) determined threshold pressures for a series of steroids. They concluded that MW and the arrangement of the functional groups in the molecule had negligible influence on

threshold pressure. They indicated that androsterone and dihydrotestosterone were not able to be fractionated. The only difference between these molecules is the position of the carbonyl and the hydroxyl groups. Fractionation of the hydroxycoumarins, if mixed together, may not be achieved since both compounds are sparingly soluble.

Table X lists the detector response at the plateau region for 4-hydroxycoumarin, 7-hydroxycoumarin and 7-hydroxy-6-methoxycoumarin. For these compounds the extraction profiles reached a plateau region with a very shallow declining slope that did not return to baseline within a reasonable time frame (<1 hr). Czubryt et al. (149) extracted polymers and reported solubilities based on the constant plateau method. With this method the height of the plateau is proportional to the solute concentration and fluid flow rate. In their study relative response factors were determined using propane as a reference compound. Since response factors for all the coumarin derivatives were not determined the detector response (Table X) at the plateau level may not be an indication of relative solubility. Relative response factors for 5 of the model compounds were determined under GC conditions (Table XI). The addition of a methoxy functionality onto coumarin did not change the detector response significantly. The addition of an hydroxy group on coumarin decreased the FID response by half while the addition of a methoxy and hydroxy group only decreased the FID response by one-quarter. Different amounts (2.3 to 4.3 mg) of 7-hydroxycoumarin charged to the extraction vessel did not change the plateau detection value significantly (136 pA ± 13 pA) indicating that a dynamic equilibrium was more than likely achieved. For 7-hydroxy-6-methoxycoumarin the plateau detector value averaged 249±24pA. The ratios of the detector response for 7-hydroxycoumarin to 7-hydroxy-6-methoxycoumarin under SFE conditions at 60°C and

Table X: Detector Response (pA) at Plateau Region as a Function of Extraction (Oven) Temperature.

| Compound | Detector Response | | | |
|---|---|---|---|---|
| | 40°C | 60°C | 80°C | 100°C |
| 4-hydroxycoumarin | - | 50±10(18)[a] | - | - |
| 7-hydroxycoumarin | 43±6(14) | 136±13(9.6) | 97±27(28) | 150±44( 29) |
| 7-hydroxy-6-methoxyycoumarin | 123±20(16) | 249±24(9.6) | 402±29(7.2) | 710±120(17) |

a. Value in () is the %RSD.

Table XI: Relative FID Response Factors under GC Conditions for 5 model compounds.

| Compound | Area counts /ng | Relative Response |
|---|---|---|
| Coumarin | 106±10 | 1.0 |
| 7-methoxycoumarin | 103±11 | 0.97 |
| 3-phenylcoumarin | 128±11 | 1.2 |
| 7-hydroxy-6-methoxycoumarin | 83±9 | 0.8 |
| 7-hydroxycoumarin | 52±9 | 0.5 |

GC conditions were 0.56±0.11 and 0.62±0.17 respectively. These values are within the error of the measurements, therefore the apparent increase in solubility of 7-hydroxy-6-methoxycoumarin over 7-hydroxycoumarin is only suggested by the decreased threshold density.

The apparent threshold density at 60°C for 7–hydroxycoumarin (0.67 ± 0.03 g/mL) agrees somewhat with the data obtained by Stahl et al. (102) where they extracted a variety of model compounds from quartz wool with $CO_2$ at 40°C. While Stahl indicated that the intensity of the TLC spot for coumarin was strong at 70 bar (0.21 g/mL), he did not indicate the extent of extraction of the 7–hydroxycoumarin at 100 bar (0.64 g/mL). The addition of a methoxy group at C6 on 7–hydroxycoumarin decreases the threshold density suggesting an increase in solubility. Muyachi et al. (99) extracted coumarins from plants at 40°C and 400 bar for 20 min. They indicated qualitatively the amount extracted with a plus (+) or minus (-) relating to the intensity of the TLC spot. Scopoletin (7-hydroxy-6-methoxycoumarin) was given a single + for extraction with 100% $CO_2$. This contrasted with two pluses when 3% ethanol was added to the fluid. Muyachi et al. (99) did not evaluate the extractability of 7–hydroxycoumarin.

While the addition of a methoxy group appears to enhance solubility, the addition of an hydroxyl group at C6 on 7–hydroxycoumarin makes the compound insoluble under the extraction conditions employed. Stahl et al. (102) were also unable to extract 6,7–dihydroxycoumarin at 40°C up to 400 bar. Muyachi et al. (99) were unable to extract the 6,7–dihydroxycoumarin at 40°C and 400 bar even with the addition of ethanol. The addition of a phenyl group at C4 on the 7–hydroxycoumarin also makes the compound insoluble under the conditions employed during this current investigation. No

threshold densities were reported for the last four compounds listed in Table IX since no significant changes above the blank run were observed. In some instances impurities manifested themselves with a baseline rise that returned to background levels. No significant change above the blank was observed when the sample was reextracted after the initial extraction.

Extraction temperature:

Four of the compounds from the model series were chosen for the evaluation of extraction temperature, detector temperature, restrictor type and matrix interaction. These compounds were 7–methoxycoumarin, 3–phenylcoumarin, 7–hydroxy–6–methoxy-coumarin and 7–hydroxycoumarin. Table XII lists the threshold densities of the 4 model compounds at different extraction temperatures. In all cases as the temperature increased the threshold density decreased. The initial temperatures investigated were 40, 60, 80 and 100°C. Figure 27 is a plot of the data and shows that for 7–methoxycoumarin and 3–phenylcoumarin there was a drastic change between the threshold density at 40 and 60°C. Therefore, threshold density values at 45 and 50°C were also obtained for these two compounds to better define the curve. The values in Table XII were not corrected for the residence time within the restrictor length. Since the experimental design employs a fixed restrictor and density programming, the linear velocity of the system is constantly changing. Thus, the residence time within the restrictor length changes from a maximum time at the initial density to a minimum time at the final density. Only linear velocity values at 80°C and 0.82 g/mL with different detector temperatures were determined for the restrictor used during the extraction

Table XII: Apparent Threshold Densities (g/mL) of Four Model Compounds as a Function of Extraction (Oven) Temperature.

| Compound | Threshold Density | | | | | |
|---|---|---|---|---|---|---|
| | 40°C | 45°C | 50°C | 60°C | 80°C | 100°C |
| 7-methoxycoumarin | 0.59±0.01 | 0.44±0.01 | 0.33±0.01 | 0.29±0.02 | 0.28±0.01 | 0.22±0.02 |
| 3-phenylcoumarin | 0.69±0.01 | 0.59±0.02 | 0.47±0.01 | 0.41±0.01 | 0.34±0.01 | 0.27±0.00 |
| 7-hydroxy-6-methoxycoumarin | 0.86±0.01 | - | - | 0.73±0.01 | 0.58±0.01 | 0.45±0.01 |
| 7-hydroxycoumarin | 0.92±0.02 | - | - | 0.76±0.01 | 0.65±0.01 | 0.51±0.02 |

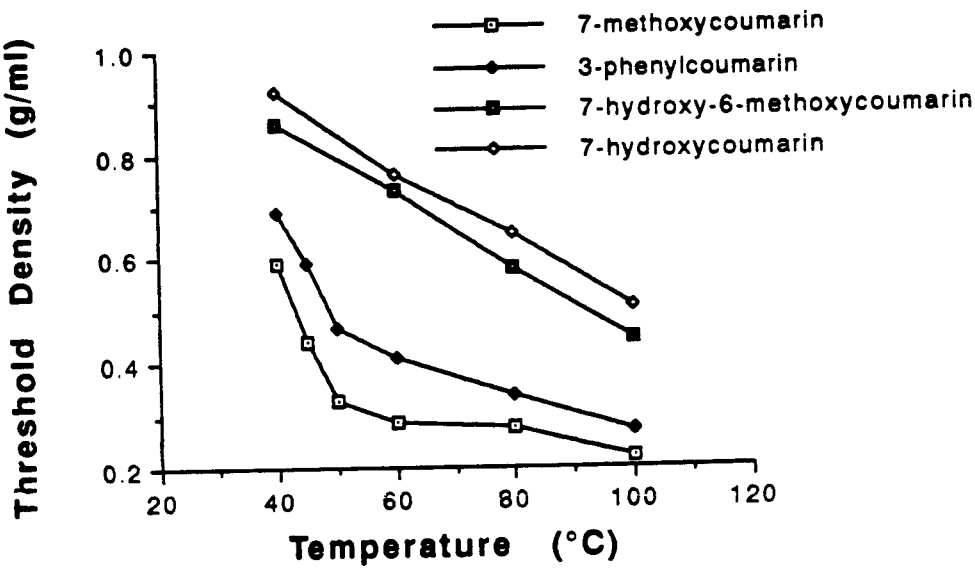



**Figure 27.** Plots of threshold density vs extraction temperature.

temperature study. Measurements of linear velocity through the temperature range 40-100°C at low and high densities were obtained using a 50% phenylmethylpolysiloxane column (100 μm i.d. x 10 m) with a frit restrictor (100 μm i.d. x 61 cm). Using this data (Appendix D) minimum and maximum residence times were estimated for the restrictor used in the extraction experiments. These times were used to calculate the minimum and maximum density changes that occurred during the residence time based on a density rate of 0.05 g/mL/min. For 40°C the maximum change in density due to residence time was 0.083 g/mL while at 80°C it was calculated to be 0.046 g/mL. Even using a correction factor for the maximum density change, similar patterns in threshold densities as a function of extraction temperature are observed.

Since an increase in temperature decreases the threshold density, increasing vapor pressure of the compounds appears to come into play. Smith et al. (145) determined threshold pressures for pyrene and benzopyrene at various temperatures. For benzopyrene as the temperature increased the threshold pressure increased. This indicated that at a given pressure the solubility of the compound is less at higher temperatures because the solvent strength is reduced. For pyrene as the temperature increased above 60°C the threshold pressure decreased. This indicated that the vapor pressure of the compound began to affect the solubility more than the solvent strength. But if the pressure data for both compounds are converted to density, the solvent density decreases with increasing temperature. Decreased solvent density means decreased solvating power. Therefore, the vapor pressure of benzopyrene also appears to affect its solubility also. When the data obtained in this study was converted to pressure units a pattern similar to that reported by Smith et al. (145) did not emerge.

Figure 28 shows the extraction profile for 7-methoxycoumarin at 40°C. The extraneous peaks on the leading edge indicate that impurities are present. Careful inspection of the other extraction profiles for 7–methoxycoumarin indicate that by 60°C these peaks are still integrated separately although off scale. At 80 and 100°C the integrator only records a single peak for 7–methoxycoumarin and the impurities. These impurities possibly could be fractionated from the bulk of the 7–methoxycoumarin by a step extraction although complete recovery of the 7–methoxycoumarin may require a preparatory chromatographic step.

Detector Temperature:

Berger and Toney (150-151) recently reported that with a fixed restrictor, column pressure, column temperature and restrictor temperature all affect the mass flow through the column and therefore, change the linear velocity. The mobile phase linear velocity can increase 10-15 fold during pressure programming (150). Berger (151) showed that with a linear restrictor, hotter restrictors have higher mass flows, as well as, larger increases in mass flow vs pressure than colder restrictors. His data suggest that the restrictor temperature should be kept as low as possible. One problem that exists with a low detector temperature (restrictor temperature) when using FID is that generally detector spiking increases. This was evident during the experiment with the detector temperature set at 175°C. For the two very soluble compounds investigated detector spiking was evident during the extraction.

The effect of detector temperature on the threshold density was determined at 80°C with a density program of 0.14 to 0.82 g/mL at 0.05 g/mL/min. The data are

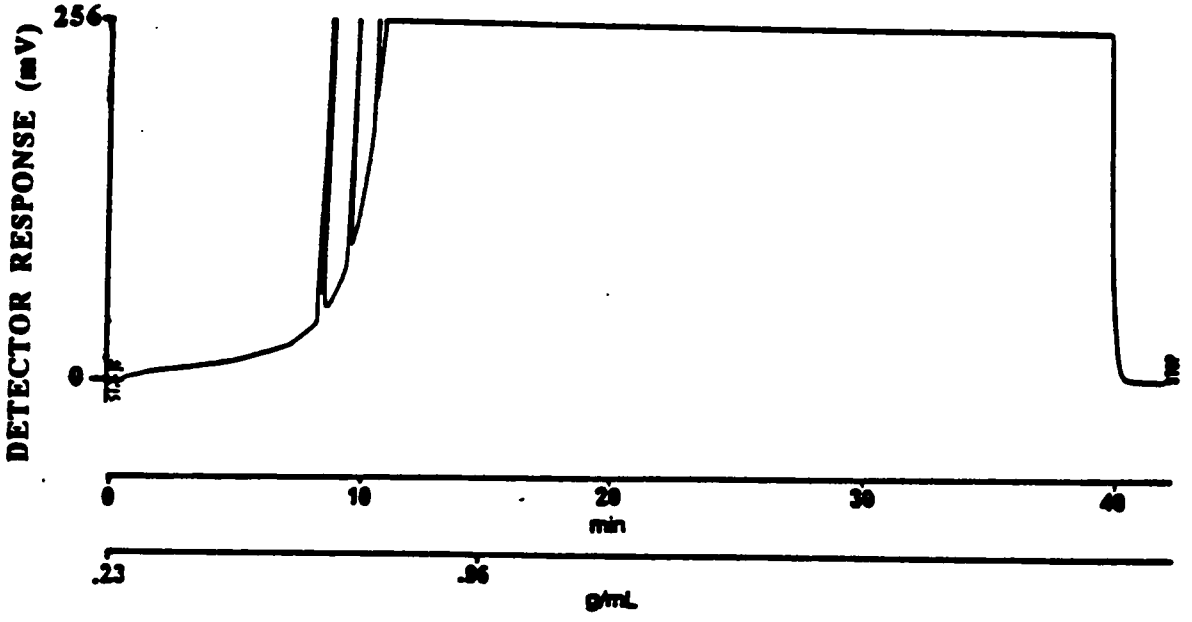



**Figure 28.** Extraction profile of 7–methoxycoumarin at 40°C, 0.23 to 0.96 g/mL at 0.05 g/mL/min.

recorded in Table XIII. The detector temperature appears to have minor influence on the measured threshold densities although values appear to be decreasing as the detector temperature decreases. This decrease could be the result of increasing linear velocity as detector temperature decreases. The linear velocity was measured at 0.82 g/mL. The extraction cell was replaced with a zero dead volume connector and $CH_2Cl_2$ was injected. The retention time of $CH_2Cl_2$ was used in the linear velocity calculations. The threshold density values for the four compounds investigated varied from 0.27 to 0.65 g/mL. Thus, a linear velocity measurement at 0.82 g/mL would not adequately reflect changes due to residence time within the restrictor. Therefore, additional linear velocity measurements were obtained at different detector temperatures and densities between 0.14 and 0.82 g/mL with an oven temperature of 80°C (Appendix D). These measurements were made using a 50% phenylmethylpolysiloxane column (100 μm i.d. x 10 m) with a frit restrictor (100 μm i.d. x 61 cm). These data contrast with Berger's data using a linear restrictor. The difference could be caused by the drop in pressure over the multipath frit restrictor as opposed to an open restrictor.

Restrictor Type:

The effect of restrictor type was evaluated at 80°C. The restrictors that were evaluated in addition to the frit restrictor (a porous polymer plug at the end of an uncoated capillary tube) were a linear and an integral restrictor. A linear restrictor is a straight piece of capillary tubing whose inner diameter is generally ≤ 15 μm. An integral restrictor is a capillary tube (≥ 25 μm i.d.) that was tapered or sealed to an opening around 2 μm i.d. The linear restrictor had too large a flow resulting in detector

Table XIII: Apparent Threshold Densities (g/mL) as a Function of Detector Temperature

| Compound | Threshold Density[a] | | |
|---|---|---|---|
| | 375°C | 275°C | 175°C |
| 7-methoxycoumarin | 0.28±0.01(0.24)[b] | 0.28±0.01(0.25) | 0.27±0.01(0.25) |
| 3-phenylcoumarin | 0.34±0.01(0.30) | 0.33±0.01(0.30) | 0.31±0.00(0.29) |
| 7-hydroxy-6-methoxycoumarin | 0.58±0.01(0.55) | 0.56±0.01(0.53) | 0.53±0.01(0.51) |
| 7-hydroxycoumarin | 0.65±0.01(0.63) | 0.64±0.01(0.62) | 0.60±0.01(0.59) |
| linear velocity (cm/sec)[c] | 3.9 | 4.9 | 6.2 |

a. Extraction conditions: 80°C, 0.14 to 0.82 g/mL at 0.05 g/mL/min.
b. Correction based on restrictor residence time. See Appendix D.
c. Measured at 0.82 g/mL without extraction cell in-line.

spiking during the extraction. The integral restrictor was commercially purchased and rated at 6 mL/min. The linear velocity measured at 80°C and 0.82 g/mL was 0.83 cm/sec. Table XIV lists the apparent threshold densities as a function of restrictor type. If the threshold density values for 7-methoxycoumarin and 3-phenylcoumarin are corrected using the value calculated for the density change during restrictor residence time, then there essentially is no difference between the threshold values for the different restrictors. The restrictor residence times were calculated using the linear velocity measured at the density closest to the threshold density (see Appendix D). The threshold density values for 7-hydroxy-6-methoxycoumarin and 7-hydroxycoumarin were also corrected. In this instance a higher threshold density is found for the integral restrictor. This increased threshold density could be explained by interaction with the restrictor surface eventually causing restrictor plugging. Figure 29 shows the extraction profile for 7-hydroxycoumarin employing an integral restrictor. The initial extraction behaved in the same manner as those extractions with a frit restrictor. After two days of use, the response for 7-hydroxycoumarin decreased significantly when using the integral restrictor. This decrease may have been caused by adsorption of the extracted material onto the restrictor internal surface. A decrease in detector response was also observed for 7-hydroxy-6-methoxycoumarin. Further evidence of adsorption was observed with the extraction profile of 7-methoxycoumarin. While no apparent change in threshold density was found using the correction factor, the profile showed tailing as the extraction went to completion. No tailing was observed with 3-phenylcoumarin when using the integral restrictor or with 7-methoxycoumarin during the previous experiments with the frit restrictor. Apparently the addition of a

Table XIV: Apparent Threshold Densities as a Function of Restrictor Type

| Compound | Threshold Density[a] frit | integral |
|---|---|---|
| 7-methoxycoumarin | 0.28±0.01(0.24)[b] | 0.41±0.02(0.23) |
| 3-phenylcoumarin | 0.34±0.01(0.30) | 0.47±0.01(0.29) |
| 7-hydroxy-6-methoxycoumarin | 0.58±0.01(0.55) | 0.73±0.04(0.63) |
| 7-hydroxycoumarin | 0.65±0.01(0.63) | 0.78±0.01(0.72) |
| linear velocity (cm/sec)[c] | 3.9 | 0.83 |

a. Extraction conditions: 80°C, 0.14 to 0.82 g/mL at 0.05 g/mL/min.
b. Correction based on restrictor residence time. See Appendix D.
c. Measured at 0.82 g/mL without extraction cell in-line.

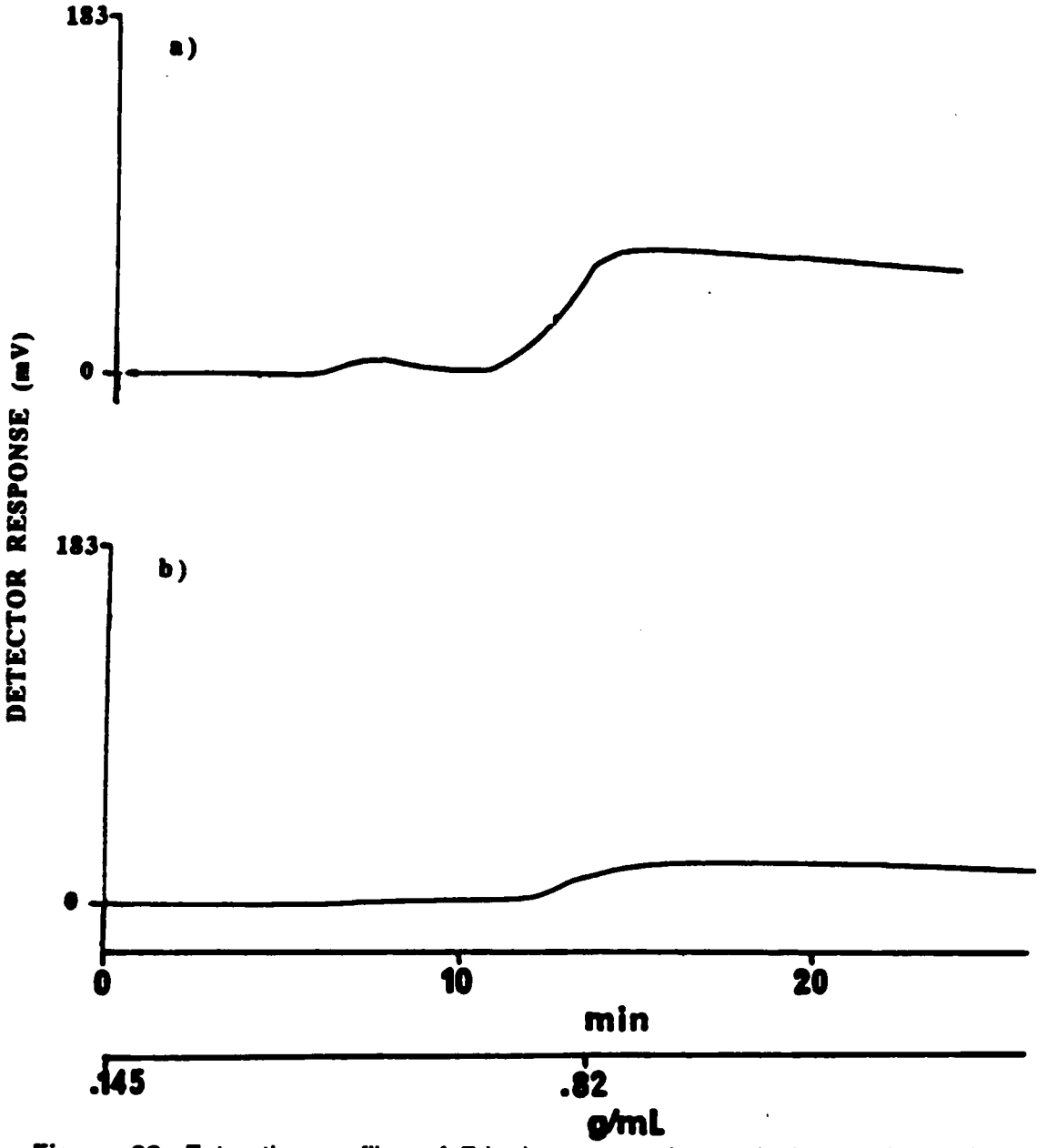



**Figure 29.** Extraction profiles of 7-hydroxycoumarin employing an integral restrictor with an extraction temperature of 80°C. a) Initial extraction; b) third extraction 2 days later.

functional group containing a polar functionality on the coumarin backbone results in adsorption onto the surface of the integral restrictor. The adsorption may have resulted from insufficient deactivation of the fused silica used for the integral restrictor. Examination of the restrictor under a microscope revealed apparent plugging. Since no indication of sample precipitation occurred with the frit restrictor, this plugging may have resulted from adsorption at the integral tip. Further examination of the integral restrictor tip showed that the tip opening was distorted which also could have resulted in plugging.

Density Gradient Rate:

The threshold density values for 3-phenylcoumarin and 7-hydroxycoumarin at two different density gradients were compared (Table XV). The threshold density value for 3–phenylcoumarin slightly decreased when the lower density rate (0.02 g/mL/min) was used. This decrease was still evident with the values corrected for density changes due to residence time in the restrictor. A possible explanation for this decrease is that the extracting fluid was in contact with the solute at a given density for a longer time when the lower density rate was employed. With 7-hydroxycoumarin a similar pattern emerged but the values are within the measurement error of the lower rate. The restrictor employed for the density gradient rate experiments was 100 μm x 20.6 cm. This might account for slight differences in the threshold densities measured during the extraction temperature evaluations that employed the same 100 μm i.d. frit restrictor but its length was 61 cm.

Table XV: Apparent Threshold Densities as a Function of Density Ramp Rate

| Compound | Threshold Density[a] 0.050 g/mL/min | 0.020 g/mL/min |
|---|---|---|
| 3-phenylcoumarin | 0.37±0.01[b](0.36)[c] | 0.31±0.01(0.30) |
| 7-hydroxycoumarin | 0.67±0.01(0.66) | 0.63±0.04(0.63) |

a. Extraction conditions: 80°C, 0.14 to 0.82 g/mL.
b. The average of duplicate runs except for 3-phenylcoumarin at density gradient of 0.05 g/mL/min which was the average of triplicate runs.
c. Correction based on residence time. See Appendix D.

Matrix Interaction:

Four matrices, carbon black, silica gel, acidic aluminum oxide and corn starch were evaluated for their effect on the extraction of 7–methoxycoumarin, 3–phenylcoumarin, 7–hydroxy–6–methoxycoumarin and 7–hydroxycoumarin. Carbon black is a quasi-graphite form of carbon of small particle size. Activated charcoal is generally prepared from wood and vegetables. Silica gel is silicic acid ($H_2SiO_3$) in the form of granules. Aluminum oxide that is suitable for chromatography is prepared from aluminum hydroxide. The hydroxide is dehydrated and calcined at about 900°C in a $CO_2$ stream. This tends to coat the $Al_2O_3$ particles with a thin layer of aluminum oxycarbonate ($[Al_2(OH)_5]_2CO_3 \cdot H_2O$. The alkalinity of the particles is adjusted by washing with dilute acids. Starch is a crystalline polymeric compound consisting of linear (amylose) and branched (amylopectin) polymers of glucose (152). Each matrix was preconditioned by filling the extraction cell and running the gradient at 80°C. Once the matrix was preconditioned, known quantities of solid sample and matrix were manually mixed. The ratio of grams matrix to grams compound ranged between 0.8 to 11. A solution of the model compound was not used because evaporation of the organic solvent could not ensure the absence of solvated molecules or matrix. Wheeler and McNally (71) showed that the simple addition of 200 µL of organic solvent to a matrix could affect the extraction of herbicide and pesticides.

The threshold densities determined for 7-methoxycoumarin and 3-phenylcoumarin did not significantly increase when a matrix was added to the extraction. In some instances the threshold density decreased (Figure 30). The lack of consistent change in threshold densities indicates that the majority of the compound present in the cell had very little interaction with the matrix. The ability for the model compounds to

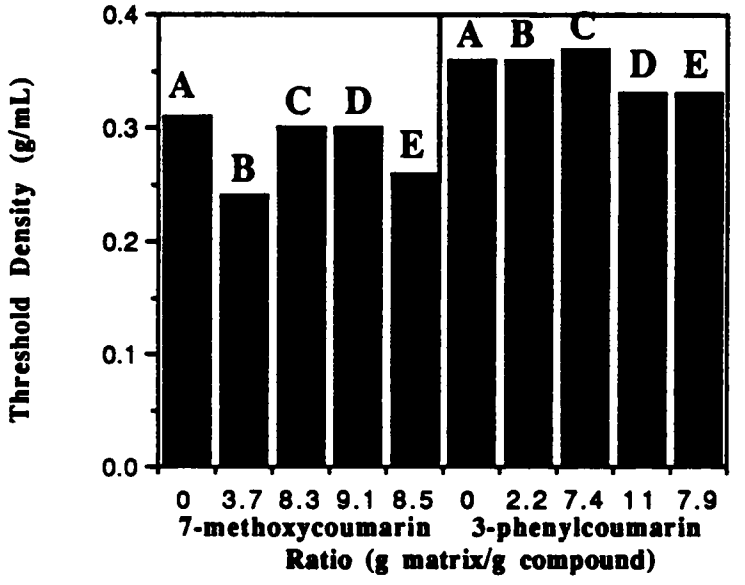



**Figure 30.** Bar graph of threshold densities determined for 7–methoxycoumarin and 3–phenylcoumarin with a variety of matrices. A) no matrix; B) carbon black; C) silica gel; D) aluminum oxide, acid; E) corn starch.

be completely extracted within the time frame of the extraction without matrix (Figure 31) was affected. Corn starch was the only matrix that permitted a complete extraction. Of the other three matrices, carbon black has the slowest desorption rate (smallest slope value) while silica gel had the fastest desorption rate in each series (Tables XVI and XVII). The results show that corn starch appears to be an ideal non-interactive matrix. Also for the 7-methoxycoumarin and 3-phenylcoumarin, silica gel may be a good matrix to use if concentration of the extracted material is desired onto a solid phase. Since desorption is the fastest, recovery would be quicker. Further studies would be required to determine the breakthrough point using the various matrices.

Since 7-hydroxy-6-methoxycoumarin and 7-hydroxycoumarin were not completely extractable under the constraints of the experimental design, only threshold density values with and without matrices were evaluated. Table XVIII lists the average value of threshold densities for duplicate runs. For 7-hydroxy-6-methoxycoumarin the %RSD (0 to 1.6) values for all matrices except carbon black are within the range found with the extraction temperature experiments. The % RSD (6.2) for the carbon black is greater. This variation results because the initial threshold value observed was significantly greater than the second value obtained without recharging the extraction vessel. The second value was within the range observed for no matrix and for the other matrices investigated. The high initial value could be caused by a coextracted material that retained the compound longer than the carbon black matrix.

For 7-hydroxycoumarin the threshold density value with carbon black present increased significantly. Also of significance was the decrease in detector response at the plateau level. With carbon black present, the detector response was 1/3 the response observed for no matrix or any of the other matrices. When silica gel was mixed with

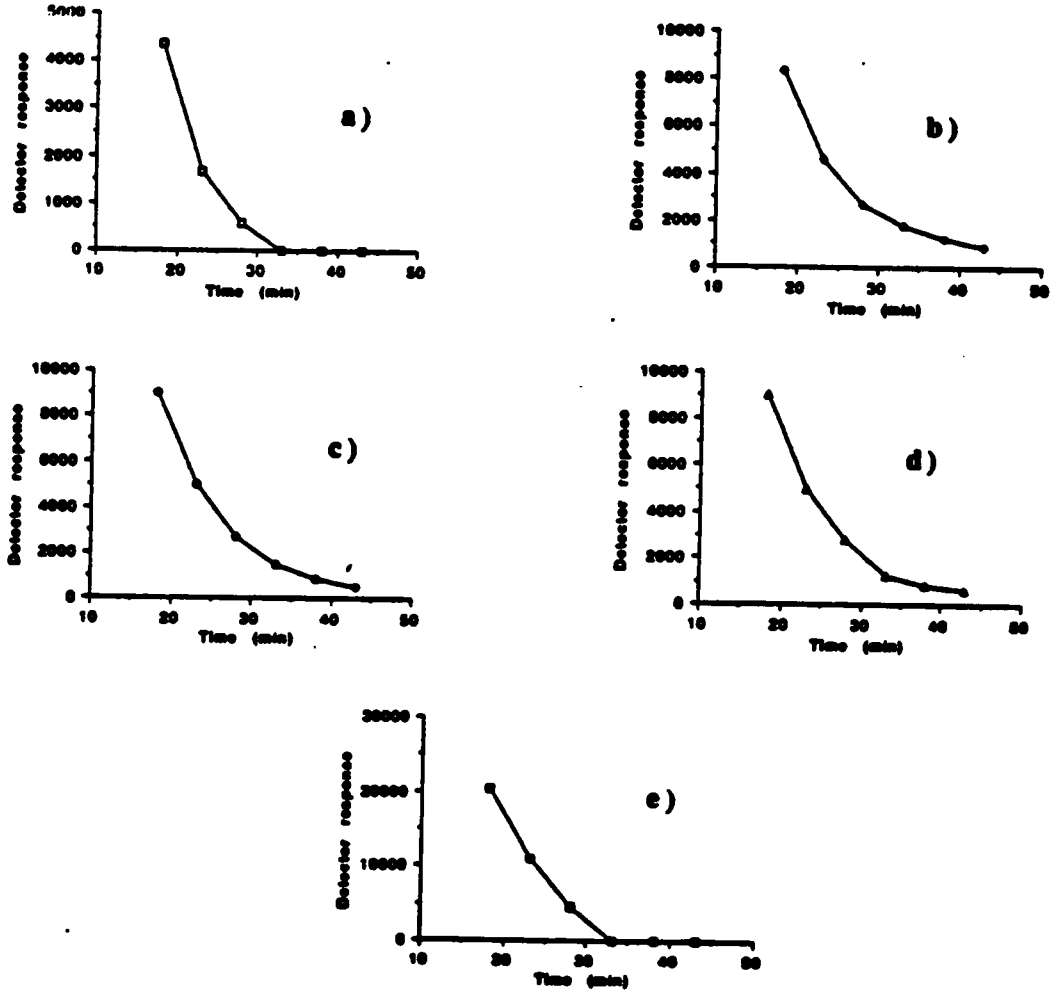



**Figure 31.** Plots of detector response vs time of extraction for 3–phenylcoumarin mixed with a variety of matrices. Extraction conditions: 80°C, 0.145 to 0.820 g/mL at 0.05 g/mL/min. a) no matrix; b) carbon black; c) silica gel; d) $Al_2O_3$; e) corn starch.

Table XVI: Linear Response Data of Log Detector Response vs Extraction Time Plot for the Interaction of 7-methoxycoumarin and a Variety of Matrices.

| Matrix | Ratio[a] | Slope | R2[b] |
|---|---|---|---|
| $Al_2O_3$,acid | 9.1:1 | -0.027[c] | 0.946 |
| | 10:1 | -0.039[d] | 0.906 |
| Silica gel | 8.3:1 | -0.031[c] | 0.991 |
| | 8.2:1 | -0.043[d] | 0.982 |
| Carbon black | 3.7:1 | -0.020[c] | 0.975 |
| | 1.8:1 | -0.029[d] | 0.939 |

a. Ratio of g matrix/ g compound.
b. Correlation coefficient - measures the "goodness of fit" of the regression line.
c. Run 1 with manual recording of detector response.
d. Run 2 with automatic recording of detector response.

Table XVII: Linear Response Data of Log Detector Response vs Extraction Time Plot for the Interaction of 3-phenylcoumarin and a Variety of Matrices.

| Matrix | Ratio[a] | Slope | $R^{2b}$ |
|---|---|---|---|
| $Al_2O_3$,acid | 11:1 | -0.030[c] | 0.968 |
| | 9.0:1 | -0.049[d] | 0.985 |
| Silica gel | 7.4:1 | -0.043[c] | 1.000 |
| | 8.1:1 | -0.051[d] | 1.000 |
| Carbon black | 2.2:1 | -0.030[c] | 0.990 |
| | 1.8:1 | -0.039[d] | 0.985 |

a. Ratio of g matrix/g compound
b. Correlation coefficient - measures the "goodness of fit" of the regression line.
c. Run 1 with manual recording of detector response.
d. Run 2 with automatic recording of detector response.

Table XVIII: Apparent Threshold Densities as a Function of Matrix

| Matrix | Threshold Density[a] | Ratio[b] |
|---|---|---|
| 7-hydroxy-6-methoxycoumarin: | | |
| none (2.2 mg) | 0.60±0.02(3.3)[c] | - |
| carbon black | 0.65±0.04(6.2) | 0.81 |
| silica gel | 0.60±0.00(0) | 1.8 |
| $Al_2O_3$ | 0.62±0.01(1.6) | 7.3 |
| corn starch | 0.61±0.01(1.6) | 7.1 |
| 7-hydroxycoumarin: | | |
| none (1.1mg) | 0.67±0.01(1.5) | - |
| carbon black | 0.80[d] | 3.7 |
| silica gel | 0.70±0.06(8.5) | 5.1 |
| $Al_2O_3$ | 0.64±0.02(3.1) | 4.7 |
| corn starch | 0.67±0.01(4.6) | 7.8 |

a. Extraction conditions: 80°C, 0.14 to 0.82 g/mL at 0.05 g/mL/min.
b. Ratio of g matrix/g compound.
c. Value in () is the %RSD.
d. Plateau region for second run below 10% FS response.

7–hydroxycoumarin the %RSD (8.5) was outside the range found with the extraction temperature experiments. As with 7-hydroxy-6-methoxycoumarin mixed with carbon black, the initial threshold density value was greater than the second value obtained without recharging the vessel. This high initial value could result from the coextraction of an impurity that retained the compound longer than the silica matrix.

## D. CONCLUSION

Functionality, extraction temperature and matrix affect the extractability of the compounds investigated in this study as demonstrated by changes in threshold density values and incomplete extraction of compounds that were readily extracted without matrix. Although the detector temperature can affect flow characteristics through a chromatographic system and therefore retention times, threshold densities for the model compounds studied are not drastically affected. For extracted materials at low densities a lower gradient rate may permit a more accurate measure of threshold density values. While the experimental design used a frit restrictor, an integral restrictor is possible to use. When using any restrictor care must be taken to ensure that the restrictor tip is deactivated. The high flow through a linear restrictor caused detector spiking. Another type of detection such as UV could possibly avoid this spiking problem.

The addition of hydroxyl or amine groups onto the coumarin structure decreases their extractability in SC–$CO_2$. This decrease is expected and has been reported by other authors for a variety of compounds (91,102,143). The position of the functional group can affect a compound's extractability. The increased threshold density of 4–hydroxycoumarin from 7–hydroxycoumarin may be due to a change in dipole moment.

While the dihydroxycoumarin may permit intra-hydrogen bonding, an extensive inter-hydrogen bonding network is possible. This may account for the inability to extract the dihydroxycoumarin using SC–$CO_2$ upto 400 atm.

The extraction temperature data suggest that vapor pressure plays a significant role in the extractability of the coumarins as well as other organic compounds (145). Although higher temperatures cause a decrease in threshold densities, fractionation between the coumarins may best be accomplished at mid-temperatures 45-60°C. Some impurities as shown with 7-methoxycoumarin are more readily fractionated at lower temperatures.

The matrix study shows that corn starch is a non-interactive matrix. The experimental design permits an easy means of evaluating desorption of compounds from a matrix. This could be useful information if the extracted material is concentrated onto a solid phase. The desired material could then be desorbed from the solid phase using SC-$CO_2$ for further analysis by chromatographic techniques such as SFC or GC.

## VI. SUMMARY AND FUTURE WORK

Three applications of chromatography or extraction employing SFs in the analysis of food components and natural products were presented. The data show that SFC and SFE have significant potential for the analysis of food components and natural products.

The first application related to the analysis of peracetylated nitrogen derivatives of carbohydrates. Generally, it is preferred to analyze the original compound instead of employing derivatization methods to enhance detectability. Since many food components are too polar to analyze using SFC or GC, derivatization of polar compounds makes them amenable to analysis by GC or SFC. The study was originally initiated to increase the MW range of the peracetylated aldonitrile (PAAN) derivatives of carbohydrates detected under GC conditions. Several laboratories have successfully detected larger MW species of derivatized polysaccharides using SFC than can be detected under GC conditions (18-19, 21). The attraction for using the PAAN derivatives is that the GC analysis is simplified (109-111). That is, one reaction product per sugar is observed. SFC analysis of the PAAN derivatives of monosaccharides resulted in the detection of multiple reaction products per sugar. The ease of coupling SFC to FT-IR and MS detectors permitted the identification of some of those reaction products not observable under conventional GC analysis.

The peracetylated nitrile and acyclic oxime were readily identified with the data from both spectroscopic techniques. MS data were required since the nitrile stretching region (2400-2100 $cm^{-1}$) is completely masked by the SC-$CO_2$ absorbance. Also, the C=N absorbance (1660-1610 $cm^{-1}$) was too weak to assign in the FT-IR spectra

obtained. MS data from ammonia chemical ionization were required to distinguish between the reaction products. Initial methane chemical ionization data showed that both the peracetylated nitrile and acyclic oxime had the same major ion. The FT-IR and MS data for the third reaction product suggested that a cyclic oxime was produced. The data were not conclusive enough to determine if the structure contained a pyranose or furanose ring.

The complex nature of the derivatization mixture containing a variety of monosaccharides indicated that this was not an ideal derivatization method to use for SFC. The PAAN derivatization procedure employed in this study demonstrated the ability of using SFC with spectroscopic detection to monitor derivatization reactions. Furthermore, the effect of modifications to derivatization procedures which are designed to eliminate additional reaction products, that may be thermally labile or non-volatile, can be quickly investigated employing SFC. This ability should hold true for other derivatization methods employed.

Future research related to carbohydrate analysis include studying different derivatization methods. The MS detection limits the molecular weight range of the polysaccharide that can be detected. By employing a derivatization method that incorporates a carbonyl moiety, FT-IR can be used as a selective detection method. That is, the carbonyl region is still available even when an organic modifier is used.

The second application related to the use of SFC/FT-IR as an analytical method in monitoring changes in chemical composition of vegetable oils due to processing. Soybean oil was studied since it is one of the most widely used vegetable oils in the US. Hydrogenation is a processing technique used on soybean oil to change the characteristics of the original oil. Hydrogenation changes the fatty acid content of the original oil.

Analytical methods that permit the identification of individual isomers resulting from partial hydrogenation is desirable. IR detection is used to monitor conversion from cis to trans isomers. The SFC literature has shown that FAMEs, FFAs and TGs can be separated. Saturated and unsaturated C18 fatty acids were partially resolved spectroscopically even though chromatographic resolution was not achieved when employing packed column SFC with on-line FT-IR detection (34). In their report the conversion of cis to trans isomers due to processing was not studied. Another laboratory (32) used SFC with a polar open tubular column and successfully separated the saturated and unsaturated C18 fatty acids. They also indicated partial separation of cis and trans isomers but they did not employ a FT-IR detector. The application reported here combined the resolving power of the polar open tubular column with the information available with FT-IR detection.

The data from this study indicate that SFC with on-line FT-IR detection shows promise in the ability to monitor some changes in the chemical composition of vegetable oils due to processing. A major advantage of SFC over LC is that on-line detection using SFC permits the monitoring of the C-H deformation region (1000-900 $cm^{-1}$) of trans $R_1HC{=}CHR_2$ groups, the C–H stretching region (3020- 2800 $cm^{-1}$) and the carbonyl region (1800-1700 $cm^{-1}$). Therefore, an opportunity to determine the extent of unsaturation and isomerization in a single analysis exists. The SFC/FT-IR on-line file spectra of TGs and FFAs from soybean oil showed that the conversion to trans isomers was readily detected in those samples that were hydrogenated with the Ni-S catalyst. The Ni catalyst resulted in a much smaller isomer conversion than the Ni-S catalyst. Therefore, it was easier to detect the trans CH deformation absorbance in the separated components of the Ni-S catalyst than the Ni catalyst samples. Generally, most uses of

processed soybean oil require a low conversion to the trans isomer (140). Therefore, on-line FT-IR detection may not be sufficient to characterize the isomers resulting from partial hydrogenation. IR absorbance below 800 cm-1 is completely masked by the SC-$CO_2$ absorbance. While the cis olefinic CH stretching absorbance (3010-3018 cm-1) was readily observed in the lipid spectra, the CH deformation vibrations of the cis $R_1HC{=}CHR_2$ groups observed at 730 $cm^{-1}$ was not detected. The SFC separations of the FAMEs, TGs, and FFAs obtained during this study resulted in only partial resolution of the cis and trans isomers. Also, the TGs in refined soybean oil were not completely resolved. The chromatographic resolution may be improved by using a series of columns with different stationary phases such as a 50% cyanopropyl polysiloxane phase in series with a liquid crystalline stationary phase that can separate isomers. Additional phases may become available that will provide better resolution of lipids as stationary phase technology improves. By coupling SFC with solvent elimination FT-IR the ability to determine cis/trans isomerization at low levels should be enhanced.

The third application dealt with the SFE of a model compound system based on the coumarin structure. The SFE literature indicated that two laboratories (98-99, 102) did limited work with coumarins. Stahl et al. (102) determined that 6,7–dihydroxycoumarin was not extractable at 40°C with pressures up to 400 bar. The "rules of thumb" they established with the data from a variety of model compounds did not account for their coumarin derivative data. Due to the availability of coumarins with a variety of functional groups, a study was initiated to determine if the empirical rules within the literature could be enhanced by studying this structural system. Threshold density values were determined using a supercritical fluid chromatograph with a flame ionization detector. The extraction cell was LC stainless steel precolumn. Milligram

quantities of the model compounds were extracted. The effects of functionality, extraction temperature, detector temperature and matrix on threshold density were evaluated.

As anticipated functionality, extraction temperature and matrix affected the extractability of the compounds investigated in this study. These effects were demonstrated by changes in threshold density values and incomplete extraction of compounds that were readily extracted without matrix. Although the detector temperature can affect flow characteristics through a chromatographic system and therefore retention times, threshold densities for the model compounds studied were not drastically affected. For extracted materials at low densities a lower gradient rate resulted in lower threshold density values.

The addition of hydroxyl or amine groups onto the coumarin structure decreases their extractability in SC-$CO_2$. This decrease is expected and has been reported by other authors for a variety of compounds (91,102,143). The position of the functional group can affect a compound's extractability. The increased threshold density of 4–hydroxycoumarin from 7–hydroxycoumarin may be due to a change in dipole moment. This contrasted with the data of Stahl and Gerard (65), who indicated that the positions of the functional groups on some steroids did not affect the extractability. For those compounds the switching of the hydroxyl and carbonyl groups did not significantly change the dipole moments of the molecules. Dange et al. (143) reported that the position of halogens on the phenol structure did affect extractability, with these compounds you would anticipate a change in dipole moment. The simple addition of a hydroxyl group onto the coumarin structure significantly reduces the extractability of the compound. The addition of a phenyl group onto 7-hydroxycoumarin resulted in the

compound not being extracted under the conditions studied. Therefore, it is not surprising that the addition of two fused rings onto 7–hydroxycoumarin, to produce coumestrol, results in the inability to extract this compound. My original assumption, based on the "rules of thumb" proposed by Stahl et al. (102), was that coumestrol contained only two phenolic hydroxyl groups that were on opposite ends of the molecule therefore it would be possible to chromatograph under SFC conditions. While the 6,7–dihydroxycoumarin may permit intra-hydrogen bonding, an extensive inter-hydrogen bonding network is possible. This may account for the inability to extract the dihydroxycoumarin using SC-$CO_2$ up to 400 atm. The results from this study indicate that one hydroxyl group added to the coumarin structure reduces the extractability significantly such that additional functional groups render the compound non-extractable below 400 atm.

The extraction temperature data suggest that vapor pressure plays a significant role in the extractability of the coumarins. Higher temperatures cause a decrease in threshold densities. Smith et al. (145) showed this with pyrene and benzopyrene. Since the greatest difference between the threshold densities of 7-methoxycoumarin, 3–phenylcoumarin, 7–hydroxy–6–methoxycoumarin and 7–hydroxycoumarin was at lower temperatures, fractionation between these compounds may best be accomplished at lower temperatures.

The matrix study shows that corn starch is a non-interactive matrix. The experimental design permits an easy means of evaluating desorption of compounds from a matrix. Of those matrices that showed an effect, desorption was fastest with the silica gel. Also, the 7–hydroxycoumarin was adsorbed to the carbon black more strongly than the other compounds. This greater interaction was evident by the increased threshold

density. This information is useful when the extracted material is concentrated onto a solid phase. The desired material could then be desorbed from the solid phase using SC-$CO_2$ for further analysis by chromatographic techniques such as SFC or GC.

Future research that should enhance the experimental SFE data include estimating the solubility parameters, vapor pressures and dipole moments of these model compounds. These data may help explain the extractability of the model compounds further. Continued work with matrix interactions involves increasing the matrix to compound ratio to determine at what concentration of compound the matrix begins to interact. Comparison between mixing the solutes with the matrix as a solid vs a solution also should be pursued.

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# APPENDIX A

**Effect of Injection Solvents on the Analysis of Triacylglycerols:**

The appearance of double or split peaks in a supercritical fluid chromatographic separation is generally attributed to a poorly installed butt connector or a misaligned injector rotor. (153) Dean and Poole (154) have shown with packed column SFC that if the injection solvent has a greater solvating strength for the analyte than the mobile phase, then peak splitting can occur. At the recent 1989 SFC symposium/workshop (Snowbird, UT) low solubility of the analyte in the organic solvent was discussed as an additional reason for split peaks (155). In gas chromatography (GC) the appearance of multiple peaks from a single compound has been attributed to a leak at the graphite ferrule around the inlet insert, low injector temperature, detector overload, or sample flashing prior to injection (156-157). During method development for the analysis of triacylglycerols by supercritical fluid chromatography (SFC) double peaks were observed with a lipid standard mixture containing tristearate, triolein, trilinolen and trilinolenin at approximately 0.25 μg/μL. Extensive troubleshooting showed that under certain circumstances mixed injection solvents can cause double peaks.

A 50% cyanopropyl polysiloxane capillary column was used for the analysis of free fatty acids in soybean oil for approximately three months. Lipid standard Solution I was analyzed before and after the FFA study. Double peaks were observed during the second analysis (Figure 32a). Reducing the oven temperature to 100°C resulted in the elimination of the double peaks (Figure 32b). A standard mixture prepared in a single solvent (Solution II) did not result in double peaks when chromatographed (Figure 32c). An 1 mL aliquot of standard Solution II was diluted with 1 mL of isooctane and subsequently analyzed. The chromatographic separation resulted in double peaks.

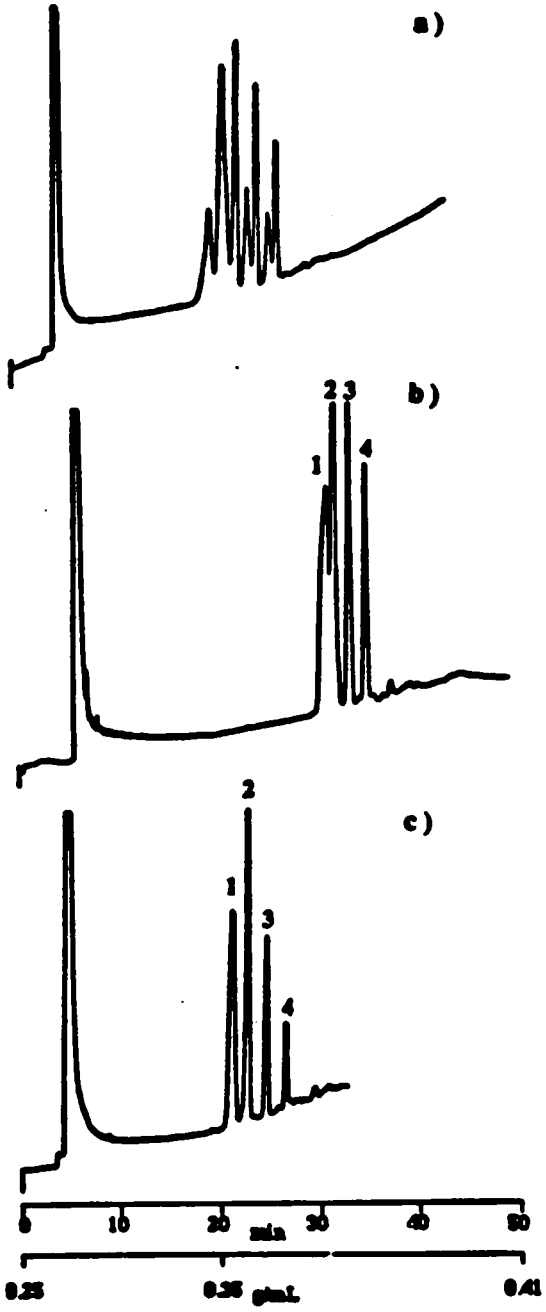


**Figure 32.** SFC chromatogram of triacylglycerol standard mixtures after FFA study. a) separation of Solution I at 140°C; b) separation of solution I at 100°C; c) separation of Solution II at 140°C. See Figure 16 for chromatographic conditions. 1) tristearin; 2) triolein; 3) trilinolein; 4) trilinolenin.

In an effort to reestablish the original column condition, the column was reconditioned in a GC oven at 240°C for 16 hr. After this reconditioning step, the column was checked using a polar test mixture (Supelco, Bellefonte, PA). No indication of excessive peak tailing was evident. When Solution I was reanalyzed under SFC conditions double peaks were still present. A split injection (1:2) of Solution I eliminated the double peaks. This could be attributed to the loss of the more volatile solvent (isooctane) through the splitter.

Using a new 50% cyanopropyl polysiloxane capillary column the two standard solutions were analyzed. Although split peaks were not observed, front shoulders were evident when standard Solution I (mixed solvent) was analyzed. Broader peaks were obtained with the mixed solvent as evidenced by the integrator ar/ht ratio. When the standard solution containing toluene alone was analyzed, the ar/ht was 0.365 for trilinolen while the ar/ht ratio for trilinolen in the mixed solvent was 0.455. No apparent peak distortion was present when a solution of methyl stearate in 50% isooctane/50% toluene was chromatographed. A 10 min hold at an initial density of 0.15 g/mL at 140°C had to be added to insure that methyl stearate was resolved from the solvent front. Under these conditions methyl stearate eluted at 16.3 min and had an ar/ht ratio of 0.24.

# APPENDIX B

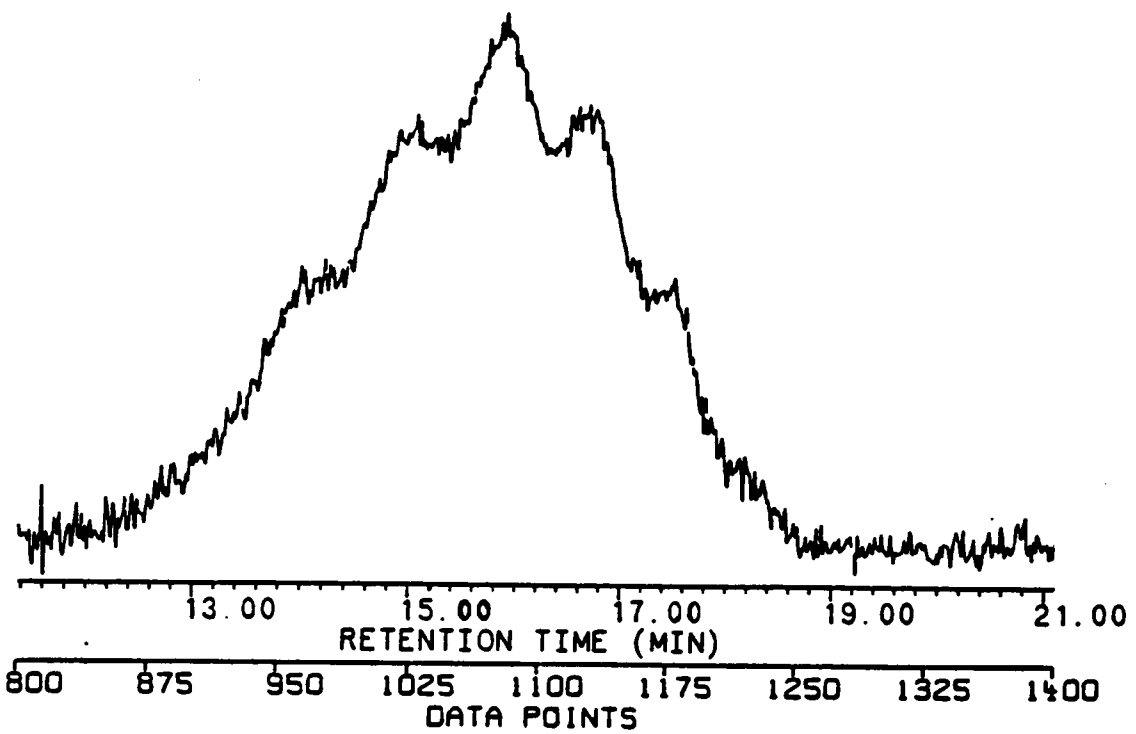


**Figure 33.** Partial GSR of triacylglycerols in Ni hydrogenated soybean oil. See Table VI.

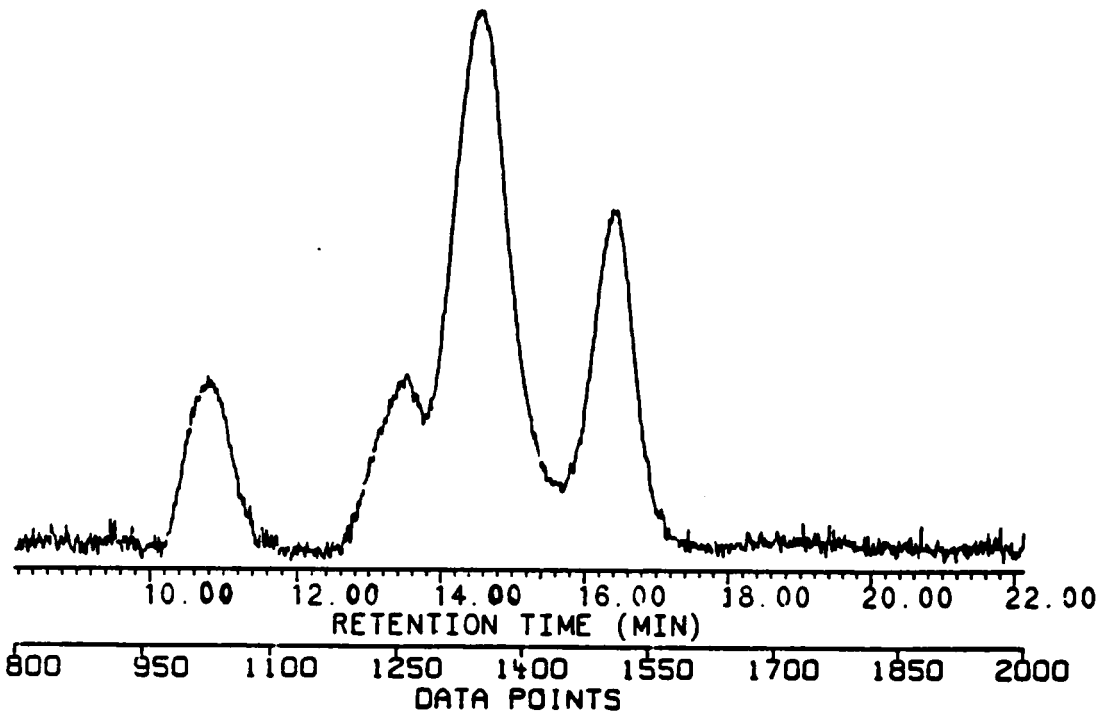


**Figure 34.** Partial GSR of free fatty acids in Ni hydrogenated soybean oil. See Table VI.

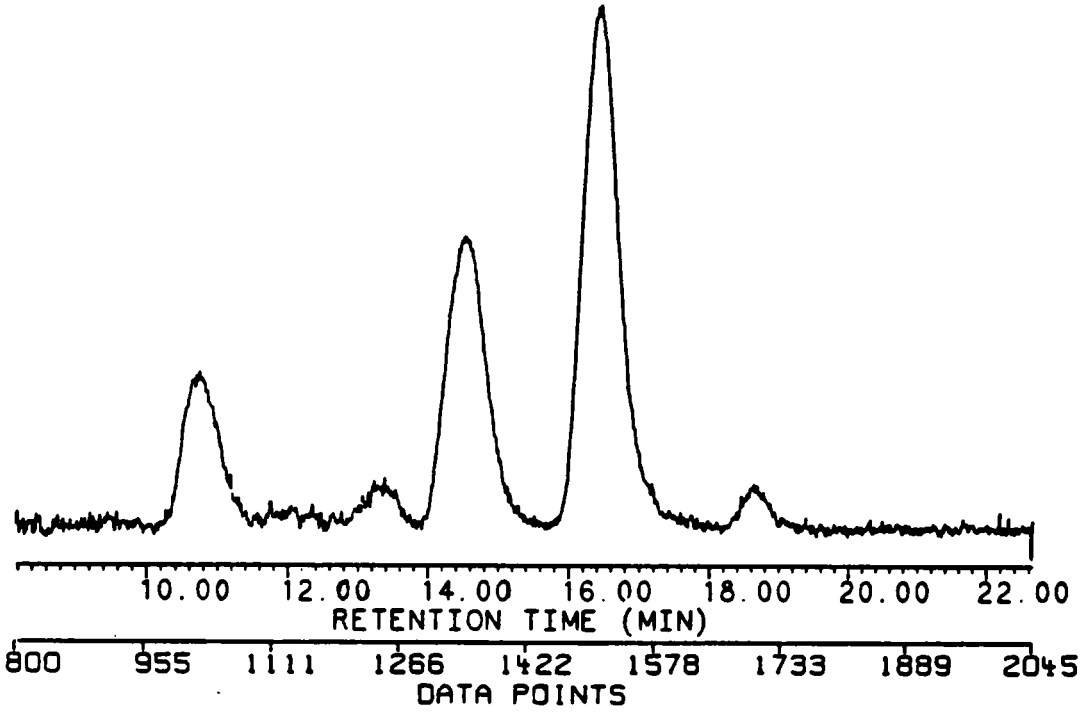


**Figure 35.** Partial GSR of free fatty acids in refined soybean oil. See Table VII.

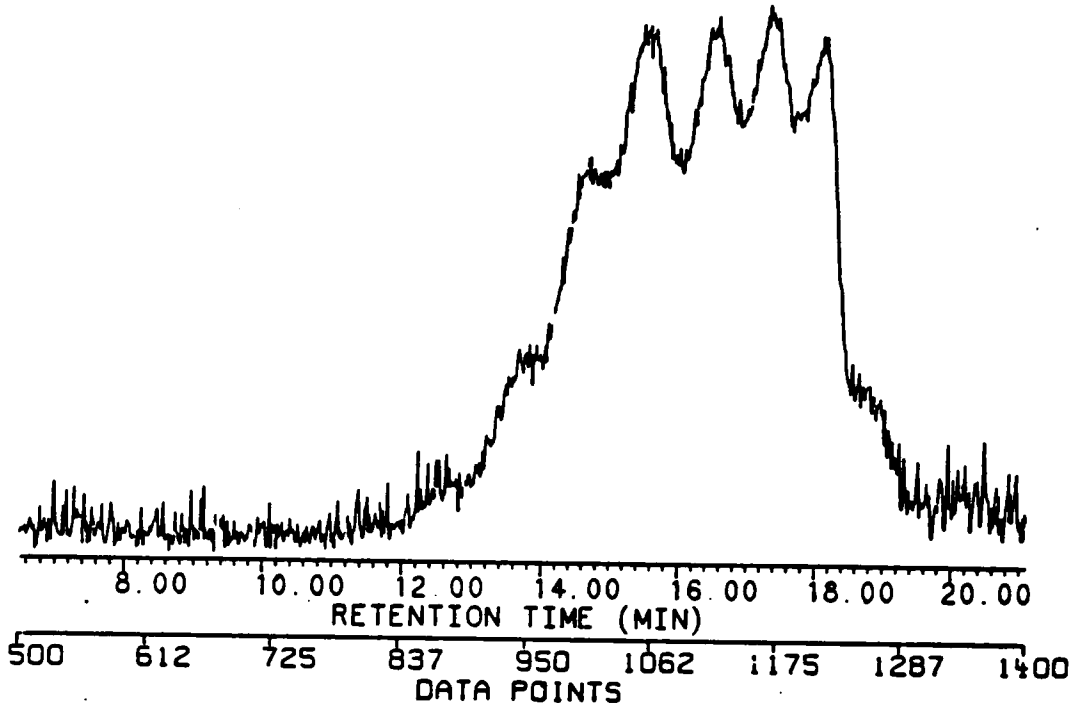


**Figure 36.** Partial GSR of triacylglycerols in refined soybean oil. See Table VIII.

# APPENDIX C

BASIC PROGRAM:

```
180   GOSUB 2290!$6;CLEV(3 AND PEEK(ADRS)),
182   GOSUB 2290: GOSUB 2280!$8.3;(2PEEK(#8A ADRS))/A(3 AND
PEEK(1ADRS))
184   RETURN
200   INPUT "TIME INTERVAL"XX
201   XX=100*XX
202   TFN"SR",1: END
210   AA=0
220   IF (2PEEK(138ADRS#1C)-AA)<XX THEN 200 ELSE AA=2PEEK(138ADRS#1C)
230   GOSUB 180
240   IF (100*TT(2)-AA)>XX THEN 220 ELSE END
```

# APPENDIX D

Calculations for the density change due to residence time in the restrictor.

1. Determine linear velocity at various densities for the restrictor used in the extraction study.

$$\bar{u}_1/\bar{u}_2 = \bar{u}_3/\bar{u}_4$$

$$\bar{u}_3 = (\bar{u}_1 \times \bar{u}_4)/\bar{u}_2$$

$\bar{u}_1$ = measured average linear velocity at 0.82 g/mL during extraction experiments.

$\bar{u}_2$ = measured average linear velocity at 0.82 g/ml using chromatographic column (see Tables XIX and XX).

$\bar{u}_3$ = calculated average linear velocity during extraction experiment at specified density.

$\bar{u}_4$ = measured average linear velocity using chromatographic column at specified density.

2. Determine residence time in restrictor.

$$L/[(60\text{sec/min}) \times (\bar{u}_3)] = RT$$

L = length of restrictor.
RT = residence time.

3. Determine density change during residence time.

RT x (density gradient rate) = density change

Table XIX: Linear Velocity Measurements at Low and High Densities

| Temperature (°C) | Density (g/mL) | $t_o$ (min ) | linear velocity[a] (cm/sec) |
|---|---|---|---|
| 40 | 0.23 | 16.42 | 1.1 |
| | 0.96 | 2.93 | 6.0 |
| 45 | 0.21 | 13.84 | 1.3 |
| | 0.95 | 2.73 | 6.5 |
| 50 | 0.19 | 12.64 | 1.4 |
| | 0.93 | 2.76 | 6.4 |
| 60 | 0.17 | 11.37 | 1.6 |
| | 0.90 | 2.65 | 6.7 |
| 80 | 0.14 | 9.26 | 1.9 |
| | 0.82 | 2.55 | 6.9 |
| 100 | 0.13 | 8.06 | 2.2 |
| | 0.76 | 2.32 | 7.6 |

a. Obtained using a 50% phenylmethylpolysiloxane column (100 μm i.d.x 10 m) with a frit restrictor (100 μm i.d. x 61 cm).

Table XX: Linear Velocity Measurements at 80°C and Different Detector Temperatures

| Detector Temperature (°C) | Density (g/mL) | $t_o$ (min) | linear velocity[a] (cm/sec) |
|---|---|---|---|
| 375 | 0.14 | 9.26 | 1.9 |
| | 0.30 | 7.76 | 2.3 |
| | 0.50 | 6.79 | 2.6 |
| | 0.70 | 4.34 | 4.1 |
| | 0.82 | 2.55 | 6.9 |
| 275 | 0.14 | 7.42 | 2.4 |
| | 0.30 | 5.93 | 3.0 |
| | 0.50 | 5.26 | 3.4 |
| | 0.70 | 3.40 | 5.2 |
| | 0.82 | 2.06 | 8.6 |
| 175 | 0.14 | 5.54 | 3.2 |
| | 0.30 | 4.37 | 4.0 |
| | 0.50 | 3.88 | 4.6 |
| | 0.70 | 2.51 | 7.0 |
| | 0.82 | 1.60 | 11.1 |

a. Obtained using a 50% phenylmethylpolysiloxane column (100 µm i.d. x 10 m) with a frit restrictor (100 µm i.d. x 61 cm).

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## ABSTRACT

Supercritical fluid (SF) technologies are being investigated extensively by the food industry for a variety of applications. Carbon dioxide in the supercritical state is of particular interest to the food industry due to its extremely low toxicity in comparison with organic solvents. Three applications of chromatography or extraction employing SFs in the analysis of food components and natural products were investigated. These applications related to carbohydrate, lipid and coumarin derivative analyses.

The peracetylated nitrogen derivatives of carbohydrates were analyzed by supercritical fluid chromatography (SFC) with flame ionization detection (FID), Fourier-transform infrared (FT-IR) detection and mass spectrometry (MS) detection. Although reports in the literature indicated that only one derivatized component was detected under GC conditions, the SFC analysis of peracetylated aldonitrile derivatives of monosaccharides resulted in detection of multiple reaction products. The identification of the peracetylated nitrile and acyclic oxime was accomplished using both SFC/FT-IR and SFC/MS. The spectroscopic data indicated that an additional reaction product was a peracetylated cyclic oxime. The data was not conclusive enough to determine if the structure contained a pyranose or furanose ring.

Changes in chemical composition of vegetable oils due to processing were observed employing SFC/FT-IR. Refined soybean oil and soybean oil that was hydrogenated with a Ni or a Ni–S catalyst were analyzed. SFC/FT-IR provided an opportunity to determine the extent of unsaturation and isomerization in a single analysis. The C–H deformation of <u>trans</u> $R_1HC{=}CHR_2$ groups was observed at 972 $cm^{-1}$. The double bond in the <u>cis</u> configuration was evident by absorptions above 3000 $cm^{-1}$. On-line FT-IR spectra of triacylglycerols and free fatty acids readily revealed conversion to <u>trans</u> isomers in those samples that were hydrogenated with the Ni–S

Quantitation was not possible due to the incomplete resolution of the cis and trans isomers present in the hydrogenated samples.

Apparent threshold densities were determined for a series of coumarin derivatives using a supercritical fluid chromatograph with a flame ionization detector. The extraction cell was a LC stainless steel precolumn. Milligram quantities of the model compounds were extracted. Functionality, extraction temperature and matrix affected the measured threshold densities of the compounds. The addition of two hydroxyl groups onto the coumarin structure caused the compound to be unextractable at 60°C and densities up to 0.90 g/mL. For those compounds that were completely extracted, coumarin, 7–methoxycoumarin, 3-phenylcoumarin and psoralen, the apparent threshold density increased with increasing melting point. The melting or decomposition points of those compounds that were partially extracted ranged between 200 and 240°C. For those compounds that were not extracted, under the constraints of the experiment, the melting or decomposition points were greater than 240°C. A higher extraction temperature resulted in a lower threshold density. Corn starch was found to be a non-interactive matrix.