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Note

Improved procedure for preparation of pentafluorobenzyl derivatives of carboxylic acids for analysis by gas chromatography with electron-capture detection

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Several workers have shown that pentafluorobenzyl (PFB) derivatives of carboxylic acids possess excellent electron absorption qualities [1,2]. Frequency-pulsed electron-capture gas chromatography (FPEC–GC) is an extremely sensitive and selective technique that has been used to detect carboxylic acids in body fluids [3–7], and PFB derivatives of these acids may also have potential for detection of those acids by FPEC–GC. PFB derivatives of tuberculostearic acid (TSA) have been reported to possess excellent qualities for negative-ion chemical ionization mass spectrometric (NICI-MS) analysis [2]. Further, analysis of the PFB derivatives of unknowns by NICI-MS could facilitate their identification.

With current PFB derivatization techniques for analysis by FPEC–GC there is not an adequate procedure for removing excess PFB reagent and reaction by-products. Failure to remove these contaminating products limits the amount of sample that can be analyzed by FPEC–GC and thus limits the sensitivity of the techniques. This problem is especially severe with detection of short-chain acids (C_2–C_{10}). The objective of this investigation was to develop a sensitive, practical procedure that would significantly reduce the background signal associated with PFB derivatives. An improved procedure would be useful for detecting both short- and long-chain acids and trace (25 fmol) amounts of acids that are disease markers by FPEC–GC. Further, the improved PFB derivatization procedure would also be useful for identifying unknowns by NICI-MS.

EXPERIMENTAL*

Preliminary studies

Initially, two derivatization methods were performed as previously described [1,2]. We used the derivatization reagent mixture described [2] and developed a more efficient derivatization technique and background clean-up procedure. First, we tested the possibilities of reducing the amount of PFB reagent by heating the reagent sample mixture at 40, 60 and 100°C for 30 min and 1 h. Second, we tested the possibilities of improving the derivative by removing water from the aqueous extract with sodium sulfate (described below) and by increasing the PFB reagent two-fold to compensate for moisture. Third, we tested the possibilities of removing excess reagent and reaction by-products by using disposable extraction columns and different solvents and solvent combinations. We tested silica (SI), aminopropyl (NH2), phenyl (PH), diol (2OH) and ethyl (C_2) columns. The derivatives were processed through the columns with each sample dissolved in hexane and eluted with acetonitrile and benzene. For the cyanopropyl (CN) column the derivatives were processed with each sample dissolved in hexane and eluted with acetonitrile, benzene, acetone, ethyl acetate, dioxane, chloroform and diethyl ether. The CN column was also evaluated by processing each sample dissolved in 10% acetone in hexane, 15, 10 and 5% acetonitrile in hexane, and eluted with diethyl ether. The best column and solvent combinations are listed below.

Sample extraction

Body fluid (2 ml), serum or cerebrospinal fluid (CSF) was placed in a 50-ml Teflon-lined screw-cap round-bottom tube and adjusted to pH 2 using 0.1 ml of sulfuric acid. An internal standard (7.6 pmol of C_7) was added [3] and the acidified sample was extracted with chloroform to obtain carboxylic acids as previously described [3–5].

PFB derivatization

For our derivatization procedures the extract was placed in a 150 mm × 20 mm screw-cap test tube and evaporated with clean, dry air filtered through calcium sulfate (W.A. Hammond Drierite, Xenia, OH, U.S.A.) to approximately 1 ml. About 200 mg of sodium sulfate were added to the sample, mixed by shaking, and the chloroform layer (which was now free of water) was quickly (1 min) removed and transferred to a 3-ml conical tube and then evaporated with air to about 10 μl. Next, 1 μl of a 35% solution of PFB bromide in acetonitrile and 10 μl of triethylamine were added to the sample; it was mixed by shaking and heated in a 60°C sand bath for 30 min. Finally, 1 ml of 5% acetonitrile in hexane was added to the sample and the tube was stoppered.

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*Use of trade names is for identification only and does not imply endorsement by the Public Health Service or U.S. Department of Health and Human Services.

Purification of PFB derivatives

A CN reversed-phase chromatography column (Analytichem International, Harbor City, CA, U.S.A.) was conditioned with two column volumes (6 ml) of hexane, the PFB derivatives prepared by our procedure were aspirated through the column using a partial vacuum of about 50 mm Hg for about 2 min and the eluate was discarded. The 3-ml conical tube was rinsed twice with 1 ml of diethyl ether, drained and placed on the collector rack. Then, the isolate was eluted from the column with 1 ml diethyl ether into the rinsed conical tube. The eluate was evaporated to 10 μl (but not to dryness) with clean, dry, filtered air in a 60°C sand bath, and 0.2–0.4 ml of xylene–ethanol (1:1) was added.

Gas chromatography

The derivatives were analyzed in 1-μl aliquots using a Perkin-Elmer 3920B gas chromatograph equipped with FPEC electrometers, 10-mCi ^{63}Ni detectors and standard 0.7-mm injection ports containing glass inserts. Non-polar OV-1 (25 m$\times$0.53 mm with 5 μm film thickness), moderately polar OV-1701 (30 m$\times$0.53 mm with 1.0 μm film thickness) and polar OV-225 (25 m$\times$0.32 mm with 0.25 μm film thickness) large-bore fused-silica capillary columns were used. The connection of the large-bore capillary columns to the packed-column injection ports were made by using 0.7 mm$\times$0.2 mm flexible graphite reducing ferrules. For the analysis of derivatives on the OV-1 and OV-1701 columns, the instrument was programmed from 100 to 275°C at 2°C/min. For the OV-225 column, the instrument was maintained at 90°C for 4 min and then programmed at 2°C/min to 220°C. Helium was used as the carrier gas for all of the capillary columns. The flow-rate was 3 ml/min for the OV-1 and OV-1701 columns and 5 ml/min for the OV-225 column. A mixture of argon–methane (95:5) was used as make-up gas to maintain a total flow-rate of 70 ml/min through the detector. The temperature of injector, manifold, and detector were 180, 250, and 300°C, respectively. The sensitivity of the instrument was set and maintained by adjusting the standing current (between 1 and 3), attenuation (256 or 512) and make-up gas (70 ml/min), so that the internal standard (C_7) produced a full-scale peak with 1 μl of sample dissolved in 0.4 ml of xylene–ethanol as the final solvent. Two Perkin-Elmer Model 56 strip chart recorders were operated with a single input of 1 mV and a chart speed of 10 mm/min. The temperature programmer, recorder and computer were activated simultaneously by pressing a foot pedal switch at the time of sample injection.

An IBM System 9000 computer equipped with CAP 2.0 software collected data from the instrument, analyzed chromatograms according to stored methods, integrated peaks, expanded sections of the chromatogram, and increased or decreased peak attenuation for easy comparison. The internal standard (C_7) was used as a reference for both quantitative data and retention times. Both palmitic (C_{16}) and stearic (C_{18}) acids, which were always present in serum and CSF, were also used as retention time references for the identification of TSA in body fluids.

The PFB-derivatized acids were analyzed by electron-impact and positive-ion and negative-ion chemical ionization mass spectrometry. A Finnigan TSQ 46 gas chromatograph–mass spectrometer–data system equipped with a splitless injec-

tor and a DB-5 bonded-phase fused-silica capillary column (30 m × 0.25 mm with 0.25 μm film thickness) was used (J&W Scientific, Folsom, CA, U.S.A.). The carrier gas was helium at a flow-rate of 1 ml/min. For analysis, 4 μl of the derivatized standard mixture were injected. The instrument was held at 90°C for 3 min, and then the injector, which had a temperature of 180°C, was vented, and the instrument was programmed at 6°C/min to 275°C and maintained at this temperature for 26 min. Methane was used as the reagent gas for chemical ionization.

RESULTS

When PFB derivatives of the standard acid mixture (C_2–C_{22}) were prepared with 10 μl of PFB reagent and 10 μl triethylamine in an unheated reaction [2], excess PFB reagent significantly reduced sensitivity and overloaded the detector (Fig. 1A). When the derivatives were prepared with 50 μl of a reagent containing 0.3% PFB bromide and 0.3% crown ether in benzene at 40°C for 90 min [1], the final product was less contaminated but the relative amounts of derivatized acids were significantly reduced (Fig. 1B). Both of these problems were avoided in our procedure, in which 10% of the amount of PFB reagent described in ref. 2 was used in a total volume of 25 μl, the reaction temperature was elevated to 60°C for 30 min, a 3-ml conical tube with a small tip was used which provided a small volume for the reaction mixture, and the excess reagent was removed on a CN column (Fig. 1C).

When PFB derivatives of the standard acid mixture were prepared by our procedure but without removing the excess reagent on the CN column, cleaner derivatives resulted, without loss of derivatized sample (Fig. 2A). By comparing Fig. 1A and Fig. 2A, it is apparent that reducing the amounts of PFB reagent and heating the reaction mixture (25 μl) in a 3-ml conical tube contributed to this result. The CN column was highly effective in further reducing background signal associated with the excess reagent. However, when benzene was used as the eluting solvent (Fig. 2B), unreacted PFB and derivatized acids with chain lengths from C_2 to C_{10} were retained on the column, while acids with chain lengths greater than C_{10} were eluted. This also occurred when the acetonitrile concentration in hexane was increased from 5 to 15%. When the sample was dissolved in 5% acetonitrile in hexane and eluted with diethyl ether, excess PFB reagent was retained by the column and both short-chain (C_2–C_{10}) and long-chain (C_{10}–C_{22}) acids were recovered (Fig. 2C).

The presence of water in the reaction mixture reduced the amount of PFB-derivatized acids produced. Drying the acidic chloroform extracts with sodium sulfate before derivatization or using twice the amount of PFB reagent (2 μl) improved derivative formation. However, using 2 μl of PFB reagent increased the reagent peaks; therefore, drying with sodium sulfate was included as a routine part of the procedure.

When we prepared a PFB derivative of an acidic chloroform extract of serum from a patient with tuberculous meningitis using our procedure, TSA (about 25 fmol) was detected (Fig. 3A, note the peak labeled TSA with an arrow at 79 min).

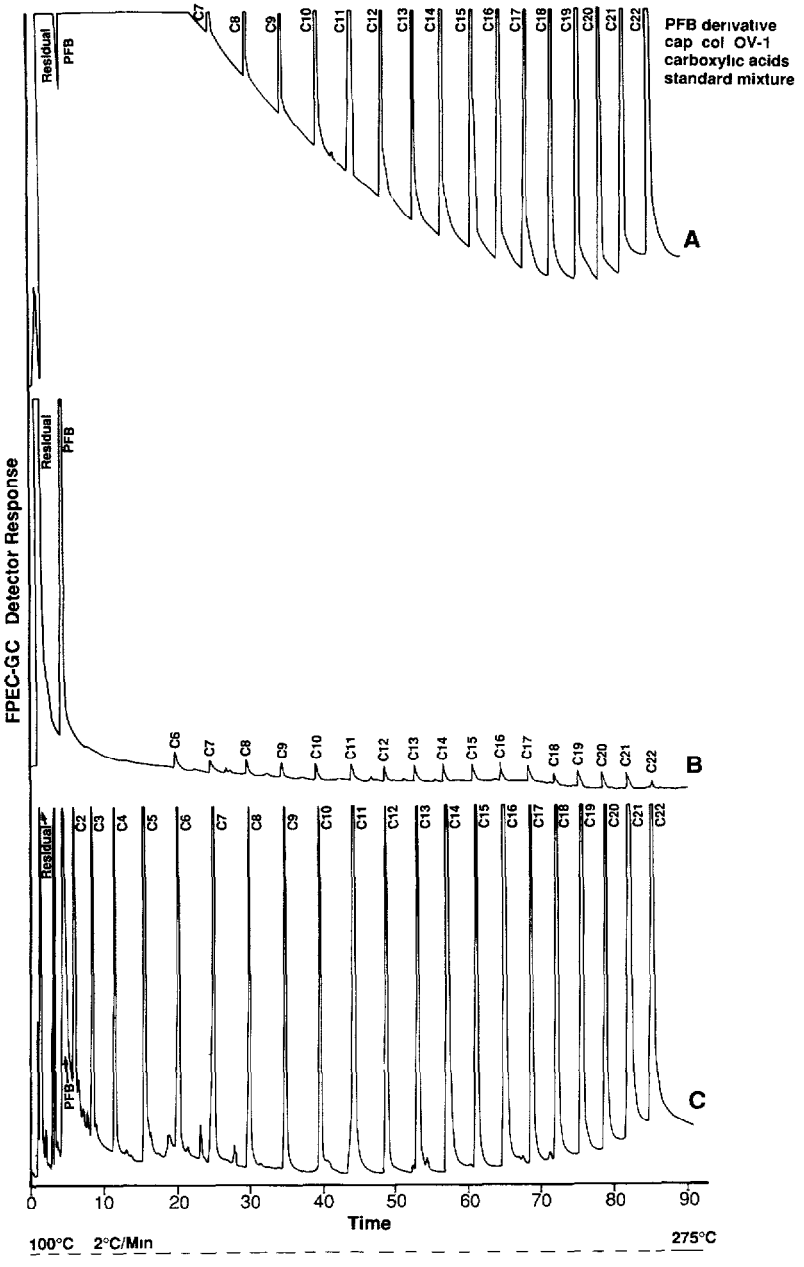

Fig. 1. FPEC-GC of PFB-derivatized samples from standard acid mixtures (C_2-C_{22}). Analyses were carried out on a 25-m OV-1 capillary column. The letter C followed by a number indicates a saturated carboxylic acid with the number of carbon atoms indicated. (A) PFB derivative prepared by adding 10 μl of 35% PFB in acetonitrile and 10 μl of triethylamine and permitting the reaction mixture to remain at room temperature for 10 min [2]. (B) PFB derivative prepared by adding 50 μl of derivatization reagent containing 0.3% PFB bromide and 0.3% crown ether in benzene and heating the reaction at 40 °C for 90 min [1]. (C) PFB derivative prepared as described in Experimental, using smaller amounts of PFB reagent and a cyanopropyl reversed-phase column clean-up procedure.

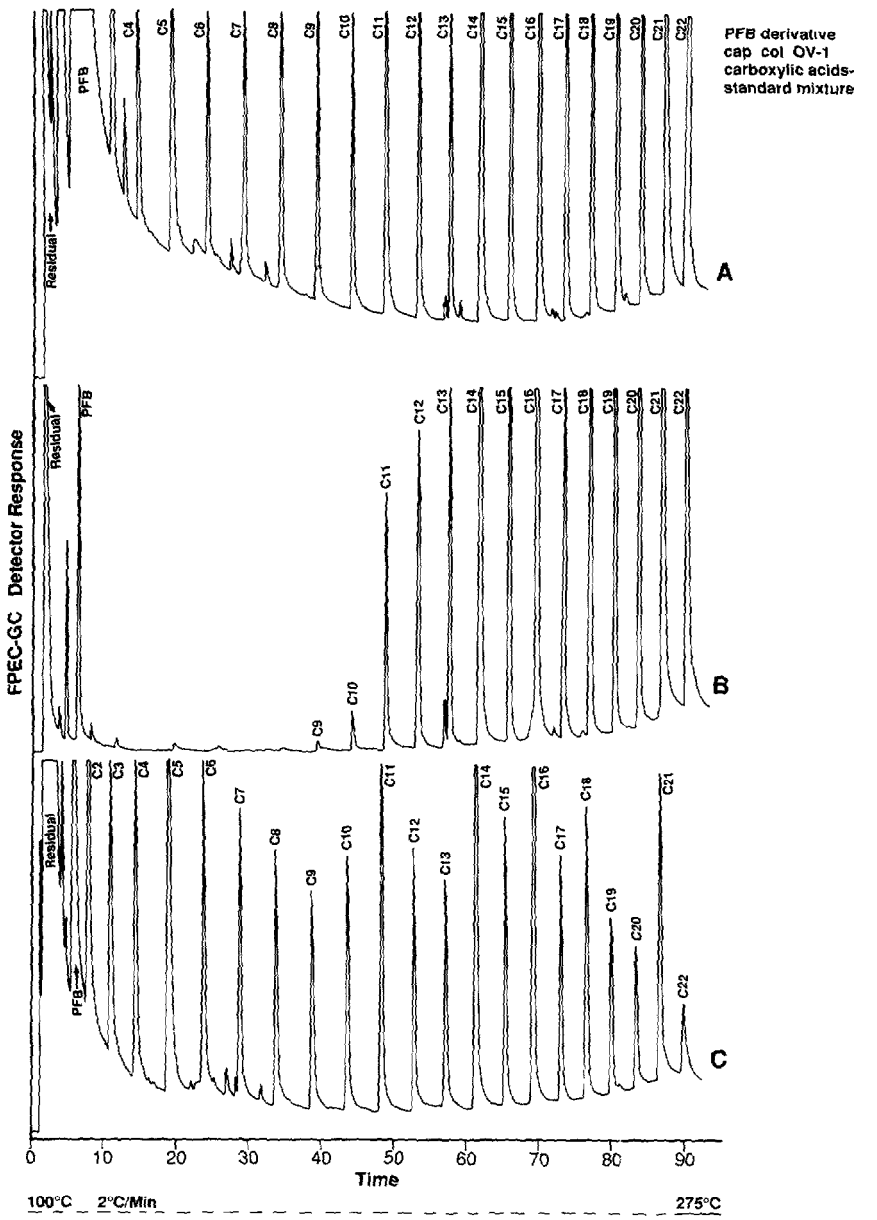

Fig. 2. FPEC-GC of PFB-derivatized samples from standard acid mixtures (C_2-C_{22}). The FPEC-GC conditions were the same as those described for Fig. 1. (A) PFB derivative analyzed without clean-up through a cyanopropyl (CN) reversed-phase column. (B) PFB derivative dissolved in hexane, processed with a CN column and eluted with benzene. (C) PFB derivative dissolved in 5% acetonitrile in hexane, processed with a CN column and eluted with 1 ml of diethyl ether.

Unknown peaks (designated UN1 and UN2) were also detected; these may be chloroform-soluble hydroxy acids. The retention times of UN1 and UN2 have been compared with those of 2-hydroxylauric and 2-hydroxymyristic acids, but

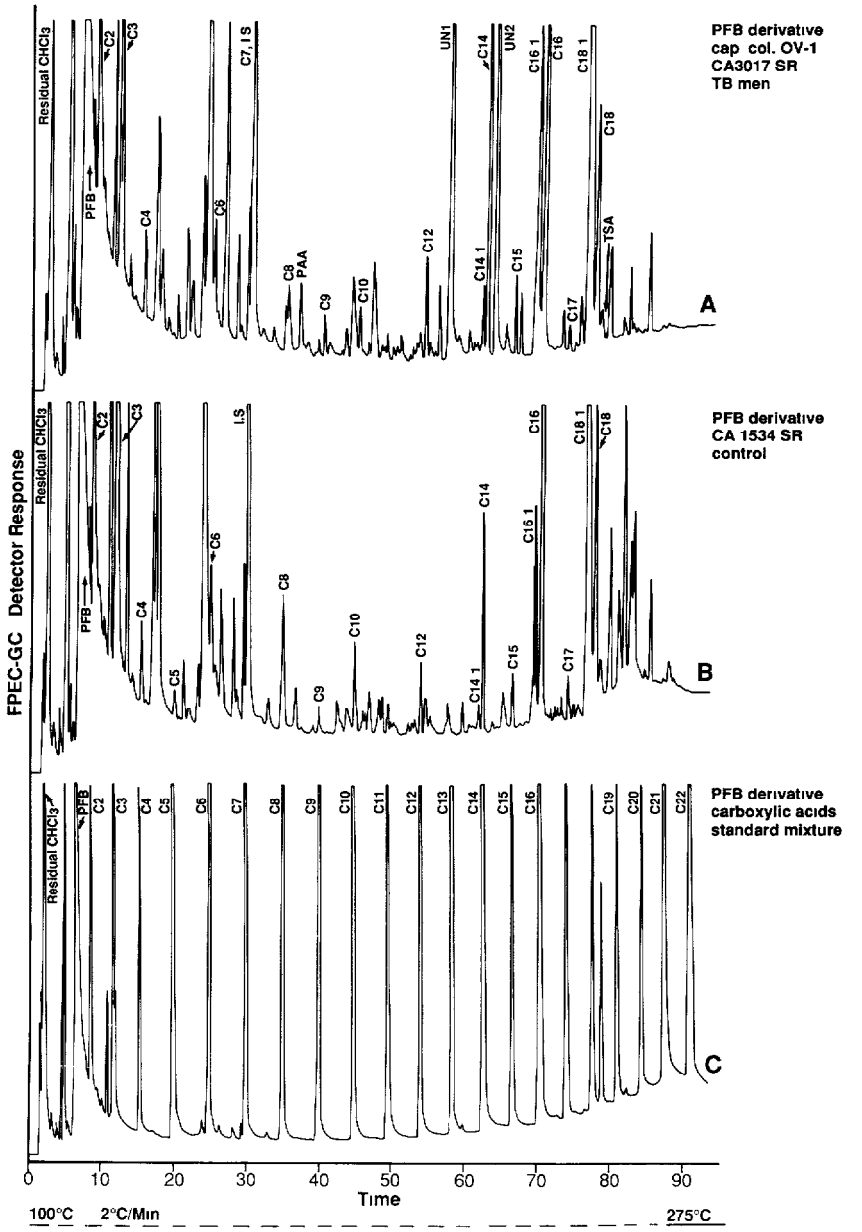

Fig. 3. FPEC-GC profiles. (A) PFB derivative of an acidic chloroform extract of serum (SR) taken from a patient with tuberculous meningitis (TB men). (B) PFB-derivatized acidic chloroform extract of a control serum. (C) PFB-derivatized standard mixture containing C_2–C_{22} and TSA. The FPEC-GC conditions were the same as those described for Fig. 1. i = iso; UN = unknown peak; PAA = phenylacetic acid; a colon between two numbers indicates unsaturation.

they do not match. Fig. 3B shows the FPEC-GC profile obtained from a PFB-derivatized acidic chloroform extract of a control serum, and Fig. 3C is a chromatogram of a standard acid mixture (C_2–C_{22}) with the addition of 100 fmol of

TSA.

PFB-derivatized samples were also analyzed and separated effectively on high-resolution, moderately polar OV-1701 and polar OV-225 capillary columns (data not shown). We were able to analyze the standard mixture at a lower temperature (90–220°C) on the OV-225 column than we did on the OV-1 and OV-1701 columns (100–275°C). In addition, the unsaturated acids had a higher retention time than did the saturated acids of the same chain length. Aromatic acids also had a much higher retention time on the OV-225 column than the straight-chain acids with similar carbon numbers (data not shown).

NICI-MS analysis produced a base peak at M − 181. Mass spectral analysis also showed that all acids (C_2–C_{22}) included in the standard mixture were derivatized with PFB.

DISCUSSION

The use of electron-absorbing reagents such as PFB, although essential to producing highly detectable electron-absorbing derivatives, presents a problem when analyzed by an FPEC detector and capillary column. The use of PFB derivatives may offer an additional sensitive method of detecting metabolites in diseased body fluids by FPEC–GC and perhaps NICI-MS. However, effective removal of unreacted PFB reagent is essential if the PFB derivatives are to be analyzed with an FPEC detector. If adequate clean-up procedures are not used, the capillary column and FPEC detector are overloaded. Diluting the sample helps; however, this greatly reduces sensitivity and reagent peaks are still present. Decreasing the amount of PFB reagent, heating the reaction mixture in a small volume and selectively removing the excess PFB reagent permit the introduction of more sample into the capillary column and result in increased sensitivity.

Removing unreacted PFB without significant loss of short-chain acids (C_2–C_{10}) was accomplished with a CN reversed-phase column. Using this procedure, carboxylic acids with chain lengths from C_2 to C_{22} were derivatized and detected in both serum and CSF in picomole and femtomole amounts.

It is necessary to use a polar column to verify identification of TSA and other acids from body fluids. The highly polar OV-225 column is preferred for this purpose, since the shift of compounds are drastic, and the eluting temperature is lower than OV-1 and OV-1701 columns. However, the OV-225 column cannot be used in the same instrument as the OV-1 column, because it is degraded at 275°C. The moderately polar OV-1701 column can be used with the same temperature program as the OV-1 column; therefore these columns can be used in the same instrument and the sample can be analyzed on both columns simultaneously. The presence of TSA, which can be used to aid in identification of tuberculous meningitis [6], in body fluid specimens was confirmed by analysis on each of the three columns. PFB derivatives were less specific for carboxylic acids than were trichloroethanol (TCE) derivatives, since 2-hydroxy acids were derivatized with PFB, but not with TCE. PFB derivatives contained more background contamination than did TCE derivatives, but the PFB derivative may be superior for identifying unknowns using NICI-MS, because the PFB derivatives produced a

base peak at M − 181 for all acids in the standard mixture. If additional studies confirm that all PFB acid derivatives produce a large M − 181 ion, then molecular mass determination of unknowns would be facilitated.

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