INSTRUCTIONS

Acylation Derivatization Reagents

PIERCE
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0765.2

Number	Description
67363	**TFAA (Trifluoroacetic Acid Anhydride),** 100 g
65193	**PFAA (Pentafluoropropionic Acid Anhydride),** 10 × 1 ml ampules
65192	**PFAA**, 25 g
65191	**PFAA**, 100 g
63164	**HFAA (Heptafluorobutyric Acid Anhydride),** 10 × 1 ml ampules
63163	**HFAA**, 25 g
63162	**HFAA**, 100 g
	Storage: Upon receipt store TFAA, PFAA and HFAA at room temperature.
48882	**TFAI (Trifluoroacetylimidazole**), 10 × 1 ml ampules
	Storage: Upon receipt store TFAI at 4°C. TFAI is shipped at ambient temperature.
49700	**MBTFA (*N*-Methyl-bis[trifluoroacetamide]),** 10 x 1 ml ampules
49701	**MBTFA,** 5 g
49703	**MBTFA**, 25 ml
49704	**MBTFA**, 100 ml
	Storage: Upon receipt store MBTFA at -20°C. MBTFA is shipped at ambient temperature.
44211	**HFBI (Heptafluorobutyrylimidazole),** 5 g
	Storage: Upon receipt store HFBI at -20°C. HFBI is shipped with an ice pack.

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Note: Please read this entire document before beginning an experimental program using any of these reagents.

Introduction

Acylation derivatization reagents are used to convert compounds containing active hydrogens such as -OH, -SH and -NH into esters, thioesters, and amides, respectively, through the action of a carboxylic acid derivative.[1] Acylation reactions are used in chromatographic applications for converting compounds into derivatives that are more easily separated[2] or that give a greater response in the detection system than the parent compound.[3] For example, insertion of perfluoroacyl groups into a molecule enhances its detectability by electron capture. The presence of a carbonyl group adjacent to halogenated carbons enhances the electron capture detector (ECD) response. In addition, acyl derivatives tend to direct the fragmentation patterns of compounds in MS applications, providing useful information on the structure of these materials.[4]

The acylation derivatization reagents are divided into three groups; perfluoro acid anhydrides, perfluoroacylimidazoles and *N*-Methyl-bis(trifluoroacetamide) (MBTFA). The most frequently used perfluoro acid anhydrides for preparing perfluoroacyl derivatives for GC/MS analysis are Trifluoroacetic Acid Anhydride (TFAA), Pentafluoropropionic Acid Anhydride (PFAA, PFPA or PFPAA) and Heptafluorobutyric Acid Anhydride (HFAA, HFBA or HFBAA). These reagents react readily with alcohols, phenols and amines to produce stable, volatile derivatives for TCD, FID and ECD techniques. (See Table 1 for the molecular structures of perflouroacyl acid anhydrides.)

The widely used perfluoroacylimidazoles are Trifluoroacetylimidazole (TFAI) and Heptafluorobutyrylimidazole (HFBI). The perfluoroimidazoles react with hydroxyl groups, both primary and secondary amines and quantitatively acylate indole alkylamines.[3,5] In many cases, the *N*-perfluoroacylimidazoles offer considerable advantage over anhydrides for preparing perfluoroacyl derivatives, as their reactions are smooth, quantitative and produce no acid by-products that must be removed from before injection. Instead, the principal by-product is imidazole, which is relatively inert. Tryptamine and metabolites present in spinal fluid have been analyzed by ECD using HFBI.[3] Fluorinated imidazoles have also been used in bifunctional derivatization schemes and in exchange reactions where TMS derivatives were converted to HFB derivatives. The hydroxyl groups of catecholamines were derivatized with TMSI, followed by conversion of amines to acylamines with HFBI.[6] (See Table 1 for the molecular structures of *N*-perfluoracylimidazoles.)

N-Methyl-bis(trifluoroacetamide) (MBTFA) trifluoroacetylates primary and secondary amines, hydroxyl and thiol groups under mild non-acidic conditions.[7] The principal by-product from the derivatization reaction is *N*-methyltrifluoroacetamide, which is stable, volatile and does not present problems in subsequent gas chromatography. Sullivan and Schewe[8] have reported using MBTFA to prepare volatile TFA derivatives of mono-, di- and tri-saccharides with good yield. Subsequent gas chromatography showed excellent separations with retention times substantially lower than the corresponding TMS derivatives, for all compounds tested. (See Table 1 for the molecular structure of MBTFA.)

Important Product Information

- Acylation reagents are moisture-sensitive: take precautions to prevent moisture introduction from the atmosphere or reagent handling.
- The toxicological properties of these materials are not well known. Regard these materials as potentially toxic. Avoid contact with these reagents.

Example Protocols for Fluorinated Anhydrides

Notes:

- When amine bases are used, perform aqueous extraction steps below pH 6, as nonprotonated amines will catalyze hydrolysis of the derivatives.
- Anhydrides produce the corresponding perfluoro acid as a by-product from the reaction with the active hydrogens from alcohols, phenols, mercaptans and amines. These acidic by-products must be removed from the reaction mixture before the derivatives are injected onto the column to prevent damage to the column.
- Many compounds can be derivatized directly with anhydride and solvent; however, for best results use amine bases (such as triethylamine) to react with the acidic by-products and drive the reaction toward completion.

Submicrogram Scale Preparation of Fluoroacyl Derivatives of Amines and Alcohols for ECD

Materials Required

- Dry reaction solvent such as benzene, toluene, or other suitable non-halogenated, water insoluble, volatile organic solvent.

- 0.05 M Triethylamine (TEA) in chosen solvent
- 5% Aqueous ammonia solution

Electron Capture Protocol

1. Combine <50 ng of sample dissolved in 500 µl solvent and 100 µl of 0.05 M TEA in solvent in a 5 ml Reacti-Vial™ Small Reaction Vial.
2. Add 10 µl of fluorinated anhydride. Cap vial and heat for 15 minutes at 50°C.
3. Cool to room temperature or below.
4. Add 1 ml of distilled water and shake for 1 minute.
5. Add 1 ml of aqueous ammonia solution and shake for 5 minutes.
6. Centrifuge for 1-5 minutes at high speed or until a clear upper organic layer is obtained to remove most of the water from the organic phase.
7. Inject 1-10 µl of the (upper) organic phase for ECD.

Note: With triethylamine as a catalyst, acylation of amines is quantitative within a few minutes. This method uses a 10-fold molar excess of TEA over the sample. Unlike other base catalysts such as pyridine, TEA does not cause disturbances in the chromatogram at high EC sensitivity.

Preparation of Fluoroacyl Derivatives of Phenols for FID or ECD

Materials Required

- Dry reaction solvent such as benzene, toluene, or other suitable non-halogenated, water insoluble, volatile organic solvent.
- 0.1 M Triethylamine (TEA) in the above solvent
- 1 M Phosphate buffer, pH 6.0
- 5% Aqueous ammonia solution

Flame Ionization Protocol

1. Combine 1 mg of sample dissolved in 500 µl of solvent with 200 µl of 0.1 M TEA in solvent in a 5 ml centrifuge tube or 5 ml Reacti-Vial™ Small Reaction Vial.
2. Add 25 µl of fluorinated anhydride. Cap vial and react at room temperature for 15 minutes.
3. Add 500 µl of 1 M phosphate buffer, pH 6.0, and shake for 30 seconds.
4. Centrifuge for 1-5 minutes at high speed or until a clear upper organic layer is obtained to remove most of the water from the organic phase.
5. Separate the organic phase from the aqueous phase and analyze the organic phase by GC.

Electron Capture Protocol

1. Combine sample dissolved in 500 µl of benzene with 100 µl of 0.1 M TEA in benzene in a 5 ml centrifuge tube or a 5 ml Reacti-Vial™ Small Reaction Vial.
2. Add 10 µl of fluorinated anhydride. Cap vial and react at room temperature for 10 minutes.
3. Add 500 µl of 1 M phosphate buffer, pH 6.0, and shake for 30 seconds.
4. Centrifuge for 1-5 minutes at high speed or until a clear upper organic layer is obtained to remove most of the water from the organic phase.
5. Analyze 2 µl of the organic phase by GC using the plug injection technique with 1 µl of benzene behind the sample.

Note: HFB esters of phenols are stable in the presence of water at pH <6. Alkaline extraction such as aqueous ammonia decomposes the HFB ester. If the pH is kept at or below 6, the TEA is maintained in the protonated form. Nonprotonated TEA will catalyze the decomposition of the esters.

Example Protocols for Fluorinated Acylimidazoles

Preparation of Fluoroacyl Derivatives for FID

Note: This protocol is for derivatization of amines, amides, alcohols, and phenols.

1. Combine 0.1-2 mg of sample with 200 µl of fluoroacylimidazole in a 1 ml Reacti-Vial™ Small Reaction Vial.
2. Heat at 60°C for 15-30 minutes or until reaction is complete as shown by GC analysis. (Hindered sterols may require 2-6 hours.)
3. Inject an appropriate-sized sample directly into the GC.

Milligram Scale Protocol for Preparation of HFB Derivatives of Indolealkylamines using HFBI for FID and ECD

1. Add 200 µl of HFBI to 2 mg of indolealkylamine in a 3 ml Reacti-Vial™ Small Reaction Vial. Cap vial and heat at 85°C for 1 hour.
2. Cool to room temperature.
3. Add 1 ml of water and 2 ml of toluene. Cap vial and shake vigorously.
4. Allow the phases to separate. Analyze an appropriate-sized sample of the toluene (upper) layer by GC.

   Note: A small amount of HFB acid remains in the toluene layer. If this acid interferes with the analysis, it can be removed by extracting the toluene layer 2-3 times with 0.5 ml of distilled water.

Microgram Scale Protocol for Preparation of HFB Derivatives of Indolealkylamines using HFBI for FID and ECD

1. Add 20 µl of HFBI for microgram to picogram quantities of sample isolated as residues in a Reacti-Vial™ Small Reaction Vial. Cap vial and heat reaction at 85°C for 1 hour.
2. Cool reaction to room temperature.
3. Add 2 ml of pure toluene and 0.5 ml of distilled water. Cap vial and shake vigorously for 2 minutes.
4. Remove aqueous (bottom) layer and discard.
5. Wash the toluene layer three times with 0.5 ml of cold, distilled water, discarding the washes.
6. Centrifuge for 2 minutes. Analyze an appropriate sample of the toluene layer.

Example Protocols for MBTFA

Note: MBTFA reacts quantitatively with amines at room temperature in ~30 minutes. Hydroxyl groups react more slowly, therefore, for best results heat the reaction mixture for 15-30 minutes at 60-100°C. Highly hindered compounds may require longer reaction times and higher temperatures.

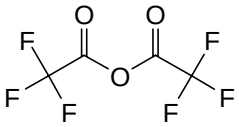
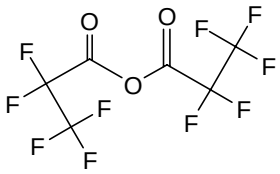
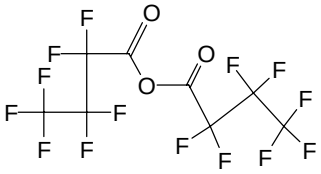
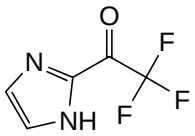
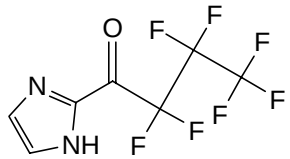
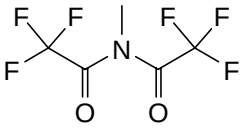
Preparation of Trifluoroacetyl Derivatives of Primary and Secondary Amines, Hydroxyls and Thiols

1. Add 100-200 µl of MBTFA to 1-10 mg of sample. If sample does not dissolve readily, add 0.5-1.0 ml of DMF, THF or acetonitrile. (MBTFA can be premixed with any of these solvents in a 1:4 ratio. Add 1 ml of the premix per 1-10 mg of sample.)
2. Cap vial and heat at 60-90°C for 10-30 minutes.
3. Cool and inject an appropriate-sized sample into the GC.

Preparation of Trifluoroacetyl Derivatives of Sugars

1. Combine 5-10 mg of dry sugar, 500 µl of MBTFA and 500 µl of pyridine in a 5 ml Reacti-Vial™ Small Reaction Vial.
2. Cap and heat at 65°C for 1 hour with occasional shaking.
3. Analyze appropriate-sized sample by GC.

Table 1. Molecular structures of acylation reagents.

Structure	Name	MW	Boiling point	d^{20}
	TFAA	210.0	39.5-40.5°C	1.490
	PFAA	310.0	69-70°C	1.571
	HFAA	410.0	106-107°C	1.665
	TFAI	164.08	38-40°C/14 mm	1.56
	HFBI	264.10	57-58°C/10 mm	1.562
	MBTFA	223.08	123-124°C	1.55

Appendix: Derivatization Overview

Chemical derivatization of a compound or a mixture of compounds is performed to make a compound suitable for analysis.[9] Often, compounds cannot be analyzed by a particular method because they are not in a form amenable to the analytical technique such as non-volatile compounds for gas chromatographic analysis[2,10]and insoluble compounds for HPLC analysis. Many materials that are not stable under the conditions of the technique are in this category.[11] The derivatization procedure modifies the chemical structure of compounds so that they can be analyzed by the desired technique.[12]

Derivatization also improves analytical efficiency.[13,14] The direct analysis of many compounds and mixtures of compounds is difficult because interactions between the compounds themselves or between the compounds and the column can lead to poor peak resolution as well as unsymmetrical peaks that make proper peak integration difficult or impractical. In many cases, conversion to derivatized products will reduce interactions interfering with analysis.[15,16] Compounds that co-elute with or that are poorly resolved from other sample components can frequently be resolved if one or more of them is converted to an appropriate derivative.[17]

Derivatization also can improve compound detectability.[18] by increasing the compound bulk or by introducing atoms or functional groups that interact strongly with the detector.[19,20] Some examples for GC applications include adding halogen

atoms for electron capture detectors[21] and the formation of TMS derivatives to produce readily identifiable fragmentation patterns and mass ions.[22]

Most analytical derivatization reactions used for gas chromatography can be classified into three general reaction types: alkylation, acylation and silylation. The ideal derivatization procedure has the following characteristics:

- Accomplish the desired modification
- Proceed quantitatively or at least reproducibly
- Produce products that are readily distinguishable and separable from starting materials
- Proceed rapidly with simple and straightforward laboratory techniques
- Be relatively selective and still applicable to a number of similar compounds
- Involve reagents and reactions that present no unusual hazards

For GC analysis, compounds containing functional groups with active hydrogens such as -COOH, -OH, $-NH_2$, -NH and -SH are of primary concern. The tendency of these functional groups to form intermolecular hydrogen bonds affects the inherent volatility of compounds containing them, their tendency to interact deleteriously with column packing materials and their thermal stability. Silylation, alkylation and acylation are all used to modify these classes of compounds.

References

1. Donike, M. (1973). Acylation with bis(acylamides). *N*-methyl-bis(trifluoroacetamide), two new reagents for trifluoroacetylation. *J. Chromatogr.* **78:**273-9.
2. Sullivan, J.E. and Schewe, L.R. (1977). Preparation and gas chromatography of highly volatile trifluoroacetylated carbohydrates using *N*-methyl-bis(trifluoroacetamide). J. Chromatogr. Sci. **15:**196-7.
3. Bennington, F. *et.al.* (1975). Identification and separation of indolealkylamines by gas-liquid chromatographic analysis of their heptafluorobutyryl derivatives. *J. Chromatogr.* **106:**435-9.
4. Borga, O. *et.al*. (1971). Quantitative determination of nortriptyline and desmethylnortriptyline in human plasma by combined gas chromatography-mass spectrometry. *J. Chromatogr.* **4(12):**837-49.
5. Seeley, S.D. and Powell, L.E. (1974). Gas chromatography and detection of microquantities of gibberellins and indoleacetic acid as their fluorinated derivatives. *Anal. Biochem.* **58:**39-46.
6. Horning, M.G. *et.al*. (1968). The GLC separation of hydroxyl-substituted amines of biological importance including the catecholamines. Preparation of derivatives for electron capture detection. *Anal. Lett*, **1(5):**311-21.
7. Donike, M. (1973). *N*-Methyl-bis(trifluoroacetamide) and bis(trifluoroacetamide). Two new reagents for trifluoroacetylation. *J. Chromatogr.* **78:**273-9.
8. Sullivan, J.E. and Schewe, L.R. (1977). Preparation and gas chromatography of highly volatile trifluoroacetylated carbohydrates using N-methyl-bis(trifluoroacetamide). *J. Chromatogr. Sci*. **15:**196-7.
9. Knapp, D.R. (1979). Handbook of Analytical Derivatization Reactions, John Wiley & Sons, New York.
10. Brittain, G.D. and Schewe, L. (1971). In Recent Advances in Gas Chromatography; Domsky, I.I., Perry, J.A., Eds., Marcel Decker; New York, N.Y.
11. Hallgren, B. and Larsson, S. (1962). The glyceryl ethers in the liver oils of elasmobranch fish. *J. Lipid Res.* **3(1):**31-8.
12. Samuelsson, K. and Samuelsson, B. (1969). Gas-liquid chromatography-mass spectrometry of cerebrosides as trimethylsilyl ether derivatives. *Biochem. Biophys. Res. Commun.* **37(1):**15-21.
13. Khalifa, S. and Mumma, R.O. (1972). Gas chrromatographic separation of the aglycone metabolites of carbaryl. *J. Agr. Food Chem.* **20(3):**632-4.
14. Sinsheimer, J.E. and Smith, R.V. (1967). Methods for the qualitative and quantitative analysis of some hydroxystilbenes. J. Pharm. Sci. 56, 1280.
15. Langer, M. *et.al*. (1960). Chem. Ind.(London), 378.
16. Neeman, M., *et al* (1959). *Tetrahedron*, **6:**36.
17. Marfey, P. (1984). Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. *Carlsberg Res. Commun.* **49:**591-596.
18. Poole, C.F. (1976). Chem. Ind. 479.
19. Ehrsson, H., *et al.* (1971). Quantitative gass chromatographic determination of picogram quantities of phenols. *Acta. Pharm. Suecica*. **8:**319-328.
20. Koshy, K.T. *et.al*. (1975). O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride as a sensitive derivatizing agent for the electron capture gas liquid chromatographic analysis of keto steroids. *J. Chrom. Sci.* **13:**97-103.
21. Walle, T. and Ehrsson, H. (1970). *Acta Pharm. Suecica.* **7:**389-406.
22. Kelly, R.W. and Taylor, L. (1976). tert-Butyldimethylsilyl ethers as derivatives for qualitative analysis of steroids and prostaglandins by gas phase methods. *Anal. Chem.* **48(3):**465.