

Analyzing Fatty Acids by Packed Column Gas Chromatography

Described here are packed column gas chromatographic analyses of C1-C7 volatile fatty acids, lactic acid, and longer chain nonvolatile acids, including *cis/trans* isomers, in various sample matrices. Packings, packed columns, and standards for these analyses are listed after the analyses.

Key Words:

- fatty acids ● volatile fatty acids
- *trans* fatty acids ● lactic acid

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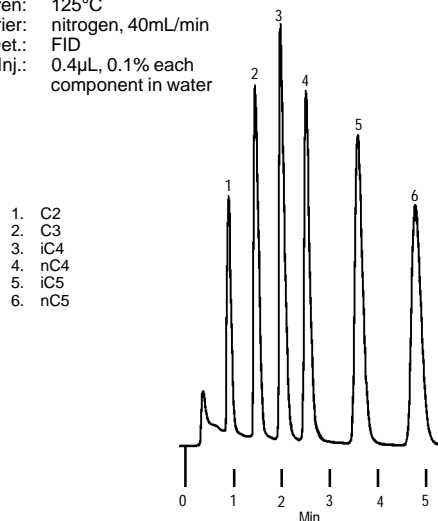
C2-C5 Volatile Fatty Acids

C2-C5 volatile fatty acids (VFAs) in dilute aqueous solution can be separated and quantified as free acids by using a column containing 10% SPTM-1200/1% H₃PO₄ on Chromosorb[®] WAW, a packing developed specifically for this purpose (1,2). The SP-1200 phase separates the free (unmethylated) acids rapidly, with minimal peak tailing, and with good separation of propionic and isobutyric acids (Figure A). Figure B shows a separation of a rumen fluid sample, and Figure C shows the C2-C5 acids in blood plasma. Acid concentrations in the rumen fluid and blood plasma samples were calculated by comparing peak heights to those in the standards. Baumgardt's informative paper on rumen fluid analysis deals with the practical aspects of quantitative GC analyses of VFAs in aqueous solution (3).

Note that 0.6μL samples were used in Figures B and C. Sample volume should be kept small to ensure optimum column performance. If sample volume is excessive, acetic and propionic acids will overload the column and elute with poor peak shape. For rumen fluid, which contains high concentrations of VFAs, we recommend samples in the range of 0.2-0.3μL. Because blood has very low concentrations of VFAs, sample volumes can be larger.

Figure A. C2-C5 Volatile Fatty Acids as Free Acids

Packing: 10% SP-1200/1% H₃PO₄ on 80/100 Chromosorb W AW
 Cat. No.: 11965 (20g)
 Column: 6' x 2mm ID glass
 Oven: 125°C
 Carrier: nitrogen, 40mL/min
 Det.: FID
 Inj.: 0.4μL, 0.1% each component in water

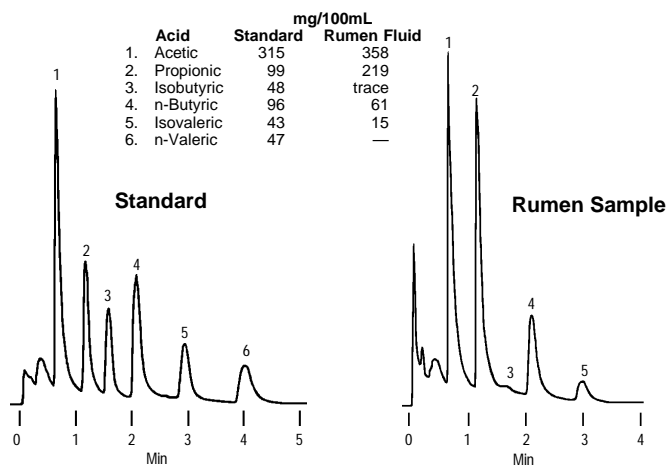


1. C2
2. C3
3. iC4
4. nC4
5. iC5
6. nC5

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Figure B. Volatile Fatty Acids in Rumen Samples

Packing: 10% SP-1200/1% H₃PO₄ on 80/100 Chromosorb W AW
 Cat. No.: 11965 (20g)
 Column: 6' x 2mm ID glass
 Oven: 130°C
 Carrier: nitrogen, 40mL/min
 Det.: FID, 175°C
 Inj.: 0.6μL (analyte concentrations on figure), 170°C



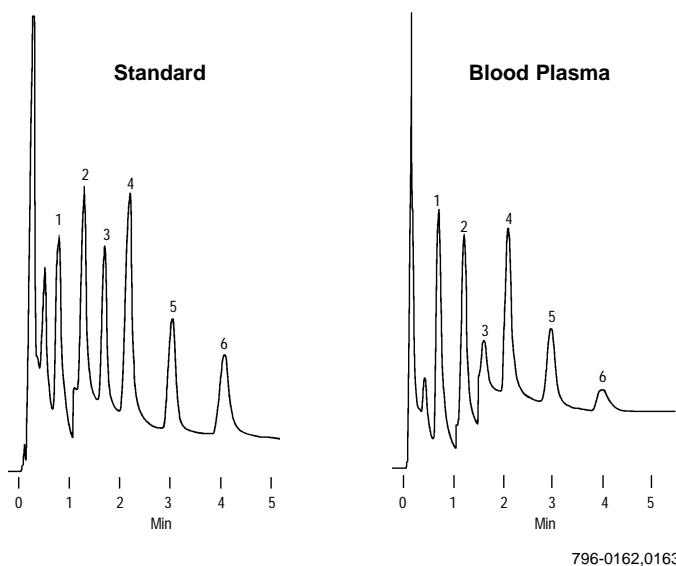
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Chromatograms provided by L.E. Chase, Dept. Animal Science, The Pennsylvania State University, University Park, PA USA.

Figure C. Volatile Fatty Acids in Blood Plasma

Packing: 10% SP-1200/1% H₃PO₄ on 80/100 Chromosorb W AW
Cat. No.: 11965 (20g)
Column: 6' x 2mm ID glass
Oven: 125°C
Carrier: nitrogen, 40mL/min
Det.: FID, 175°C
Inj.: 0.6µL (analyte concentrations on figure), 170°C

	Acid	Standard	Blood Plasma
1.	Acetic	12.6	14.5
2.	Propionic	4.0	5.3
3.	Isobutyric	1.9	0.2
4.	n-Butyric	3.8	1.3
5.	Isovaleric	1.7	0.5
6.	n-Valeric	1.9	0.2



Chromatograms provided by L.E. Chase, Dept. Animal Science, The Pennsylvania State University, University Park, PA USA.

The 10% SP-1200/1% H₃PO₄ packing can be used in either glass or stainless steel tubing. The phosphoric acid incorporated into the packing deactivates metal tubing, as well as the support material, thus ensuring that acid peaks do not tail. When preparing the column, plug the ends with H₃PO₄-treated glass wool, rather than with silanized or untreated glass wool. The latter tends to cause free acid peaks to tail. Do not inject samples into a metal inlet. This also will cause the peaks to tail.

Sample Preparation

Various procedures have been described for determining volatile fatty acids in rumen fluid and blood plasma. In many of these procedures, steam distillation is needed to obtain the maximum VFA values, and to eliminate or minimize interference with the GC analysis (4,5). The major shortcomings of such procedures are the lengthy steam distillation step, and the need for 5mL or more of plasma.

In a micro method, VFAs are extracted from 200µL of blood plasma using 1mL of ethanol, with 20µL of 14mM isobutyrate added as an internal standard (6). After agitation and centrifugation, the supernatant is transferred to a 4mL flask, made alkaline with 20µL of 0.2M NaOH, and evaporated in an air current at 20°C. The residue is redissolved in 15µL of water a few minutes before the analysis. Immediately before the material is injected onto the column, 5µL of 25% (v/v) orthophosphoric acid is added. Recovery studies show that the VFAs are quantitatively extracted by ethanol.

The following procedures for sample work-up were adapted by L.E. Chase, Department of Animal Science, The Pennsylvania State University, University Park, Pennsylvania, from procedures used by Baumgardt (3), Erwin, *et al.* (7), and Simkins (8). When samples prepared according to these procedures are used, nonvolatiles in the samples accumulate at the column inlet. In time, these will cause column performance to deteriorate. To avoid this, use a replaceable glass-lined inlet to trap the nonvolatiles, and periodically replace this liner (daily, with heavy usage). Alternatively, you can clean the sample using the steam distillation method described by Ross and Kitte (9). Do not inject samples containing nonvolatiles directly into the column, because it will be very difficult to remove the accumulated material from the column.

Preparation of Samples for VFA Analysis by GC

Rumen Fluid

1. Shake sample of strained rumen fluid. Pipette 5mL into a centrifuge tube.
2. Add 1mL 25% metaphosphoric acid. Mix thoroughly and allow to stand for 30 minutes.
3. Centrifuge at 2000 rpm (International Centrifuge, size 2, model head 250A) for 10 minutes. Supernatant can be analyzed without further preparation.

Blood Plasma

1. Pipette 8mL 0.2N sulfuric acid into a 50mL Erlenmeyer flask.
2. Add 2mL blood plasma, using an Ostwald-Foin pipette. Mix contents by swirling and allow to stand for 10 minutes.
3. While gently swirling flask, add 2mL 10% sodium tungstate (w/v). Continue swirling flask for 30 seconds to ensure complete mixing, then allow mixture to stand for 10 minutes.
4. Centrifuge mixture at 5000rpm for 10 minutes. Transfer 7.5mL of supernatant to a 6" x 3/4" test tube.
5. Make liquid alkaline by adding 0.20mL 3N NaOH.
6. Freeze liquid in a dry ice-ethanol mixture.
7. Place test tube in a lyophilizer and freeze dry contents. When all water has been removed from sample, a fine white powder will remain in tube.
8. Add 0.2-0.5mL metaphosphoric acid reagent (prepared by diluting 250g 36% HPO₃ to one liter with distilled water). Inject resulting solution onto column for analysis.

Silage

Prepare water extract according to (10), using only sufficient water to cover the silage, to keep the VFAs as concentrated as possible. Acidify and centrifuge the extract as in the procedure for rumen fluid. The supernatant is ready for GC analysis.

1. Weigh out 50-100g material and transfer to 4oz. (250mL) screw-cap bottle or jar.
2. Tamp sample and barely cover with distilled water (unless otherwise specified) containing a small amount of thymol as an antiseptic. Secure cap and record size of sample and amount of water used. Store at 4°C for 6-7 days.
3. Squeeze material through two layers of cheesecloth or through glass wool in a funnel. Centrifuge liquid at about 2000rpm for 5 minutes. Place in tubes or bottles, cap, and store at 4°C.

- Pipette 5mL sample and 1mL 25% metaphosphoric acid into a 10mL centrifuge tube. Mix and allow to stand for at least 30 minutes.
- Centrifuge mixture at 4000rpm for 20-30 minutes. Monitor VFAs in supernatant.

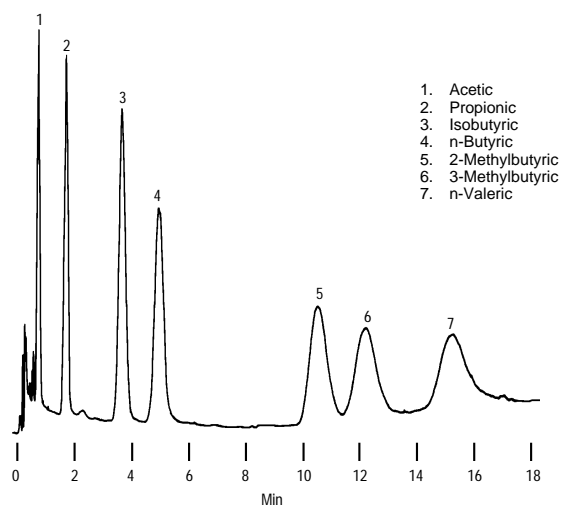
Note: Silage samples prepared in this manner will be heavily laden with nonvolatiles. To prevent these materials from accumulating in the column inlet and eventually appearing as extraneous peaks, change the glass liner frequently and/or make 2-3 injections of distilled water after every 2-3 injections of sample.

C2-C5 Volatile Fatty Acids at ppm Concentrations in Water

An inert GC column is needed for analyses of trace concentrations of free C2-C5 VFAs in water. The acids can be strongly adsorbed to the support, the injector system, or the column tubing. At trace levels, this can significantly reduce the accuracy of quantitative data. Several suitable packings have been developed by DiCorcia and Samperi (11). The packing we currently recommend is 60/80 Carbopack™ C/0.3% Carbowax® 20M/0.1% H₃PO₄. The support is deactivated by the H₃PO₄ in the packing. Consequently, the acids do not interact with the inert column, and even low ppm amounts of VFAs can be monitored reliably. In Figure D a Carbopack C/Carbowax 20M/H₃PO₄ column has resolved 50ppm of each acid, including 2-methylbutyric and 3-methylbutyric acids. Peaks are symmetrical, and thus are easier to quantify accurately. To prevent analyte adsorption to the inlet and the tubing surface, use a glass column and inject samples into a glass-lined inlet or directly onto the column.

Figure D. C2-C5 Free Acids at 50ppm in Water

Packing: 60/80 Carbopack C/0.3% Carbowax 20M/0.1% H₃PO₄
 Cat. No.: 11825-U (15g)
 Column: 3' x 1/4" OD x 4mm ID glass
 Oven: 120°C
 Carrier: nitrogen, 50mL/min
 Det.: FID, 200°C
 Inj.: 1µL, 50ppm each acid, 200°C



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Another problem that can arise in these analyses is hydrolytic decomposition of the stationary phase when water passes through a new column. Phase decomposition products are generally eluted with or shortly after the water front, producing a peak that can interfere with the acid peaks. If a new Carbopack C/0.3% Carbowax 20M/0.1% H₃PO₄ column is treated at an elevated

temperature with a series of water injections, most of the decomposition products will be virtually steam cleaned from the column. With subsequent analytical samples, the peak from decomposition products will be smaller and will not interfere with the analysis. Condition the new column according to the procedure that accompanies the packing, using carbon dioxide-free deionized water containing 1% formic acid. This treatment will not interfere with the analysis because it is not seen by a flame ionization detector. Repeat the 1% formic acid treatment when peaks begin to tail, or if the column is unused for an extended time. Also use formic acid or, if necessary, H₃PO₄ to acidify samples. Stronger acids (e.g., hydrochloric, sulfuric, nitric) will damage the column.

We recommend using a 3' x 4mm ID glass column packed with Carbopack C/0.3% Carbowax 20M/0.1% H₃PO₄ for monitoring the C2-C5 free acids at trace levels in water. Leave 4cm of the column inlet unpacked to allow for sample expansion and use phosphoric acid-treated glass wool plugs at the column inlet and outlet. Untreated or silanized glass wool can cause the acid peaks to tail. Minimize the water peak by injecting small samples (1µL) at high sensitivity, rather than larger samples at low sensitivity.

C1-C7 Volatile and Nonvolatile Fatty Acids in Anaerobic Fermentation Products

Two column packings – 15% SP-1220/1% H₃PO₄ on Chromosorb W AW and 10% SP-1000/1% H₃PO₄ on Chromosorb W AW – provide excellent resolution of VFAs and nonvolatile acid methyl esters in fermentation products. For greater accuracy with difficult samples, use both packings to confirm results. The SP-1220 packing offers more rapid separations, while the SP-1000 packing provides better separations of the nonvolatile acid methyl esters and isolates the acids from short chain alcohols which may be present. (To isolate lactic acid from fermentation products, see the next section of this bulletin.)

Gas chromatography is used to help identify anaerobic bacteria through determination of the C1-C7 volatile fatty acids, nonvolatile acids (which must be analyzed as methyl esters), and alcohols in the fermentation broth. Procedures for anaerobe identification have been developed by L.V. Holdeman, W.E.C. Moore, and the staff of the Anaerobe Laboratory of the Virginia Polytechnic Institute and State University, by the Anaerobic Bacterial Diseases Branch of the U.S. Department of Health and Human Services, and by others (see *Additional Information* section of this bulletin).

Both the SP-1220 and the SP-1000 packing resolve the VFAs well and provide symmetrical peaks for quantitative analyses (Figures E and F). Each packing readily separates propionic and isobutyric acids, compounds usually poorly resolved on other packings. These packings even provide a tail-free peak for formic acid, which generally is lost or tails badly on other materials. When each column is used under optimum operating conditions, analyses on the SP-1220 packing are slightly faster than those on the SP-1000 packing, despite the higher temperature and carrier gas flow rate used with the latter (Figures E and F). Both packings resolve the nonvolatile acid methyl esters, but the SP-1000 packing's performance is superior (Figure G).

Investigators at Milwaukee's Mount Sinai Medical Center evaluated the performance of the SP-1220 packing (12). In comparison, a Resoflex® column (also used to analyze fermentation products) separated propionic and isobutyric acids poorly and

Figure E. C1-C7 Volatile Fatty Acids on 15% SP-1220 Packing

Packing: 15% SP-1220/1% H₃PO₄ on 100/120 Chromosorb W AW
 Cat. No.: 12144 (20g)
 Column: 6' x 4mm ID glass
 Oven: 145°C
 Carrier: nitrogen, 70mL/min
 Det.: TCD (required to detect formic acid)
 Inj.: 14µL ether extract

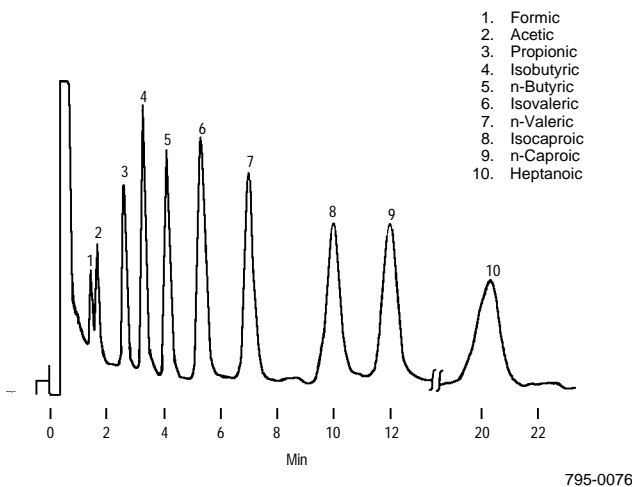


Figure F. C1-C7 Volatile Fatty Acids on 10% SP-1000 Packing

Packing: 10% SP-1000/1% H₃PO₄ on 100/120 Chromosorb W AW
 Cat. No.: 11841 (20g)
 Column: 6' x 4mm ID glass
 Oven: 155°C
 Carrier: helium, 86mL/min
 Det.: TCD (required to detect formic acid)
 Inj.: 14µL ether extract

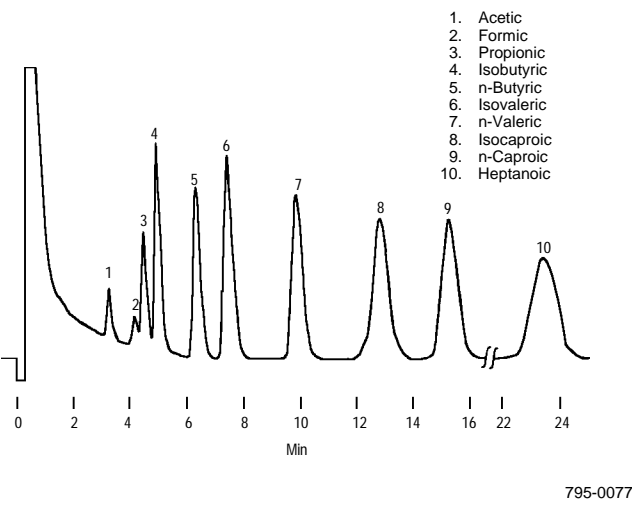
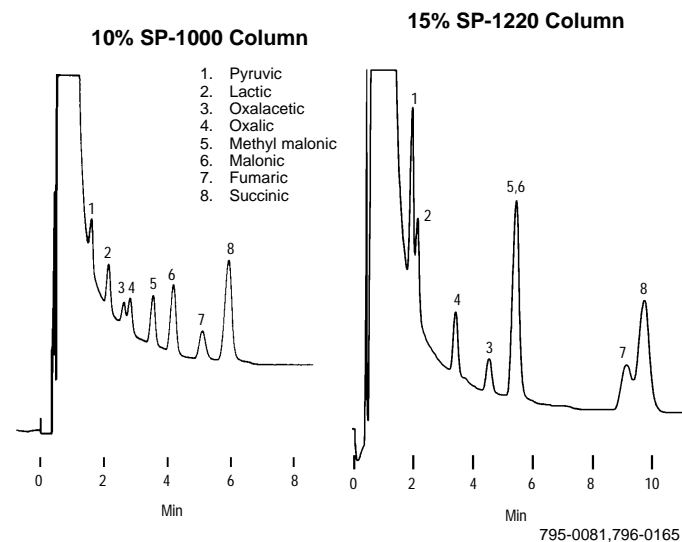


Figure G. Methyl Esters of Nonvolatile Acids from Fermentation Media

Packing: 10% SP-1000/1% H₃PO₄ on 100/120 Chromosorb W AW
 Cat. No.: 11841 (20g)
 Packing: 15% SP-1220/1% H₃PO₄ on 100/120 Chromosorb W AW
 Cat. No.: 12144 (20g)
 Column: 6' x 4mm ID glass
 Oven: 155°C (SP-1000) or 135°C (SP-1220)
 Carrier: helium, 86mL/min (SP-1000) or 70mL/min (SP-1220)
 Det.: TCD
 Inj.: 14µL ether extract



did not yield a formic acid peak. The SP-1220 packing also provided faster analyses. Subsequently, these analysts used an SP-1220 column to separate VFAs in cultures of many bacteria species. Three example are shown in Figure H. Bacteria also can be identified on the basis of cellular fatty acid methyl ester profiles.

W.E.C. Moore of the Anaerobe Laboratory of the Virginia Polytechnic Institute and State University duplicated the results obtained by Hauser and Zabransky (13). Moore agreed that the SP-1220 packing provided better separations, greater sensitivity, and faster elution for the VFAs.

Although the SP-1220 packing provides excellent separations of C1-C7 VFAs, separation of the alcohols in unsatisfactory. This can be a problem in analyses involving genera such as *Clostridium*, *Eubacterium*, and *Fusobacterium*. In contrast, the alcohols elute from the 10% SP-1000 packing well before the acids, so with this packing there is no chance of confusing alcohols and acids. Moore found that the SP-1000 packing provided a nonvolatiles separation comparable to that from a Resoflex column (13).

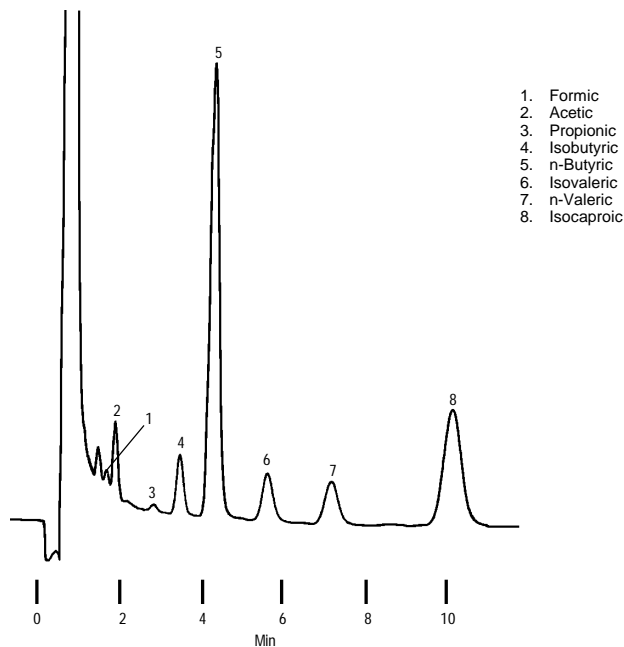
According to the CDC Lab Manual (14), the 10% SP-1000 and 15% SP-1220 columns are standard columns for use at the Centers for Disease Control of the U.S. Department of Health and Human Services, SP-1220 columns for free acids analyses and SP-1000 columns for methyl esters analyses.

Although 1/4" OD metal columns are often used to analyze fermentation products, we recommend glass columns for best results. Peaks separate better and the analysis is slightly faster (Figure I). The smaller inside diameter of a glass column (4mm vs. 5.33mm for a 1/4" OD metal column) reduces the amount of packing (7g vs. 11.8g) and carrier gas needed, thus reducing the cost per analysis. The glass tubing also enables you to observe the

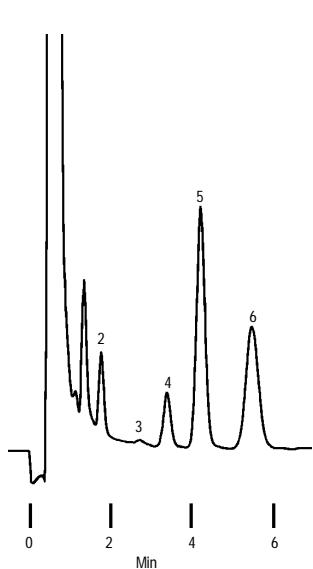
Figure H. Differing Volatile Fatty Acid Profiles from Bacterial Culture Media

Packing: 15% SP-1220/1% H₃PO₄ on 100/120 Chromosorb W AW
 Cat. No.: 12144 (20g)
 Column: 6' x 4mm ID glass
 Oven: 145°C
 Carrier: helium, 60mL/min
 Det.: TCD, 160°C
 Inj.: 15µL ether extracts, 145°C

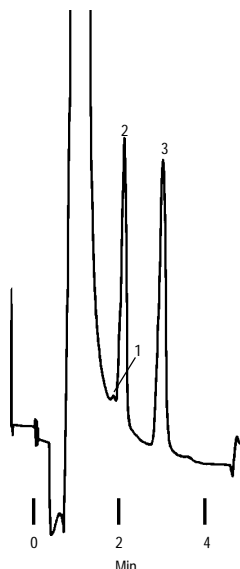
Clostridium difficile



Clostridium subterminale



Veillonella sp.



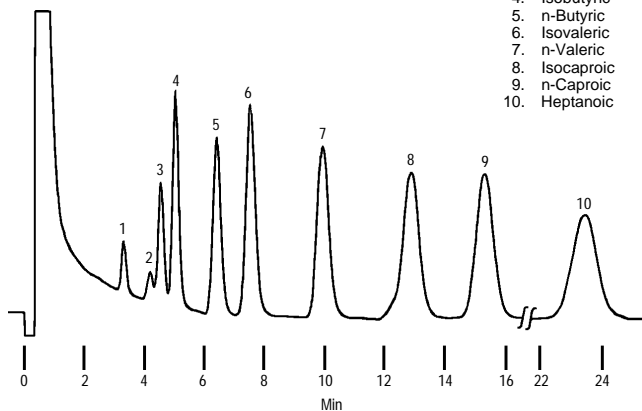
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Chromatograms provided by K.J. Hauser, Dept. Pathology, Mount Sinai Medical Center, Milwaukee, WI USA.

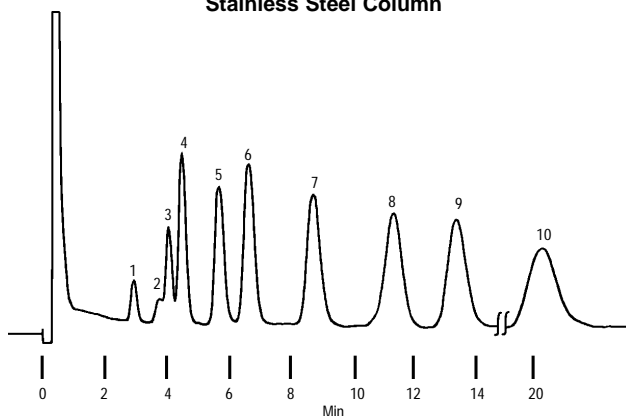
Figure I. Volatile Acids Separate Better on Glass Columns

Packing: 10% SP-1000/1% H₃PO₄ on 100/120 Chromosorb W AW
 Cat. No.: 11841 (20g)
 Column: 6' x 4mm ID glass or 6' x 1/4" OD stainless steel
 Oven: 155°C
 Carrier: helium, 86mL/min (glass) or 130mL/min (stainless steel)
 Det.: TCD
 Inj.: 14µL ether extract

Glass Column



Stainless Steel Column



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packing for discoloration that may indicate deterioration. Periodically replace the glass wool plug in the column inlet to prevent tar buildup that causes peaks to tail. Use phosphoric acid-treated glass wool. Silanized or other types of wool will cause peak tailing.

To detect VFAs and alcohol products in aqueous media, cultures are acidified and extracted with ethyl ether, and the extracts are injected onto the chromatographic column (14). Methyl derivatives of nonvolatile Krebs cycle acids and lactic acid are extracted with chloroform (14). In either process, it is important to acidify the culture to pH 2 or below, using approximately 0.1 mL of 50% aqueous H₂SO₄ per 6mL of medium (v/v). The salt forms of the fermentation acids (R-COONa) are soluble in water, but not in ether. At pH 2 the acids are in the free acid form (R-COOH), and are soluble in both water and ether.

For species identification, methylated products of cocci and Gram-positive bacilli must be analyzed if only acetic or acetic and formic acids are detected in the ether (free acid) extract. Methylated products of Gram-negative bacilli must be analyzed if butyric acid is not a major peak in the ether extract.

Extraction of Volatile Acids and Alcohols (14)

1. Pipette approximately 2mL acidified culture into a conical centrifuge tube. Retain some material for methylation.
2. Add 1mL ethyl ether, seal tube, and mix contents.
3. Centrifuge briefly to break ether-culture emulsion.
4. Use a pipette to transfer ether layer to a 12 x 75mm test tube

OR

Place tube in freezer until aqueous phase is frozen. Pour ether layer into a 12 x 75mm test tube.

5. Add anhydrous $MgSO_4$ or Na_2SO_4 equal to about 1/2 volume of ether in tube. Let stand for 10 minutes to remove all traces of water from ether.
6. Inject appropriate quantity of extract onto column (e.g., 14 μ L in Centers for Disease Control or Virginia Polytechnic Institute methods).

Derivatization and Extraction of Nonvolatile Acids (14)

1. Pipette 1mL acidified culture into a 12 x 75mm test tube.
2. Add 2mL methanol and 0.4mL 50% aqueous H_2SO_4 . Seal tube, mix, and heat at 55°C for 30 minutes or hold at room temperature overnight.
3. Add 1mL water and 0.5mL chloroform, reseal, and mix. If an emulsion forms, centrifuge briefly.
4. Draw a sample from chloroform (bottom) layer into syringe.
5. Wipe needle and inject appropriate quantity of extract onto column (e.g., 14 μ L in Centers for Disease Control or Virginia Polytechnic Institute methods). After injecting approximately 20 methylated samples, recondition column by injecting 14 μ L methanol.

Note: When samples contain very large amounts of VFAs, trace amounts of free acids might remain after methylation. These can be identified from their retention times.

Analyzing Lactic Acid in the Presence of Other Short Chain Free Acids

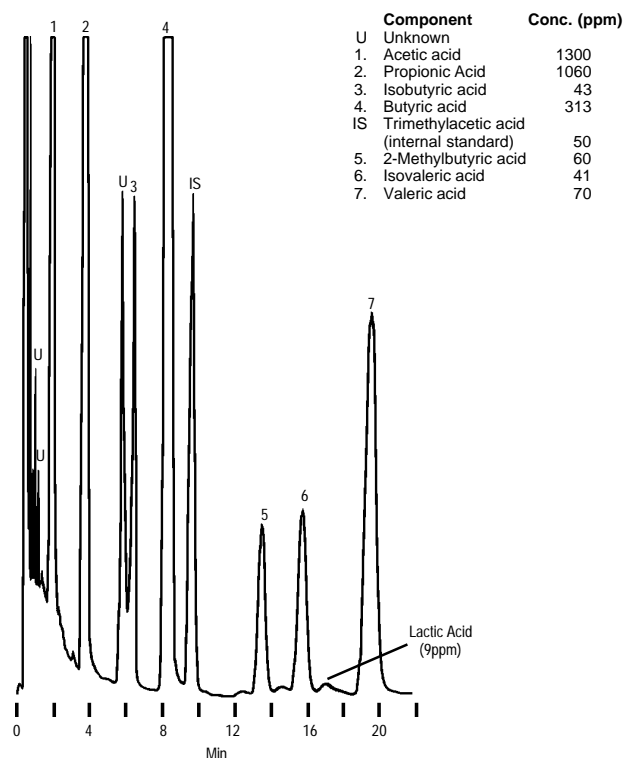
Lactic acid is often present in samples containing volatile or other nonvolatile short chain acids, but it is not eluted from most GC columns. In the few situations in which lactic acid is eluted, the peak tails severely. Developed from work by A. DiCorcia of the University of Rome, Carbowax 20M columns have a unique ability to isolate lactic acid as a symmetrical peak.

Lactic acid is an important component of silage and other fermentation products. It is also produced in the rumen of cattle and other ruminants. In normal rumen fluid, lactic acid is present in low concentrations, 25-150ppm. When cattle eat excessive amounts of feed rich in starch or sugars, abnormally large amounts of lactic acid are produced in the rumen, then transferred to the bloodstream, lowering the pH. This condition, lactic acidosis, causes cattle to go off feed and, in severe cases, can cause death.

Lactic acidosis is diagnosed by analyzing blood serum or rumen fluid. In Figure J, we resolved lactic acid and the C2-C5 VFAs in rumen fluid, using 80/120 Carbowax 20M packing. The analysis was accomplished in 25 minutes on a 2m x 2mm TIGHTSPEC™ glass column. Because the peak is symmetri-

Figure J. Lactic Acid in Rumen Fluid

Packing: 80/120 Carbowax 20M
Cat. No.: 11889 (15g)
Column: 2m x 2mm ID TIGHTSPEC glass
Oven: 175°C
Carrier: nitrogen, 24mL/min
Det.: FID, 200°C
Inj.: 1 μ L water (analyte concentrations on figure), 200°C



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cal, levels of lactic acid as low as 25ppm can be accurately quantified in the presence of much higher levels of VFAs (~800ppm).

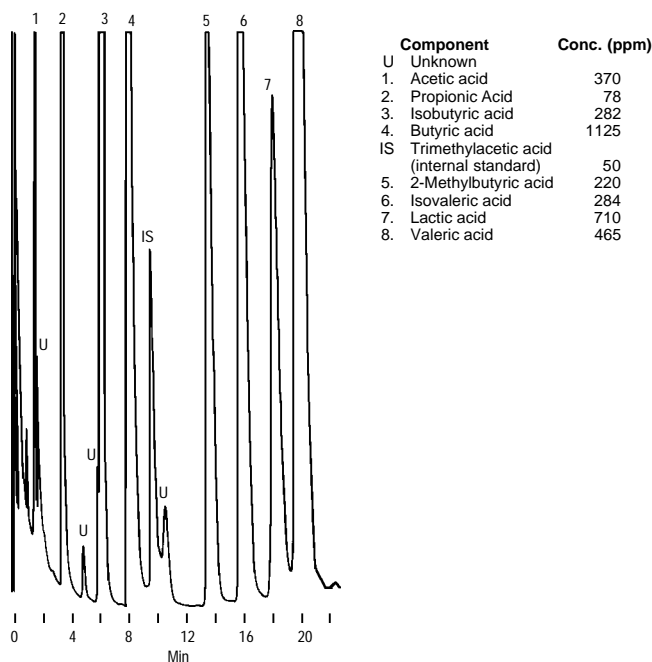
At proper levels in silage, lactic acid helps prevent spoilage and aids in fermentation to conserve the original nutrients with minimal loss. Figure K shows corn silage analyzed on a Carbowax 20M column. The lactic acid concentration in silage is much higher (50-700ppm) than is usual for rumen fluid, thus detector sensitivity must be varied. If the lactic acid concentration is above 25ppm, acidic components can be analyzed by using a 1 μ L injection and a detector sensitivity of 2×10^{-11} AFS. If lactic acid concentration is below 25ppm, however, a detector attenuation of 1×10^{-11} AFS must be used. We recommend an injection volume of 1 μ L or less, since larger volumes may cause peak splitting.

Calibration curves for Carbowax 20M columns indicate detector response is linear for lactic acid concentrations of 25-450ppm, and for VFA concentrations of 25-900ppm. Repeatability studies also show that lactic acid and VFAs can be quantified consistently well on this packing.

The level of lactic acid ghosting on Carbowax 20M packing is 3-5%. This is minimal and should not interfere with the analysis. Ghosting for VFAs is practically undetectable, averaging less than 0.5% over a concentration range of 25-900ppm. For most of the acids, ghosting was not seen at

Figure K. Lactic Acid in Corn Silage

Packing: **80/120 Carbowax B-DA/4% Carbowax 20M**
 Cat. No.: **11889** (15g)
 Column: 2m x 2mm ID TightSpec glass
 Oven: 175°C
 Carrier: nitrogen, 24mL/min
 Det.: FID, 200°C
 Inj.: 1µL water (analyte concentrations on figure), 200°C



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concentrations below 400ppm. At the extremely high concentration of 700ppm, average ghosting of lactic acid or VFAs was 20ppm. Acetic and propionic acids showed slightly more ghosting than the other acids.

Needle depth on injection is important when analyzing lactic acid and VFAs. In our laboratories, ghosting increased when the distance between the needle tip and the column packing was greater than 5mm. Apparently, some of the sample is deposited on the glass wall above the packing. The acids are then washed off and eluted with the next sample. We also recommend using only glass columns, since metal columns will adsorb the VFAs.

A 0.03M oxalic acid solution is used to condition the Carbowax B-DA/Carbowax 20M column, and must be included for proper analysis in all lactic acid standards and samples. Our oxalic acid conditioning solution is prepared at a concentration of 0.3M, therefore sample dilution will be minimal.

Carbowax B-DA/4% Carbowax 20M packing, in a 2m x 2mm ID TightSpec column, also can be used for analyses of C2-C5 VFAs, in place of 10% SP-1200/1% H₃PO₄ on 80/100 Chromosorb W AW. The Carbowax/Carbowax 20M column will provide accurate, reproducible results in these analyses, as well as for lactic acid.

C14-C20 Free Fatty Acids

A special packing is needed for analyses of C14-C20 free acids, because these analytes tail severely on most columns. The stationary phase should be acidic, to deactivate the support. Active sites on the support will adsorb the acids, causing the peaks to tail. The packing also should be highly polar, to favor separation of the acids by degree of unsaturation and to hasten their elution (15). C14-C20 acids can be separated on 5% DEGS-PS on 100/120 SUPELCOPORT™ or 10% SP-216-PS on 100/120 SUPELCOPORT. Both packings are highly polar polyesters stabilized with H₃PO₄ to improve their thermal stability and eliminate tailing of the acid peaks. Both packings have an upper temperature limit of 200°C, but the DEGS-PS packing is somewhat more thermally stable. Applications for these packings are described below. Processing of large numbers of samples can be hastened by eliminating the methylation step.

Performance of the two packings is compared in Figure L. All of the free fatty acids are separated on the DEGS-PS packing. On the SP-216-PS packing, resolution of the C16:0 and C16:1 peaks is slightly greater, but the C20:0 and C18:2 acids coelute. The two packings also can be used to separate these acids as methyl esters. Separation of either the free acids or methyl esters is by degree of unsaturation, with the saturated member of a series eluted first (e.g., C18:0, C18:1, C18:2, etc.).

Figure L. Free C14-C20 Fatty Acids on Highly Polar Columns

Packing: **5% DEGS-PS on 100/120 SUPELCOPORT**
 Cat. No.: **11870-U** (20g)

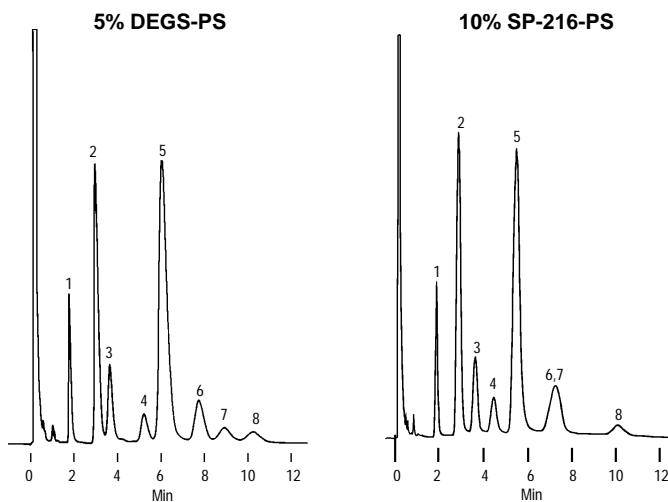
Packing: **10% SP-216-PS on 100/120 SUPELCOPORT**
 Cat. No.: **11879** (20g)

Column: 3' x 2mm ID glass
 Oven: 200°C

Carrier: nitrogen, 20mL/min
 Det.: FID, 210°C

Inj.: 1µL methylene chloride (100µg mixed acids), 210°C

- | | |
|----------------|-------|
| 1. Myristic | C14:0 |
| 2. Palmitic | C16:0 |
| 3. Palmitoleic | C16:1 |
| 4. Stearic | C18:0 |
| 5. Oleic | C18:1 |
| 6. Linoleic | C18:2 |
| 7. Arachidic | C20:0 |
| 8. Linolenic | C18:3 |



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Table 1. Relative Retention of C14-C20 Fatty Acids

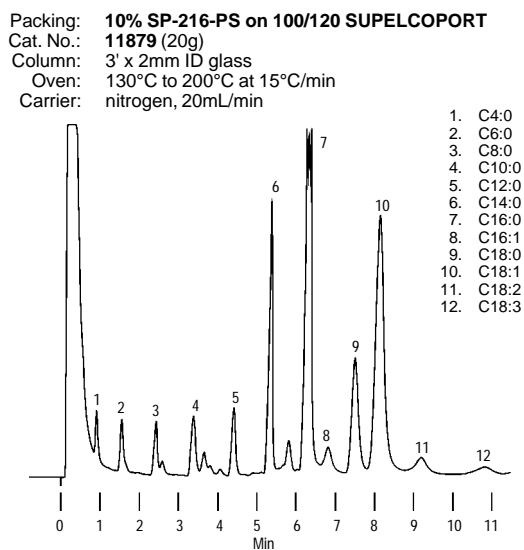
Acid	SP-216-DS	DEGS-PS	DEGA-PS	FFAP	Carbowax 20M-PTA
C14:0	0.41	0.34	0.29	0.27	0.26
C16:0	0.64	0.59	0.54	0.52	0.51
C16:1	0.80	0.71	0.62	0.58	0.57
C18:0	1.00	1.00	1.00	1.00	1.00
C18:1	1.23	1.20	1.12	1.09	1.07
C18:2	1.60	1.49	1.35	1.27	1.25
C18:3	2.22	1.96	1.74	1.58	1.54
C20:0	1.60	1.71	1.87	1.95	1.92
C18:0 (Min) McReynolds Factor (x)	5.3	11.5	22.8	44.3	49.5
	632	496	378	340	321

10% stationary phase on 80/100 mesh SUPELCOPORT, 3' x 2mm ID glass column, 200°C, 20mL/min nitrogen

We recommend using these packings in 3' columns for separating the free fatty acids, and in 6' columns for separating the methyl esters. For greatest efficiency, and to prevent tailing of the acid peaks, use glass tubing. A new column should be conditioned overnight (for at least 3 hours) at 200°C, with a 20mL/min carrier gas flow for 2mm ID columns or an 80mL/min flow for 4mm ID columns. You must use dry carrier gas with these columns, or the stationary phases will deteriorate in a relatively short time – often overnight. Furthermore, small amounts of oxygen in the carrier gas can oxidize the packing, gradually decreasing column polarity. (For information on purifying carrier gas, request Bulletin 848.) When analyzing free acids, inject the sample into a glass-lined inlet or directly onto the column. Acids can adsorb to a metal inlet, causing peaks to tail.

Whole Milk Free Acids

G. Porter of the Department of Dairy Science, The Pennsylvania State University, established conditions for separating the C4-C18:3 fatty acids from whole milk (Figure M). By analyzing the fatty acids in free form, he eliminated the chance of losing the short chain acids during conversion to the methyl esters. This analysis on an SP-216-PS column is faster than analysis of methyl esters on a conventional column. For the free fatty acids analysis, Porter used a 130°C-200°C temperature program. For methyl esters, he used an initial temperature of 40°C and waited longer for the sample to elute. A DEGS-PS column also can be used for these analyses.

Figure M. Whole Milk Free Acids

Preparation of Whole Milk Samples for Free Acids Analysis

- To a separatory funnel, add 10mL whole milk, 10mL ethanol, 3mL 28% ammonium hydroxide, 25mL petroleum ether, and 25mL diethyl ether (ethyl ether).
- Shake mixture for 5 minutes and allow to stand for 20 minutes.
- Drain off bottom phase and carefully pour ether phase out of top of funnel.
- Dry ether phase under nitrogen, add 3mL 0.5N NaOH in methanol, and heat on steam bath for 15 minutes.
- Add 5mL water, slowly add 2N HCl until pH is approximately 2.
- Extract fatty acids with 5mL petroleum ether and 5mL diethyl ether.

Column and Stationary Phase Characteristics in Analyses of Free Acids

The polarity of the stationary phase controls the order of elution and the speed of the analysis, as shown in Table 1. As the polarity of the stationary phase increases (increasing value for the McReynolds factor, x), the column's ability to separate by degree of unsaturation also increases. For example, stearic (C18:0) and oleic (C18:1) acids are increasingly separated. With more polar phases, linolenic acid (C18:3) elutes after arachidic acid (C20:0), while with less polar phases the C20:0 acid elutes last.

As column polarity increases, analysis time decreases. When the amount of stationary phase and the column temperature are constant, stearic acid is eluted in 5.3 minutes from the highly polar SP-216-PS phase, and in 49.5 minutes from the moderately polar Carbowax 20M-TPA (terephthalic acid) phase. Remember: the effective polarity of a stationary phase is affected by its concentration and by the type of support it is coated on (16).

The second important consideration in developing a stationary phase for free acid analysis is that it contain a free acid. The acid ties up active sites on the support, allowing acid peaks to be eluted without tailing. A number of acids have been used for this purpose. The most common are terephthalic (TPA) and orthophosphoric acids (H₃PO₄). We believe phosphoric acid is more effective because it deactivates the support and improves the thermal stability of the polyester stationary phase. TPA is frequently reacted with the terminal-OH group of a polyglycol (e.g., Carbowax 20M), giving the half ester and leaving a carboxyl group free in the stationary phase. Variations of this process are used to produce FFAP and SP-1000 phases.

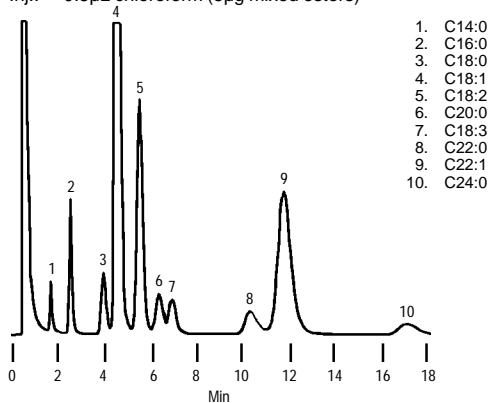
C14+ Fatty Acid Methyl Esters

C14 and larger fatty acid methyl esters are best separated by polar stationary phases, because these phases separate by the degree of unsaturation, with the saturated member of the series eluted first, e.g., stearate (18:0), oleate (18:1), linoleate (18:2), linolenate (18:3). Two classes of stationary phases are used for this separation: polyesters and cyanosilicones. The SP-2300 series cyanosilicones and the polyesters have similar separating characteristics, but the cyanosilicones have the potential for a much longer column life.

SP-2330 cyanosilicone phase has chromatographic properties very similar to DEGS and other polyesters, but with an upper temperature limit of 275°C. Therefore, when an SP-2330 column is used for analyzing fatty acid esters at 200°C, bleed is much less

Figure N. C14-C24 Fatty Acid Methyl Esters

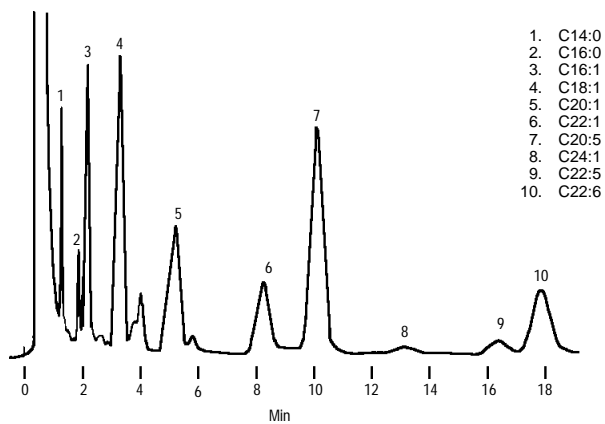
Packing: **GP 10% SP-2330 on 100/120 Chromosorb W AW**
Cat. No.: **11851 (20g)**
Column: **6' x 1/8" stainless steel**
Oven: **200°C**
Carrier: **nitrogen, 20mL/min**
Det.: **FID**
Inj.: **0.5µL chloroform (5µg mixed esters)**



796-0178

Figure O. Polyunsaturated Fatty Acid Methyl Esters

Packing: **10% SP-2330 on 100/120 Chromosorb W AW**
Cat. No.: **11851 (20g)**
Column: **6' x 1/8" stainless steel**
Oven: **210°C**
Inj.: **1.5µL iso-octane (15µg mixed esters)**



795-0199

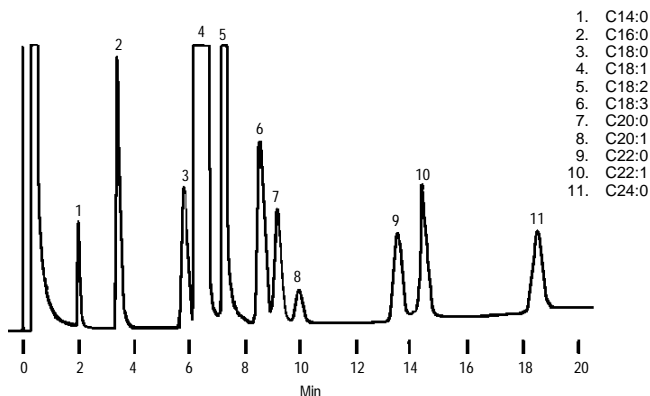
than with the polyesters, and the column will last longer. An SP-2330 column will separate the RM-3 and PUFA 1 test mixtures in a manner similar to a DEGS-PS column, as shown in Figures N and O.

Rapeseed oil methyl esters can be separated in 20 minutes, with good resolution of methyl linolenate (C18:3), methyl arachidate (C20:0), and methyl eicosenoate (C20:1), on a mixed cyanosilicone phase packing, 3% SP-2310/2% SP-2330 (Figure P). On columns of higher or lower polarity, the peaks overlap. This packing also separates the three methyl esters in the least time. Stainless steel (ss) or glass columns can be used with cyanosilicone phase packings, but 2mm ID glass ensures the best sample resolution.

Our GP 10% DEGS-PS polyester packing contains H₃PO₄ to improve its thermal stability. The maximum temperature for isothermal operation is approximately 200°C. The packing is

Figure P. C18:3, C20:0, and C20:1 Methyl Esters Resolved

Packing: **GP 3% SP-2310/2% SP-2330 on 100/120 Chromosorb W AW**
Cat. No.: **11833 (20g)**
Column: **6' x 1/8" stainless steel**
Oven: **190°C (2 min) to 220°C at 2°C/min**
Carrier: **nitrogen, 20mL/min**
Det.: **FID**
Inj.: **0.5µL methylene chloride (5µg mixed esters)**



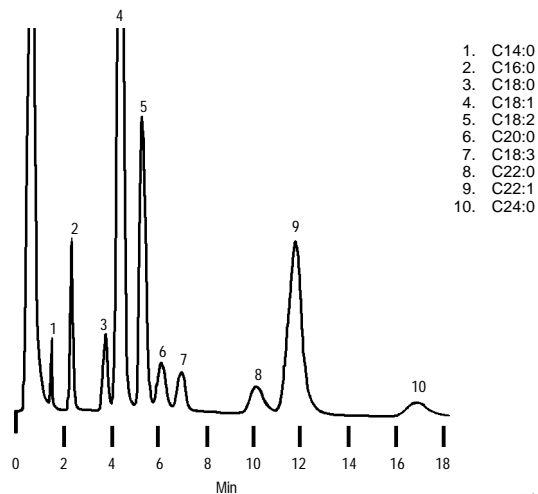
795-0260

tested for analysis of fatty acid methyl esters, to ensure results will be consistent from lot to lot. Figure Q shows the separation of a test mixture on a 6' x 1/8" stainless steel column. When analysis time is important, analyses can be completed in half the time required with the 5% phase (compare Figures Q and R). Either stainless steel or glass columns can be used with these packings but, again, we recommend 2mm ID glass columns for best sample resolution.

Two factors contribute to generally shorter lifespans for polyester columns. Polyester stationary phases are normally operated at or near their upper temperature limit of 200°C. Consequently, you should use a reliable temperature-measuring device. Do not rely solely on a device that is part of the chromatograph, particularly

Figure Q. C14-C24 Fatty Acid Methyl Esters

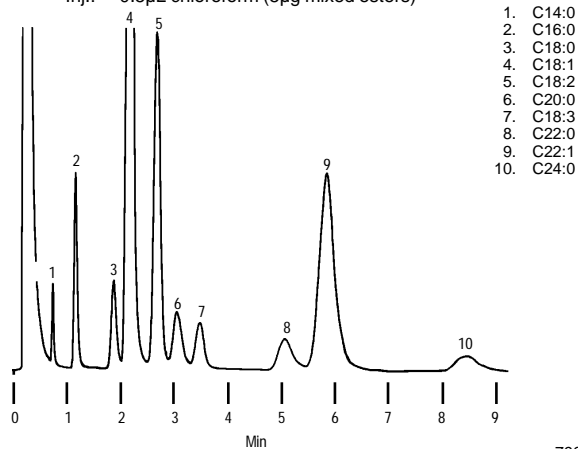
Packing: **GP 10% DEGS-PS on 80/100 SUPELCOPORT**
Cat. No.: **11999 (20g)**
Column: **6' x 1/8" stainless steel**
Oven: **200°C**
Carrier: **nitrogen, 20mL/min**
Det.: **FID**
Inj.: **0.5µL chloroform (5µg mixed esters)**



796-0174

Figure R. 5% DEGS-PS Packing Provides Rapid Analyses

Packing: **GP 5% DEGS-PS on 100/120 SUPELCOPORT**
Cat. No.: **11870-U (20g)**
Column: 6' x 1/8" stainless steel
Oven: 200°C
Carrier: nitrogen, 20mL/min
Det.: FID
Inj.: 0.5µL chloroform (5µg mixed esters)



796-0174

with older models. Also, polyesters can be damaged by oxygen or moisture in the carrier gas, or in the atmosphere if a hot column is disconnected from the chromatograph. It is imperative that these contaminants be removed from the carrier gas, or your columns will not last very long. For details on carrier gas purification, request Bulletin 848.

SP-2330 cyanosilicone also is used at approximately 200°C for fatty acid methyl ester separations. But, in contrast to the polyester phases, it has an upper temperature limit of 275°C. Because the phase is used at a temperature well below its limit, you can expect extended column life. Silicones also are generally much more resistant to degradation caused by oxygen and moisture in the carrier gas.

Fatty acid methyl esters can be separated on either glass or metal columns, but for columns of the same dimensions a glass column generally will provide better separations. For highest efficiency from a glass column, we recommend a 6' x 1/4" OD with 2mm ID, rather than 4mm ID. Similarly, for higher efficiency, we prefer stainless steel columns (6' x 1/8" OD) to aluminum or copper. If a 6' x 1/8" stainless steel column does not provide adequate separation, try a 7' or 8' length. If your chromatograph is designed for 1/4" columns, order appropriate parts for accommodating 1/8" columns from the chromatograph manufacturer. You can seriously reduce efficiency if you connect 1/8" columns using reducing unions.

The carrier gas flow rate is very important in obtaining good performance from these columns. With nitrogen as the carrier gas, use a 20mL/min flow with a 1/8" OD or 2mm ID column and 80mL/min with a 1/4" OD or 4mm ID column. Allowing for the internal diameter differences between the columns, these flows are equivalent. Reducing the carrier gas flow by half (to 10mL/min or 40mL/min, respectively) will improve column efficiency, but double analysis time. Further reduction in the flow rate could drastically reduce efficiency. On the other hand, increasing the flow rate to 40mL/min or 160mL/min, reduces column efficiency somewhat, but cuts analysis time by half.

For the packings described here, with a 20mL/min flow in a 1/8" OD or 2mm ID column, column backpressure will be 20-30psi at

200°C. Set the second stage of the regulator on the carrier gas cylinder to at least 40psi. If the pressure on the second stage is set too low, the flow controller will not function properly and the carrier gas flow rate will not be maintained, particularly during temperature programming. See the instruction manual for your chromatograph for details regarding controlling flow of carrier gas.

Fatty acid concentrations of approximately 10mg/mL are good to work with, and 1µL samples are accepted readily by these columns. We recommend diluting standards and samples with isoctane. If you use chloroform, ethanol used as a preservative in the chloroform can react with the fatty acid methyl esters, forming a mixture of the methyl and ethyl esters.

cis-trans Isomers

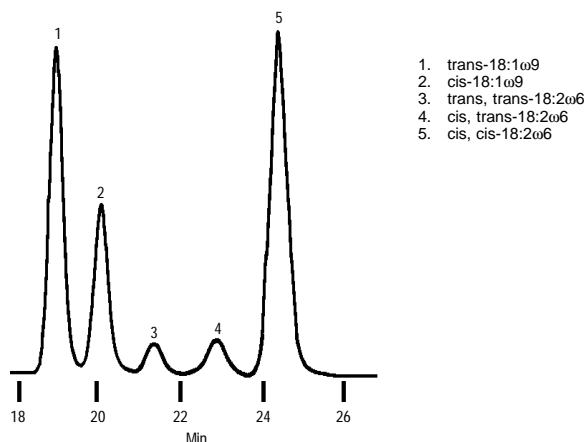
Fatty acids containing *trans* double bonds are normal constituents of the human diet. Some are formed by rumen microorganisms and appear in the milk and tissues of ruminants (17,18). *trans* fatty acids also are produced during commercial hydrogenation of liquid vegetable oils to produce oleomargarines, cooking oils, salad oils, and shortenings. A 20' column containing either SP-2340 or OV®-275 phase is needed to resolve *trans* fatty acid isomers from *cis* isomers (Figures S and T).

Ottenstein, *et al.* showed that oleate and elaidate can be separated on SP-2340 or OV-275 columns (19). Subsequently, analysts in this laboratory determined that OV-275 columns could be used to analyze mixtures of *cis* and *trans* positional isomers of octadecenoate (Figure T) and octadecadienoate in commercial oleomargarines (20).

At 220°C, these phases are sensitive to traces of oxygen in the carrier gas. The phase (and therefore the separation) will be rapidly degraded by this contaminant. Removing oxygen and water from the carrier gas will prolong column life. (For details, request Bulletin 848.) A particularly well-packed column can be used at 230°C, and will separate elaidate and oleate to baseline in 19.5 minutes.

Figure S. Fatty Acid Methyl Ester Standards

Packing: **15% OV-275 on 100/120 Chromosorb P AW-DMCS**
Cat. No.: **11844-U (20g)**
Column: 20' x 1/8" stainless steel
Oven: 230°C



796-0179

Figure T. Elaidate/Oleate Isomers in Margarines

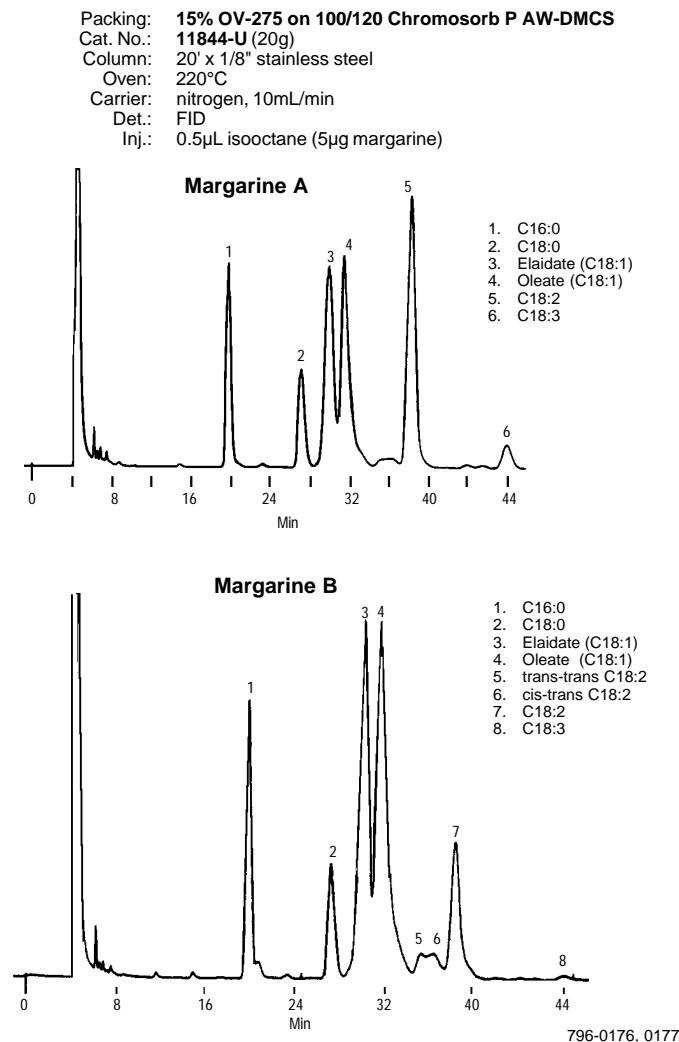
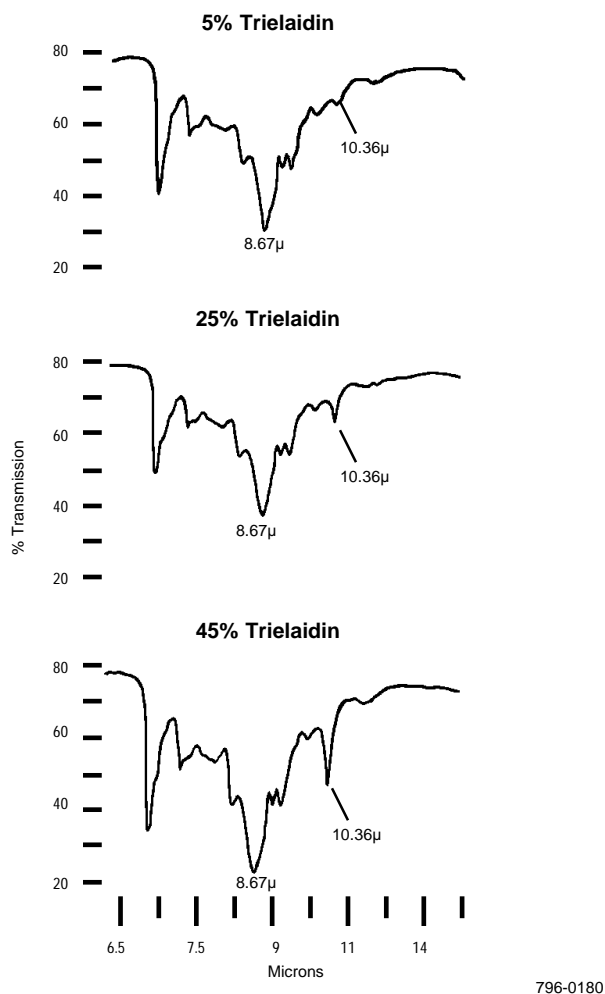


Figure U. IR Spectra for Trielaidin in Triolein

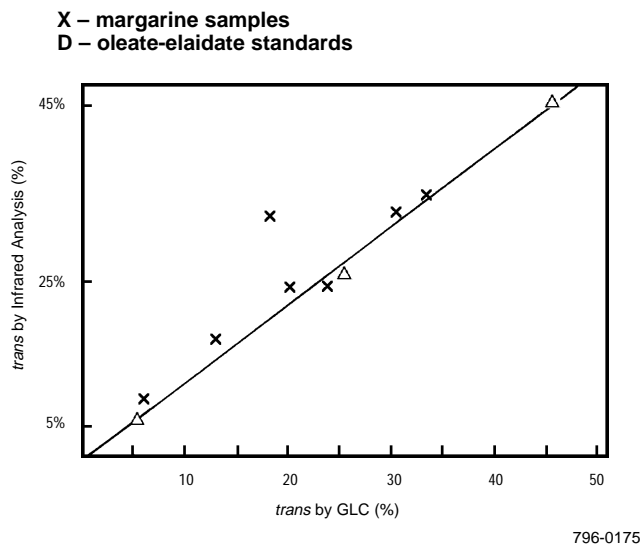


Infrared analysis has commonly been used to measure *trans* unsaturation (Figure U, ref. 21). We have found that analyses on OV-275 packed columns provide *trans* content data that is generally in excellent agreement with IR data (Figure V).

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Figure V. Correlation of *trans* Fatty Acid Content of Commercial Margarines, as Determined by Infrared Analysis and Packed Column GC



15. Ottenstein, D.M. and W.R. Supina, *J. Chromatogr.*, **91**: 119 (1974).
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 21. Allen, R.R., *JAOCS*, **46**: 552 (1969).
- References not available from Supelco.

Additional Information

The following sources offer information about specific procedures for identifying bacteria from fermentation products.

Gas-Liquid Chromatography Analysis of the Acid Products of Bacteria
G.L. Lombard and V.R. Dowell, Jr.

The "CDC Lab Manual" describes standardized procedures used by the Anaerobic Bacterial Diseases Branch of the U.S. Department of Health and Human Services. The authors stress the importance of using standardized media, reagents, and techniques. Request this publication from: U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta, GA 30333 USA.

The Anaerobe Laboratory Manual

This manual tabulates the distribution of acids and alcohols for a large number of anaerobes in different culture media. Direct inquiries about availability to: Virginia Polytechnic Institute and State University, Department of Anaerobe Microbiology, Research Division, Blacksburg, VA 24061 USA.

Wadsworth Anaerobic Bacteriology Manual (3rd edition)

V.L. Sutter, D.M. Citron, and S.M. Finegold

Direct inquiries about availability to: Anaerobic Bacteriology Laboratory, Wadsworth Hospital Center, Veterans Administration, Los Angeles, CA 90073 USA.

Ordering Information:

Description	Cat. No.
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Packings for C1-C7 Acids

GP 10% SP-1200/1% H ₃ PO ₄ on 80/100 Chromosorb W AW, 20g	11965
60/80 Carbowax C/0.3% Carbowax 20M/0.1% H ₃ PO ₄ , 15g	11825-U
GP 15% SP-1220/1% H ₃ PO ₄ on 100/120 Chromosorb W AW, 20g	12144
GP 10% SP-1001/1% H ₃ PO ₄ on 100/120 Chromosorb W AW, 20g	11841
80/120 Carbowax B-DA/4% Carbowax 20M, 15g	11889
80/120 Carbowax B-DA/4% Carbowax 20M in 2m x 2m ID TightSpec Column	
HP 5830, 5840	23109
HP 5880, 5987 (config. A)	23110-U
Perkin Elmer 900, 990, F-30, 3920	23111
Perkin Elmer Sigma Series	23112
Varian 3700, Vista Series	23113
Oxalic acid, 0.3M in water, 1mL For conditioning Carbowax B-DA/ Carbowax 20M columns	48886

Description	Cat. No.
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Packings for C14+ Fatty Acid Methyl Esters

Cyanosilicone Phases, 20g

GP 10% SP-2330 on 100/120 Chromosorb W AW	11851
GP 3% SP-2310/2% SP-2330 on 100/120 Chromosorb W AW	11833
GP 10% SP-2300 on 80/100 SUPELCOPORT	12091

Polyester Phases, 20g

GP 5% DEGS-PS on 100/120 SUPELCOPORT	11870-U
GP 10% DEGS-PS on 80/100 SUPELCOPORT	11999
10% DEGS on 80/100 Chromosorb W AW	11903
15% DEGS on 80/100 Chromosorb W AW	11904
GP 10% SP-216-PS on 100/120 SUPELCOPORT	11879

Packing for cis-trans Isomers, 20g

15% OV-275 on 100/120 Chromosorb P AW-DMCS	11844-U
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Packing for C2-C10 Free Acids, 20g

GP 10% SP-1200/1% H ₃ PO ₄ on 80/100 Chromosorb W AW	11965
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Packings for C6 and Higher Free Acids, 20g

(in order of increasing polarity)

10% Carbowax 20M-TPA on 80/100 Chromosorb W AW	11874
10% SP-1000 on 80/100 SUPELCOPORT	11872
10% SP-1000 on 100/120 Chromosorb W AW	11962
10% FFAP on 80/100 Chromosorb W AW	11873
GP 5% DEGS-PS on 100/120 SUPELCOPORT	11870-U
GP 10% SP-216-PS on 100/120 SUPELCOPORT	11879

GP – Indicates packing is tested for applications described in this bulletin.

For columns filled with any of these packings, please refer to our catalog or call our Sales Department.

For fatty acid and related standards, please refer to our catalog.

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