5

Derivatization Reactions and Reagents for Gas Chromatography Analysis

Francis Orata

Masinde Muliro University of Science and Technology,
Kenya

1. Introduction

Derivatization reactions are meant to transform an analyte for detectability in Gas Chromatography (GC) or other instrumental analytical methods. Derivatization in GC analysis can be defined as a procedural technique that primarily modifies an analyte's functionality in order to enable chromatographic separations. A modified analyte in this case will be the product, which is known as the derivative. The derivative may have similar or closely related structure, but not the same as the original non-modified chemical compound.

Volatility of sample is a requirement for GC analysis. Derivatization will render highly polar materials to be sufficiently volatile so that they can be eluted at reasonable temperatures without thermal decomposition (Knapp, 1979) or molecular re-arrangement (Kühnel et al., 2007; Blau and King 1979). Understanding the chemistry of the analytes, derivatizing reagents used in sample preparation, and the detailed functionality of Gas Chromatography are important to get reliable results. For GC analysis, compounds containing functional groups with active hydrogens such as -SH, -OH, -NH and -COOH are of primary concern because of the tendency of these functional groups to form intermolecular hydrogen bonds (Zaikin and Halket, 2003). These intermolecular hydrogen bonds affect the inherent volatility of compounds containing them, their tendency to interact with column packing materials and their thermal stability (Sobolevsky et al., 2003). Since GC is used to separate volatile organic compounds, modification of the functional group of a molecule by derivatization enables the analysis of compounds that otherwise can not be readily monitored by GC. Derivatization process either increases or decreases the volatility of the compound of interest. It also reduces analyte adsorption in the GC system and improves detector response, peak separations and peak symmetry.

In addition to particular analytes such as pharmaceuticals, biomolecules such us organic acids, amides, poly-hydroxy compounds, amino acids, pesticides and other persistent organic compounds, new classes of compounds of interest for example fluorinated alkylated substances and polycyclic aromatic hydrocarbons continue to emerge. It is necessary to develop and/or improve on chemical analytical methods and hence the need to familiarize with derivatization methods that are applicable to GG analysis. Generally derivatization is aimed at improving on the following aspects in Gas Chromatography**.**

i.   Suitability

Suitability is the form of compounds that is amenable to the analytical technique. For GC, it is a requirement that the compound to be analyzed should be volatile with respect to gas chromatographic analysis conditions, as compared to liquid chromatography (LC), where the compound of interest should be soluble in the mobile phase. Therefore, derivatization procedure modifies the chemical structure of the compounds so that they can be analyzed by the desired technique.

ii.   Efficiency

Efficiency is the ability of the compound of interest to produce good peak resolution and symmetry for easy identification and practicability in GC analysis. Interactions between the compounds themselves or between the compounds and the GC column may reduce the separation efficiency of many compounds s and mixtures (Knapp, 1979). Derivatization of analyte molecules can reduce these interactions that interfere with analysis. Also, compounds that co-elute or have poor resolution from other sample components during separation in GC can frequently be resolved by an appropriate derivative.

iii.   Detectability

Detectability is the outcome signal that emanates from the interaction between the analyte and the GC detector. Increasing the amounts of materials will impact the range at which they can be detected in Gas chromatography. This can be achieved either by increasing the bulk of the compound or by introducing onto the analyte compound, atoms or functional groups that interact strongly with the detector and hence improve signal identification. For example the addition of halogen atoms to analyte molecules for electron capture detectors (ECD) and the formation of trimethylsilyl (TMS) ether derivatives to produce readily identifiable fragmentation patterns and mass ions (Knapp, 1979).

1.1 Derivatization reagent

Derivatization reagent is the substance that is used to chemically modify a compound to produce a new compound which has properties that are suitable for analysis in GC or LC. The following criteria must be used as guidelines in choosing a suitable derivatization reagent for GC analysis.

i.   The reagent should produce more than 95 % complete derivatives.
ii.   It should not cause any rearrangements or structural alterations of compounds during formation of the derivative.
iii.   It should not contribute to loss of the sample during the reaction.
iv.   It should produce a derivative that will not interact with the GC column.
v.   It should produce a derivative that is stable with respect to time.

1.2 Objectives for derivatization

The following outlined objectives among others can be achieved by application of proper derivatization procedures;

i.   Improvement of resolution and reduce tailing of polar compounds which may contain –OH, –COOH, =NH, –NH$_2$, –SH, and other functional groups.

ii. Analysis of relatively nonvolatile compounds.
iii. Reduction of volatility of compounds prior to GC analysis.
iv. Improvement of analytical efficiency and hence increase detectability.
v. Stabilization of compounds for GC analysis.

2. Types of derivatization reactions

Derivatization reactions used for gas chromatography (GC) fall into three general reaction types namely; Alkylation of which the general process is esterification, Acylation and Silylation. Through these three processes, highly polar materials such as organic acids, amides, poly-hydroxy compounds, amino acids are rendered suitable for GC analysis by making them sufficiently volatile. These general processes are discussed below.

2.1 Alkylation

Alkylation is mostly used as the first step for further derivatizations or as a method of protection of certain active hydrogens in a sample molecule. It represents the replacement of active hydrogen by an aliphatic or aliphatic-aromatic (e.g., benzyl) group in process referred to as esterification. Equation 1 below shows the general reaction equation representing the esterification process.

$$RCOOH + PhCH_2X \rightarrow RCOOCH_2Ph + HX$$
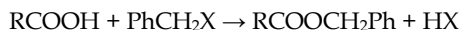
Equation 1: General reaction for esterification process: X = halogen or alkyl group R, H = another alkyl group R.

The principal chromatographic use of this reaction is the conversion of organic acids into esters, especially methyl esters that produce better chromatograms than the free acids. Alkylation reactions can also be used to prepare ethers, thioethers and thioesters, N-alkylamines, amides and sulphonamides (Danielson, 2000). In general, the products of alkylation are less polar than the starting materials because active hydrogen has been replaced by an alkyl group. The alkyl esters formed offer excellent stability and can be isolated and stored for extended periods if necessary. In esterification an acid reacts with an alcohol to form an ester. In the reaction, a catalyst more often an inorganic acid such as hydrochloric acid or thionyl chloride (Zenkevich, 2009) is recommended for example, in the trans-esterification of fats or oils (Sobolevsky et al., 2003).

2.1.1 Derivatization reagents used in alkylation

Common derivatization reagents for the Alkylation type of reactions are Dialkylacetals, Diazoalkales, Pentafluorobenzyl bromide (PFBBr), Benzylbromide, Boron trifluoride (BF_3) in methanol or butanol and Tetrabutylammonium hydroxide (TBH) among others. Alkylation reagents can be used alone to form esters, ethers and amides or they can be used in conjunction with acylation or silylation reagents. The reaction conditions can vary from strongly acidic to strongly basic with both generating stable derivatives. However, it must be noted that the reagents are more limited to amines and acidic hydroxyls and that the reaction conditions are frequently severe while the reagents are often toxic. Some derivatization reagents and their respective derivatization procedures in alkylation reactions are discussed below.

2.1.1.1 Dialkylacetals

Dimethylformamide (DMF) is an example of dialkylacetals with a general formula $CH_3CH_3NCHOROR$ are used to esterify acids to their methyl esters. Dialkylacetals have a wider applicability for the derivatization of a number of functional groups containing reactive hydrogens. Because the principal reaction product is dialkylacetals (DMF), the isolation of the derivative is not required and the reaction mixture can be injected directly into the gas chromatograph (Regis, 1999). This reagent is an excellent first choice for derivatization of a compound for which there is no published method available. The reaction between N, N-dimethylformamide dimethylacetal and Carboxylic acid is as follows (Equation 2).

$$CH_3CH_3NCHOROR + R`COOH \rightarrow R`COOR + ROH + CH_3CH_3NCHO$$
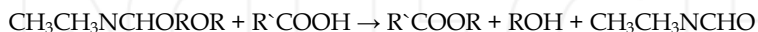
Equation 2: The reaction between N, N-dimethylformamide dimethylacetal and Carboxylic acid.

Although carboxylic acids, phenols, and thiols react quickly with DMF, to give the corresponding alkyl derivatives, hydroxyl groups are not readily methylated. During derivatization procedure, care should be taken because *N*, *N*-dimethylformamide dimethylacetals are moisture sensitive. The reagents work quickly with derivatization taking place just upon dissolution. The reaction is suitable for flash alkylation, where derivatization takes place in the injection port. Since adjustment of polarity and volatility of the sample is possible, this can allow change of retention time. It is worth noting that the reagents will react with water to give the corresponding alcohol though traces of water will not affect the reaction as long as you have an excess of the acid.

A rapid procedure for the derivatization of both carboxylic and amino acids using DMF-Dialkylacetal reagents is by dissolving the sample in 0.5 mL of a 1:1 solvent of choice/ reagent mixture by heating to 100 °C. For example, it is advisable to use pyridine for fatty acids, acetonitrile for amino acids as solvent of choice (Thenot and Horning, (1972).

Alternative method comprises of combining 50 mg fatty acid and 1 ml DMF in a reaction vial. Cap the vial and heat at 60 °C for 10 - 15 minutes or until dissolution is complete and analyze the cooled sample in gas chromatography (Thenot et al., 1972).

2.1.1.2 Diazoalkales

The main reagent in the diazoalkales group is diazomethane. Diazomethane (N_2CH_2) is the quickest and cleanest method available for the preparation of analytical quantities of methyl esters. The reaction of diazomethane with a carboxylic acid is quantitative and essentially instantaneous in ether solutions. In the presence of a small amount of methanol as catalyst, diazomethane reacts rapidly with fatty acids, forming methyl esters. Elimination of gaseous nitrogen drives the reaction forward. The reaction for the conversion of carboxylic acids to methyl esters (Equation 3) is outlined below:

$$RCOOH + \cdot CH_2N^+N \rightarrow RCOOCH_3 + N_2$$
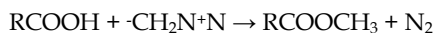
Equation 3: The reaction for the conversion of carboxylic acids to methyl esters

The yield is high and side reactions are minimal. Sample sizes of 100 - 500 µl are easily derivatized and the isolation of the methyl esters is simple and quantitative when dealing

with acids having chain lengths from C8 to C24. However, care should be taken in handling diazomethane because it is carcinogenic, highly toxic, and potentially explosive.

2.1.1.3 Pentafluorobenzyl bromide (PFBBr) and Pentafluorobenzyl-hydroxylamine hydrochloride (PFBHA)

Pentafluorobenzyl bromide ($C_7H_2F_5Br$) and also Pentafluorobenzyl-hydroxylamine hydrochloride can be used to esterify phenols, thiols, and carboxylic acids. Equation 4 below is an example for PFBBr derivatization process.

$$R`OR + C_7H_2F_5Br \rightarrow R`OC_7H_2F_5 + RBr$$
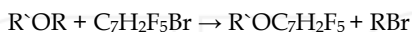
Equation 4: Reaction of PFBBr with either phenols, thiols or carboxylic acids: R = Hydrogen
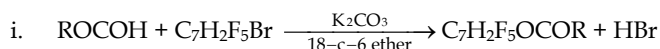
Derivatization can be done with O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride (PFBHA) for carbonyls and pentafluorobenzyl bromide (PFBBr) for carboxylic acid and phenol groups for gas (GC/MS) in an electron impact mode (EI) and a gas chromatograph/ion trap mass spectrometry (GC/ITMS) in both chemical impact and EI modes. To confirm different isomers, the PFBHA-derivatives of analytes can be rederivatized by silylation using N, O-bis (trimethylsilyl)-trifluoroacetamide (BSTFA) (Jang and Kamens, 2001).
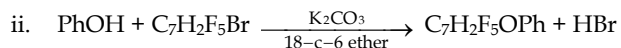
Example for the application of this method is from the work of Allmyr et al., (2006) where the analysis of Triclosan in human plasma and milk was accomplished by the conversion of Triclosan into its pentafluorobenzyl ester by adding 2 ml of H_2O (Milli-Q), 50 µl of 2M KOH, 10 µl pentafluorobenzyl chloride in 10% toluene and 0.3 g NaCl (more sodium chloride was added if emulsion formed upon mixing) to the sample extract and shaking the tube vigorously for 2 minutes. This was followed by extraction of the aqueous phase with 2 ml of n-hexane. Then, 3 ml of 98 % H_2SO_4 was added to the extract, and the tube inverted 60 times and another extraction using 2 ml of n-hexane followed. The final extract was reduced to 2 ml under a gentle stream of nitrogen gas at room temperature. Approximately 0.5 ml of the extract was injected to GC for analysis.

i. Organic Acids derivatisation

The following procedure by Chien et al., (1998) and Galceran et al., (1995) is a variation of methods previously utilized for organic acid and phenol analysis. In the method, sample extracts for organic acid/phenol derivatization are first dried by passing a gentle stream of nitrogen in the sample. Once the solvent has been evaporated, acetone is added to bring each sample to a volume of 500 µL. Each sample is added 20 µL of 10% PFBBr solution and 50 µL of 1,4,7,10,13,16-hexaoxacyclooctadecane $[C_2H_4O]_6$ (18-crown-6 ether solution), both in acetone. Approximately 10 mg of potassium is added to each extract, and the extracts are capped and sonicated for three hours. Upon completion of sonication the acetone is evaporated by passing a gentle stream of nitrogen, and the residue dissolved into hexane for GC analysis.

Equation 5 below shows the chemical reactions for the conversion of organic acids and phenols into their pentafluorobenzyl esters and ether respectively using pentafluorobenzyl bromide (PFBBr) derivatization.

$$i. \quad ROCOH + C_7H_2F_5Br \xrightarrow[\text{18-c-6 ether}]{K_2CO_3} C_7H_2F_5OCOR + HBr$$

ii. $PhOH + C_7H_2F_5Br \xrightarrow[\text{18−c−6 ether}]{\text{K}_2\text{CO}_3} C_7H_2F_5OPh + HBr$

Equation 5: Reactions for the conversion of organic acids and phenols into their pentafluorobenzyl esters (i) and ether (ii) respectively using pentafluorobenzyl bromide (PFBBr).

In another example of pentafluorobenzylation procedure (Naritisin and Markey, 1996 & Kawahara, 1968), 20 µl of PFBBr is added to the vial containing already extracted and cleaned sample in methyl chloride. The vial is then capped and shaken for 20 – 30 minutes. Gas chromatography analysis can then be accomplished using either of the two methods depending on the available detector. A portion of methylene chloride phase can be injected into the chromatograph for FID analysis or it can be evaporated using a gentle stream of nitrogen gas and re-dissolve in benzene for ECD analysis. Reagents containing fluorinated benzoyl groups are good for ECD analysis.

ii.   Carbonyls

The derivatization process performed by Spaulding et al., (2003), Destaillats et al., (2001) & Rao et al., (2001) for the analysis of carbonyl involves first, the reduction in volume of sample extracts to < 50 µL under a gentle stream of nitrogen. Once the extract volume has been reduced, a 9:1 (v/v) mixture of carbonyl-free Acetonitrile: Dichloromethane (DCM) is added to bring the sample to a volume of 500 µL. This is followed by the addition of 50 mgmL^{-1} solution of O-(2, 3, 4, 5, 6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA) in methanol that will result to a target PFBHA concentration of 5 mM. The sample is left at room temperature for a period of 24 hours then subsequently analyzed using GC. The balanced chemical reaction where O-(2, 3, 4, 5, 6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA) is used for the conversion of carbonyls into their pentafluorobenzyl oximes is provided below (Equation 6).

$$R´OCR´´ + C_6F_5\text{-}ONH_2 \rightarrow C_6F_5\text{-}ONR´CR´´ + Isomer(s) + H_2O$$
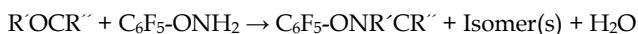
Equation 6: Chemical reaction for the conversion of carbonyls into pentafluorobenzyl oximes using O-(2, 3, 4, 5, 6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA).

The PFBHA derivatization process for most of the carbonyl compounds could be completed in 2 h at room temperature (Bao et al., 1998).

2.1.1.4 Benzylbromide

Benzyl bromide reacts with the acid part of an alkyl acid to form an ester, and therefore increase the volatility of the analyte of interest. Orata et al., (2009) determined long chain perfluorinated acids namely; perfluoro-n-octanoic acid (PFOA), 2H-perfluoro-2-octenoic acid (FHUEA), 2H-perfluoro-2-decenoic acid (FOUEA) and 2H-perfluoro-2-dodecenoic acid (FNUEA) in biota (fish) and abiota (water) by derivatization and subsequent analysis by GC/MS. The method involved derivatization of long chain perfluorinated compounds using benzyl bromide solution and acetone to form benzylperfluorooctanoate (benzyl ester) as presented by the equation 7 below which shows the modification of perfluorooctanoic acid to the respective ester.

$$CF_3(CF_2)_6COOH + C_7H_7Br \rightarrow C_{15}H_7F_{15}O_2 + HBr$$
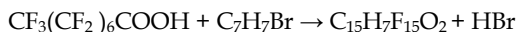
Equation 7: Convertion of Perfluorooctanoic acid to Benzylperfluorooctanoate using benzyl bromide.

Long Chain Perfluorinated alkyl acids derivatization procedure by Orata et al (2009) is as follows: Following extraction and sample clean up procedure, the sample is dried by a gentle stream of nitrogen. The sample residue is then dissolved in 0.5 mL of acetone and 0.1 mL of benzyl bromide solution is added to it. The tube is heated at 80 °C for 15 min, cooled, and evaporated to dryness with nitrogen. The residue is then dissolved in 1 mL of methylene chloride for GC analysis. Results obtained in the study (Orata et al, 2009) demonstrate that GC/MS can be an alternative to the LC/MS method for quantification of perfluorinated acids in contaminated areas, where expected higher concentration of the analyte is expected. It was in the view of the author (Orata et al, 2009) that the method can be further improved to lower the detection limits.

2.1.1.5 Tetrabutylammonium hydroxide (TBH)

Derivatization of a carboxylic acid with tetrabutylammonium hydroxide (TBH) forms butyl ester, which will allow a longer retention times in a GC column (Lin et al., 2008). The reagent is most commonly used for low molecular weight acids and is especially suitable for low molecular weight amines (Regis, 1999). Equation 8 represents the derivatization reaction for the conversion of carboxylic acid to alkyl esters using TBH.

$$[N(CH_3)_4]^+[OH]^- + RCOOH \rightarrow RCOOC_4H_9$$

Equation 8: Conversion of carboxylic acid to alkyl esters using TBH.

The following derivatization procedure can be used for flash alkylation which is suitable for biological fluids and thermally stable fatty acids analysis. Biological fluid or tissue is first extracted using toluene. Then 4 mL of the extract is transferred into a nipple tube and evaporate under a gentle stream of nitrogen gas at 60 °C. 25 µL of TBH solution is added to dissolve the residue. After 30 minutes, 4 µL of the dissolved residue is injected directly onto the chromatograph. The injection port temperature is set at 260 °C or above in GC instrument.

2.1.1.6 Boron trifluoride (BF3) in methanol or butanol

Boron trifluoride (BF$_3$) in methanol or n-Butanol has a general formula is F$_3$B: HO–C$_n$H$_{2n+1}$ where (n = 1 or 4). This reagent is convenient and inexpensive method for forming esters. It is most commonly used to form methyl (butyl) ester by reacting it with acids, as shown by the following general equation 9.

$$F_3B: HO–C_nH_{2n+1} + RCOOH \rightarrow RCOOC_nH_{2n+1}$$

Equation 9: General equation for formation of alkyl esters using Boron trifluoride (BF$_3$) as a derivatization reagent.

Boron trifluoride reagent forms fluoroboron compounds by reaction with atmospheric oxygen and methanol and therefore making it prone to instability (Christie 1993). Therefore, it should always be stored at the refrigerator temperature discarded after a few months use.

In a study by Lough (1964) were polyunsaturated fatty acids were analyzed, it was observed that boron trifluoride-methanol will cleave the rings in cyclopropane fatty acids (commonly encountered in microorganisms), and it causes cis-trans isomerization of double bonds in conjugated fatty acids. In addition, it reacts with the antioxidant Butylated hydroxytolune (BHT) to produce spurious peaks in chromatograms.

The following esterification procedure can be used in derivatization using Boron trifluoride. To a 100 mg organic acid in a vial, 3 mL BF_3 in methanol or BF_3 in n-butanol is added and heated to 60 °C for 5 to 10 minutes. Heating temperature and time may vary i.e. Ribeiro et al., (2009) used 90 °C for 10 minutes for the process. This is followed by cooling and transferring the mixture to a separating funnel with 25 mL hexane. Further, the sample is washed 2 times with saturated NaCl solution, and then dried over anhydrous Na_2SO_4. The solvent is evaporated to concentrate the sample and lastly injected onto column for GC analysis.

2.2 Silylation

Silylation is the most prevalent derivatization method as it readily volatates the sample and therefore very suitable for non-volatile samples for GC analysis. Silylation is the introduction of a silyl group into a molecule, usually in substitution for active hydrogen such as dimethylsilyl $[SiH(CH_3)_2]$, t-butyldimethylsilyl $[Si\ (CH_3)_2C(CH_3)_3]$ and chloromethyldimethylsilyl $[SiCH_2Cl(CH_3)_2]$. Replacement of active hydrogen by a silyl group reduces the polarity of the compound and reduces hydrogen bonding (Pierce, 1968). Many hydroxyl and amino compounds regarded as nonvolatile or unstable at 200 – 300 °C have been successfully analyzed in GC after silylation (Lin et al., 2008 & Chen et al., 2007). The silylated derivatives are more volatile and more stable and thus yielding narrow and symmetrical peaks (Kataoka, 2005).

2.2.1 Silylation reaction and mechanism

The silylation reaction is driven by a good leaving group, which means a leaving group with a low basicity, ability to stabilize a negative charge in the transitional state, and little or no back bonding between the leaving group and silicon atom (Knapp, 1979). The mechanism involves the replacement of the active hydrogens (in -OH, -COOH, -NH, -NH$_2$, and -SH groups) with a trimethylsilyl group. Silylation then occurs through nucleophilic attack (SN$_2$), where the better the leaving group, the better the siliylation. This results to the production of a bimolecular transition state (Kühnel et al., 2007) in the intermediate step of reaction mechanism. The general reaction for the formation of trialkylsilyl derivatives is shown by equation 10. The leaving group in the case of trimethylchlorosilane (TMCS) is the Cl atom.
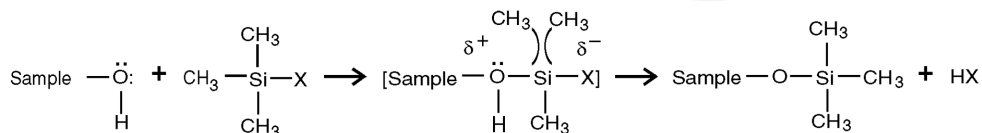
Equation 10: General reaction mechanism for the formation of trialkylsilyl derivatives for trimethylchlorosilane, X = Cl

In silylation derivatisation, care must be taken to ensure that both sample and solvents are dry. Silyl reagents generally are moisture sensitive, and should be stored in tightly sealed containers (Sobolevsky et al., 2003) and therefore the solvents used should be as pure and as little as possible. This will eliminate excessive peaks and prevent a large solvent peak. In silylation, pyridine is the most commonly used solvent. Although pyridine may produce peak tailing, it is an acid scavenger and will drive the reaction forward. In many cases, the need for a solvent is eliminated with silylating reagents. The completion of the derivatization process in silylation is usually observed when a sample readily dissolves in the reagent. According to Regis (1999) the ease of reactivity of the functional group toward silylation follows the order:

Alcohol > Phenol > Carboxyl > Amine > Amide /hydroxyl

For alcohols, the order will be as follows:

Primary > Secondary > Tertiary

Many reagents will require heating that is not in excess of 60 °C for about 10 - 15 minutes, to prevent breakdown of the derivative. Although hindered products may require long term heating.

2.2.2 Derivatization reagents used in Silylation

Reagents used for the silylation derivatization process include Hexamethyldisilzane (HMDS), Trimethylchlorosilane (TMCS), Trimethylsilylimidazole (TMSI), Bistrimethylsilylacetamide (BSA), Bistrimethylsilyltrifluoroacetamide (BSTFA), N-methyl-trimethylsilyltrifluoroacetamide (MSTFA), Trimethylsilyldiethylamine (TMS-DEA), N-methyl-N-t-butyldimethylsilyltrifluoroacetamide (MTBSTFA), and Halo-methylsilyl derivatization reagents. Halo-methylsilyl derivatization reagents which although not discussed in this chapter can produce both silylated and halogenated derivatives for ECD. Silyl reagents will react with both alcohols and acids to form trimethylsilyl ethers and trimethylsilyl esters respectively. These derivatives formed are volatile and for the most part, are easily separated (Scott, 2003). Silyl reagents are compatible with most detection systems but, if they are used in excess, they can cause difficulties with flame ionization detectors (FID) (Sobolevsky et al, 2003). Silyl reagents are influenced by both the solvent system and the addition of a catalyst. A catalyst (e.g., trimethylchlorosilane or pyridine) increases the reactivity of the reagent.

Reagents that introduce a t-butyldimethylsilyl group in place of the trimethylsilyl group were developed to impart greater hydrolytic stability to the derivatives. These t-butyldimethylsilyl derivatives not only have improved stability against hydrolysis, but they also have the added advantage of distinctive fragmentation patterns, which makes them useful in a variety of GC/MS applications. Most trimethylsilyl and t-butyldimethylsilyl derivatives have excellent thermal stability and are amenable to a wide range of injection and column conditions (Pierce 2004). Silylation gives ability to derivatize a wide variety of compounds for GC analysis. In addition a large number of silylating reagents available.

2.2.2.1 Bis(trimethylsilyl)–acetamide (BSA)

Bis(trimethylsilyl)–acetamide (BSA) was the first widely used silylating reagent. The strength of BSA as a strong silylating reagent is enhanced more because acetamide is a good leaving group. BSA reacts under mild conditions and produces relatively stable by-products. However, the by-product which is trimethylsilyl -acetamide sometimes produces peaks that overlap those of other volatile derivatives as shown in the equation 11 below.
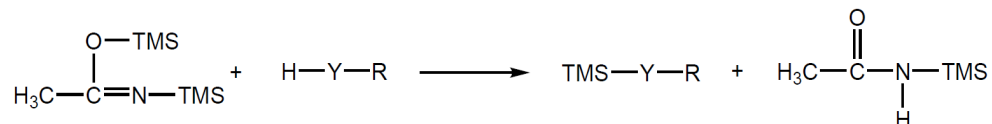
Equation 11: Equation showing the by-product which is trimethylsilyl –acetamide from Bis(trimethylsilyl)–acetamide silylation reagent: TMS = $Si(CH_3)_3$, Y = O, S, NH, NR`, COO, R, R` = Alk, Ar.

BSA forms highly stable trimethylsilyl derivatives with most organic functional groups under mild reaction conditions but their mixtures also oxidize to form silicon dioxide, which can foul FID detectors. Experimentally, BSA has been found to be very efficient and only a small amount of reagent is required. Moreso the derivatization process takes a shorter time at room temperature. Saraji and Mirmahdieh, (2009) studied the influence of different parameters on the in-syringe derivatization process using BSA were three parameters, the amount of the BSA, the reaction time and the reaction temperature were investigated to achieve the highest derivatization reaction yield. The results showed that 0.2 µL volume of BSA derivatization reagent is enough to obtain the maximum derivatization reaction yield for all compounds in 2 min time at room temperature.

2.2.2.2 Bis(trimethylsilyl)trifluoroacetamide (BSTFA)

N, N–bis(trimethyl–silyl)trifluoro-acetamide (BSTFA) just like bis(trimethylsilyl)–acetamide (BSA) are the two most popular reagents for Silylation type of derivatization. They react rapidly with organic acids to give high yields. Gerhke, (1968) developed a method in which a few milligram of an acid is placed in a vial and about 50 µl of BSA or BSTFA is added to it. The reaction can be expected to be complete directly within the solution, but the mixture can also be heated for 5 to 10 min. at 60 °C to ensure that reaction is really complete, before GC analysis. BSTFA reacts faster (as shown using the reaction 12 below) and more completely than BSA, due to presence of trifluoroacetyl group.
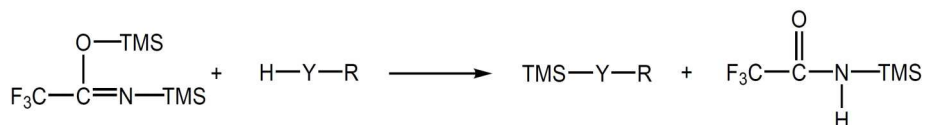
Equation 12: Silylation reaction using N, N–bis(tri methyl–silyl)trifluoro-acetamide: TMS = $Si(CH_3)_3$, Y = O, S, NH, NR`, COO, R, R` = Alk, Ar.

The high volatility of BSTFA and its byproducts results in separation of early eluting peaks. In addition, its stable products result in low detector noise and fouling. Addition of

trimethylchlorosilane (TMCS) catalyzes reactions of hindered functional groups in secondary alcohols and amines.

In the method used by Schauer et al., (2002), sample extracts for analysis of hydroxylated polycyclic aromatic hydrocarbons (Hydroxy-PAHs), the sample extracts are first reduced in volume to 200 µL under a gentle stream of nitrogen. To each sample 20 µL of freshly prepared solution of 10% (v/v) Trimethylchlorosilane (TMCS) in N-O-bis (trimethylsilyl)-trifluoroacetamide (BSTFA) is added. The samples are capped, wrapped with Teflon tape, and heated at 45 °C for 24 hours to convert the targeted analytes to their trimethylsilyl derivatives. The balanced reaction (Equation 13) for this conversion to trimethyl silyl (TMS) ethers using N-O-bis (trimethylsilyl)-trifluoroacetamide (BSTFA) is provided in below.
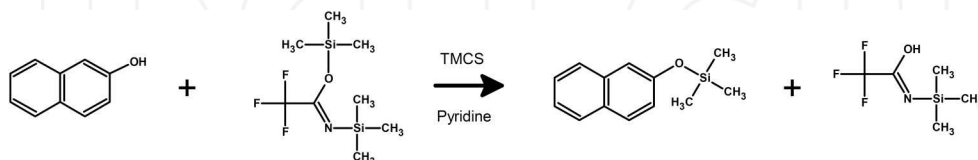
Equation 13: Derivatization of Hydroxylated polycyclic aromatic hydrocarbons (Hydroxy-PAHs) into trimethyl silyl (TMS) ethers using BSTFA.

Caution must be exercised to prevent any water from entering the samples as this will lead to hydrolysis of the BSTFA reagent and prevent any of the targeted analytes from undergoing derivatization.

Szyrwińska et al., (2007) and Kuo and Ding, (2004) used N, O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), and bromoacetonitrile (BAN) reagents to analyze standard solutions of Bisphenol-A (BPA) and extracts of powdered milk.

The method was by derivatization using Trimethylsilylation with the derivatizing reagent which was BSTFA + 1% TMCS. Bisphenol-A standard solution (200 µL) was placed in a vial (1 mL) and evaporated to dryness under a gentle stream of nitrogen at 60 °C. Silylating agent (BSTFA containing 1 % TMCS; 100 µL) was added to the residue and the vial was vortex mixed and heated at 80 °C for 30 min. After cooling, the derivatized solution was evaporated to dryness and the residue was re-dissolved in 100 µL chloroform. This solution (1 µL) was analyzed by GC–MS.

2.2.2.3 N-methyl-trimethylsilyltrifluoroacetamide (MSTFA)

N-methyl-trimethylsilyltrifluoroacetamide (MSTFA) is the most volatile of the trimethylsilyl acetamides. It is most useful for the analysis of volatile trace materials where the derivatives may be near the reagent or by-product peak. The general equation of the reaction (Equation 14) using MSTFA is shown below;
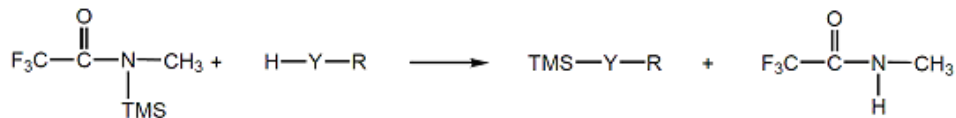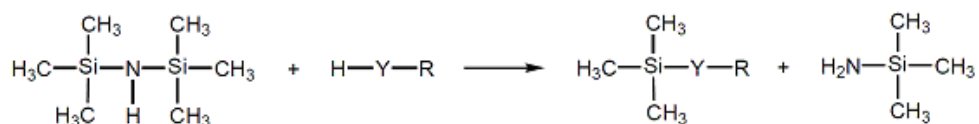

Equation 14: Silylation reaction using N-methyl-trimethylsilyltrifluoroacetamide (MSTFA): TMS = Si(CH$_3$)$_3$, Y = O, S, NH, NR`, COO, R, R` = Alk, Ar.

Silylation derivatization procedures by Butts, (1972) using BSTFA, BSA, or MSTFA formed the basis for derivatisation application that was used to analyze over 200 organic compounds including carboxylic acids, amines, alcohols, phenols, and nucleic acids. The basis of the procedure is as follows: to a 1 - 5 mg sample, 100 µL each of pyridine as a solvent and silylating reagent are added. The mixture is capped and heated to 60 °C for 20 minutes. For moderately hindered or slowly reacting compounds, BSTFA is recommended for use with 1 or 10 % TMCS catalyst. Under extreme conditions compounds may require heating for up to 16 hours. When Ketones are derivatized using this procedure, they may form 15 – 20 % enol trimethylsilyl esters. The esters can be eliminated by first forming a methoxime. In the case of Amino acids the reaction may be required to be in a sealed tube or vial (Butts, 1972). The samples are heated cautiously, near the boiling point of the mixture until a clear solution is obtained.

2.2.2.4 Hexamethyldisilzane (HMDS)

Hexamethyldisilzane (HMDS) is a weak donor, as it has symmetry. If it is used for derivatization, it will attack only easily silylated hydroxyl groups. Equation 15 shows the reaction using hexamethyldisilzane.
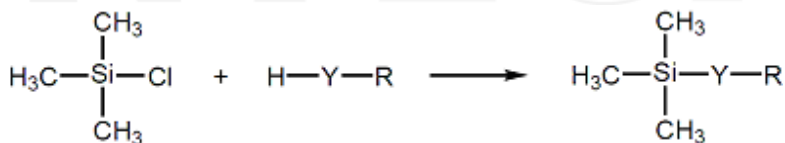

Equation 15: Silylation reaction using Hexamethyldisilzane (HMDS): Y = O, S, NH, NR`, COO, R, R` = Alk, Ar.

Hexamethyldisilzane is a weak TMS donor that can be used for silylation of carbohydrates. It can be used as mixture with pyridine and trifluoroacetic acid. The derivatization procedure is simple as shown in this procedure where 2 ml of hexamethyldisilazane, 2 ml of pyridine and 175 µL of trifluoroacetic acid are added to the samples. Silylation is then performed at 60 °C for 1 hour.

2.2.2.5 Trimethylchlorosilane (TMCS)

Trimethylchlorosilane (TMCS) is also a weak donor. In addition, it produces hydrochloric acid as a by product with is acidic. It is therefore not commonly used. However, it is often found as a catalyst to increase TMS donor potential. An example of derivatization reaction using Trimethylchlorosilane (TMCS) is shown in equation 16.

Equation 16: Silylation reaction using Trimethylchlorosilane (TMCS): Y = O, S, NH, NR`, COO, R, R` = Alk, Ar.

2.2.2.6 Trimethylsilylimidazole (TMSI)

Trimethylsilylimidazole (TMSI) is not a weak donor, but it is selective as it reacts with alcohols and phenols but not amines or amides (nitrogen groups). Since it is selective, it will target the hydroxyls in wet sugars and also derivatize the acid sites of amino acids. It will leave the amino group free for fluorinated derivatization. An example of reaction equation using TMSI is shown below (Equation 17).
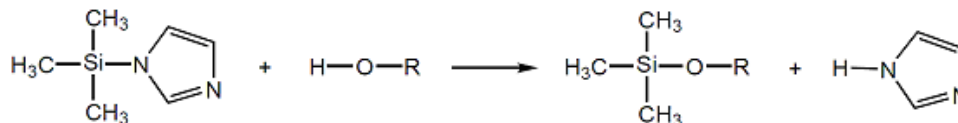
Equation 17: Silylation reaction using Trimethylsilylimidazole (TMSI): TMS = R, R` = Alk, Ar.

The derivatives produced are suitable for ECD analysis. Isoherranen and Soback, (2000) used the following derivatization procedure for the determination of Gentamicin. In the procedure, the sample solution was evaporated to dryness under a stream of nitrogen in an auto sampler vial and the dry residue was dissolved in 50 ml of anhydrous pyridine. To the pyridine solution, 100 ml of TMSI were added and the vial was closed and incubated for 15 min at 60 °C. Thereafter, 70 ml of Trifluoroacetic anhydride (TFAA) were added and the vial was closed and incubated for 60 min at 60 °C.

2.2.2.7 Trimethylsilyldiethylamine (TMS-DEA)

Trimethylsilyldiethylamine (TMS-DEA) reagent is used for derivatizing amino acids, antibiotics, urea-formaldehyde condensates, steroids and carboxylic acids and it also targets hindered compounds. Hydrolysis of TMS derivatives and reagents produces hexamethyldisiloxane $[(CH_3)_3SiOSi(CH_3)]$. Hexamethyldisiloxane is quite inert and does not interfere in the reaction or produce byproducts with the sample. The reaction by product diethylamine is very volatile and the reaction can be driven to completion by evaporating the diethylamine produced. Equation 18 shows the derivatisation reaction using TMS-DEA reagent.

$$TMS\text{-}N\ (C_2H_5)_2 + H\text{-}Y\text{-}R \rightarrow TMS\text{-}Y\text{-}R + H\text{-}N\ (C_2H_5)_2$$

Equation 18: Silylation reaction using Trimethylsilyldiethylamine (TMS-DEA) reagent: TMS = $Si(CH_3)_3$, Y = O, S, NH, NR`, COO, R, R` = Alk, Ar.

Because of its high volatility, it is eluted with the solvent or reagent and usually does not interfere with the chromatogram.

2.2.2.8 N-methyl-N-t-butyldimethylsilyltrifluoroacetamide (MTBSTFA)

Silylation derivatisaion using N-methyl-N-t-butyldimethylsilyltrifluoroacetamide (MTBSTFA) replaces the active hydrogen with tert-Butyldimethylsilyl (t-BDMS) group. The tert-Butyldimethylsilyl derivatives which are more resistant to hydrolysis and can be up to 10,000 times more stable than TMS derivatives. N-methyl-N-t-butyldimethylsilyltrifluoroacetamide will target sulfonic and phosphoric groups if present

and it is suitable for GC with Mass spectroscopy detector as it produces easily interpreted mass spectra. A typical (shown as equation 19) reaction involving MTBSTFA as the derivatization reagent is shown below.
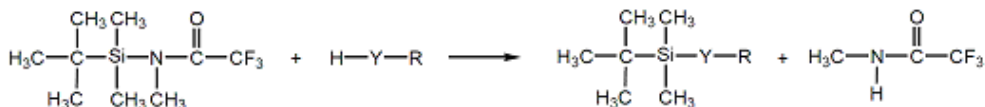
Equation 19: Silylation reaction using N-methyl-N-t-butyldimethylsilyltrifluoroacetamide (MTBSTFA) reagent: Y = O, S, NH, NR`, COO, R, R` = Alk, Ar.

Silylation derivatization procedure using MTBSTFA can be carried out in the laboratory as follows. To a 1 - 5 mg sample, 100 μL each of reagent and solvent is added and shaken on a vortex mixture for five minutes. In case of hindered compounds, they should be heated in a closed vial at 60 °C for 1 hour. For alcohols and amines, MTBSTFA with 1 % tert-butyl dimethylchlorosilane (t-BDMCS) catalyst is used.

In another method where dicarboxylic acids are produced, 150 μL of sample, 100 μL of acetonitrile and 10 uL of MTBSTFA are added. The vial is sealed and the mixture is allowed to stand overnight. Then 100 μL of water is added to hydrolyze any unreacted MTBSTFA, followed with 250 μL of hexane. Vortex mixing and centrifuging follows before the upper hexane layer is decanted and dried to approximately 5 μL under a gentle stream of nitrogen. The sample is finally injected onto GC column for analysis.

2.3 Acylation

Derivatization by acylation is a type of reaction in which an acyl group is introduced to an organic compound. In the case of a carboxylic acid, the reaction involves the introduction of the acyl group and the loss of the hydroxyl group. Compounds that contain active hydrogens (e.g., -OH, -SH and -NH) can be converted into esters, thioesters and amides, respectively, through acylation (Zenkevich, 2009). Acylation is also a popular reaction for the production of volatile derivatives of highly polar and in volatile organic materials (Zaikin and Halket, 2003). Acylation also improves the stability of those compounds that are thermally labile by inserting protecting groups into the molecule. Acylation can render extremely polar materials such as sugars amenable to separation by GC and, consequently, are a useful alternative or complimentary to the silylation. Equation 20 shows an example of an acylation is the reaction between acetic anhydride and an alcohol.

$$CH_3OCOCOCH_3 + HOR \xrightarrow{H^+(Catalyst)} CH_3OCOR' + HOCOCH_3$$

Equation 20: Reaction between acetic anhydride and an alcohol to produce acetate ester and acetic acid. (Blau and Halket, 1993).

Acylation has the following benefits in GC analysis.

i.   It improves analyte stability by protecting unstable groups.
ii.   It can provide volatility on substances such as carbohydrates or amino acids, which have many polar groups that they are nonvolatile and normally decompose on heating.

iii. It assists in chromatographic separations which might not be possible with compounds that are not suitable for GC analysis.

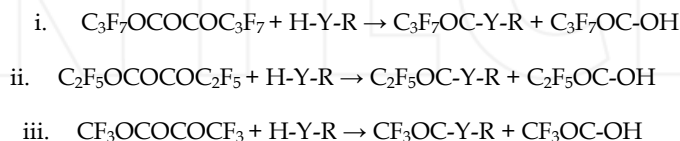iv. Compounds are detectable at very low levels with an electron capture detector (ECD).

In addition, acyl derivatives tend to produce fragmentation patterns of compounds in MS applications which are clear to interpret and provide useful information on the structure of these materials. Acylation can be used as a first step to activate carboxylic acids prior to esterfication. However, acylation derivatives can be difficult to prepare especially because of interference of other reaction products (acid by-products) which need to be removed before GC separation. Acylation reagents are moisture sensitive, hazardous and odorous.

2.3.1 Derivatization reagents used in acylation

Common reagents for the Alkylation process are Fluoracylimidazoles, Fluorinated Anhydrides, N-Methyl-bis(trifluoroacetamide) (MBTFA), Pentafluorobenzoyl Chloride (PFBCI) and Pentafluoropropanol (PFPOH). Acylating reagents readily target highly polar, multi-functional compounds, such as carbohydrates and amino acids. In addition, acylating reagents offer the distinct advantage of introducing electron-capturing groups (Kataoka, 2005), and therefore enhancing detectability during analysis. These reagents are available as acid anhydrides, acyl derivatives, or acyl halides. The acyl halides and acyl derivatives are highly reactive and may be suited for use where issues of steric hindrance may be a factor. Acid anhydrides are available in a number of fluorinated configurations, which can improve detection. These fluorinated anhydride derivatives are used primarily for electron capture detection (ECD), but can also be used for flame ionization detection (FID). Fluorinated anhydrides are often used in derivatizing samples to confirm drugs of abuse. Despite this special use, their acidic nature requires that any excess or byproducts be removed prior to GC analysis to prevent deterioration of the column. Because of the acidic byproducts, the derivatization process has been carried out in pyridine, tetrahydrofuran or another solvent capable of accepting the acid by-product.

2.3.1.1 Fluorinated anhydrides

Fluorinated Anhydrides include the following compounds; Trifluoroacetoic Anhydride (TFAA), Pentafluoropropionic Anhydride (PFPA) and Heptafluorobutyric Anhydride (HFBA) which are suitable for both Flame ionization Detectors (FID) and Electron Capture Detectors (ECD). Equations i, ii, and iii representing acylation reactions using HFBA, PFPA and TFAA derivatization reagents respectively are shown below (Equation 21).

i. $C_3F_7OCOCOC_3F_7 + H\text{-}Y\text{-}R \rightarrow C_3F_7OC\text{-}Y\text{-}R + C_3F_7OC\text{-}OH$

ii. $C_2F_5OCOCOC_2F_5 + H\text{-}Y\text{-}R \rightarrow C_2F_5OC\text{-}Y\text{-}R + C_2F_5OC\text{-}OH$

iii. $CF_3OCOCOCF_3 + H\text{-}Y\text{-}R \rightarrow CF_3OC\text{-}Y\text{-}R + CF_3OC\text{-}OH$

Equation 21: Derivatization reactions using HFBA, PFPA and TFAA respectively: Y = O, NH, NR´, R, R´ = Alk, Ar.

The perfluoroacid anhydrides and acyl halide reagents react to form acidic byproducts which must be removed prior to the GC analysis in order to prevent damage to the

chromatography column. The reagents basically react with alcohols, amines, and phenols to produce stable and highly volatile derivatives. Bases, such as triethylamine, can be added as an acid receptor and promote reactivity (Lin et al., 2008). Amine bases also may be used as catalysts/acid acceptors.

The following procedure by Palmer et al., (2000) for derivatization using Fluorinated Anhydrides involves the use of Triethylamine (TMA), as a catalyst. In a 5 mL vial, 50 µg (or 250 µg in case of FID) of sample is dissolve in 0.5 mL benzene. Then, 0.1 mL of 0.05 M triethylamine in benzene is added followed by 10 µL of desired anhydride such as heptafluorobutyric Anhydride (HFBA). The vial is capped and heat to 50 °C for 15 minutes. This is followed by cooling and addition of 1 mL of a 5 % aqueous ammonia solution. The cool mixture is shaken for 5 minutes, to separate the benzene layer, and injected directly onto the GC column.

2.3.1.2 Fluoracylimidazoles

Fluoracylimidazoles include Trifluoroacetylimidazole (TFAI), Pentafluoropropanylimidazole (PFPI) and Heptafluorobutyrylimidazole (HFBI) which reacts under mild conditions. The by-products (imidazole and/or N-methytrifluoroacetamide) are not acidic and therefore do not harm the column. Care must be taken because these reagents react violently with water. Fluoracylimidazoles work best with amines and hydroxy compounds (Kataoka, 2005). For example Heptafluorobutyrylimidazole readily forms derivatives with phenols, alcohols and amines (as shown in equation 22) and the derivatives are suitable for ECD.

$$\text{NOCC}_3\text{F}_7 + \text{H-Y-R} \longrightarrow \text{C}_3\text{F}_7\text{OC-Y-R} + \text{NH}$$
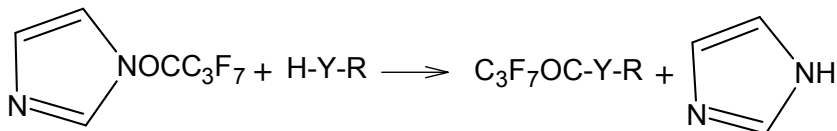
Equation 22: Derivatization reaction using Heptafluorobutyrylimidazole: Y = O, NH, NR´, R, R´ = Alk, Ar.

The following procedure can be used for the derivatization of alcohols, amines, amides, and phenols with imidazoles. To 1- 2 mg of sample in a vial, 2 mL toluene is added followed by 0.2 mL of imidazole (HFBI). The vial is capped and heat to 60 °C for 20 minutes. This is followed by cooling the mixture and then washing it 3 times with 2 mL of H_2O. The mixture is then dried over $MgSO_4$ and inject onto the GC for separation.

2.3.1.3 N-Methyl-bis(trifluoroacetamide) (MBTFA)

N-Methyl-bis(trifluoroacetamide) (MBTFA) reagent reacts rapidly with primary and secondary amines, and also slowly with hydroxyl groups and thiols. Reaction conditions are mild with relatively inert and non acidic by-products and therefore do not damage the GC column (Lelowx et al., 1989). The general reaction is presented in equation 23:

$$F_3COCN (CH_3) OCCF_3 + H\text{-}Y\text{-}R \rightarrow F_3OC\text{-}Y\text{-}R + CH_3NHOCCF_3$$
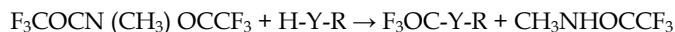
Equation 23: Representative reaction of the derivatization of amines, hydroxyl groups and thiols using N-Methyl-bis(trifluoroacetamide) (MBTFA) reagent: Y = O, S, NH, NR´, R, R´ = Alk, Ar.

N-Methyl-N-bis(trifluoroacetamide is recommended for the analysis of sugars (Matsuhisa, 2000; Hannestad, 1997) and as an acylation reaction is often used for amine drugs, such as stimulants, amino acids, and alcohols.

In the method used by Matsuhisa, (2000), 20 µL of D-glucose standard aqueous solution (10 µg/mL) was sampled and freeze-dried. Trifluoroacetylation was performed by adding 10 µL of pyridine and 10 µL of MBTFA (N-methyl-bis-trifluoroacetamide) to the dried sample, and then heating at 60 °C for 60 minutes. The cooled sample was ready for GC analysis.

Donike, (1973) applied the following procedure for the rapid derivatization of amines. To 10 mg sample, 0.2 mL MBTFA and 0.5 mL solvent of choice is added in a reaction vial. The vial is capped and heat to 60 - 100 °C for 15 to 30 minutes. It was also observed that some compounds with steric hindrance require additional heating.

2.3.1.4 Pentafluorobenzoyl Chloride (PFBCl)

Pentafluorobenzoyl chloride (PFBCl) is used in making derivatives of alcohols and secondary amines of which secondary amines are the most highly reactive, forming the most sensitive ECD derivatives of amine and phenol. Phenols are the most receptive site for this reagent (Regis, 1999). Pentafluorobenzoyl chloride (PFBCl) is suitable for functional groups that are sterically hindered. A base such as NaOH is often used to remove the HCl that is produced as byproduct. This derivatization procedure which is presented by the reaction below (equation 24) basically uses a pentafluorobenzoyl chloride (PFBCl) to provide rapid formation of the derivatives of amines and phenols.

$$C_6 F_5\text{-OCCl} + C_6H_5\text{-OH} \rightarrow C_6 F_5\text{-OCO-} C_6H_5 + HCl$$
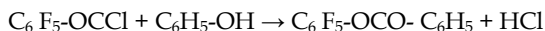
Equation 24: Formation of derivatives of amines and phenols using Pentafluorobenzoyl chloride (PFBCl).

The following derivatization procedure can be used for PFBCl reagent. In a 5 mL vial, 25 - 50 mg of sample and 2.5 mL of 2.5 N NaOH are dissolved. Then, 0.1 g of PFBCl, is added and the vial is capped, shaken vigorously for 5 minutes. The derivative formed is extracted into methyl chloride and dried over $MgSO_4$. The sample is then injected directly onto the GC column.

2.3.1.5 Pentafluoropropanol (PFPOH)

Pentafluoropropanol ($CF_3CF_2CH_2OH$) is used in combination with pentafluoropropionic anhydride (PFPA) and is applied commonly with polyfunctional bio-organic compounds (Regis, 1999). For example, 2,2,3,3,3-Pentafluoropropanol is used in combination with PFPA to make derivatives of the most common functional groups, especially polyfunctional bio-organic compounds (Regis, 1999). The formed derivatives are highly suitable for ECD.

Sample analysis and derivatization can be done through the following sequential derivatization procedure, in which 1 mg of sample is added in a reaction vial, followed by the addition of 50 µL PFPOH and 200 µL of PFPA. The sample is then heated to 75 °C for 15 minutes and then evaporated to dryness under N_2. An additional 100 µL PFPA is added and the sample is again heated to 75 °C for 5 minutes. The sample is finally evaporated to dryness and dissolved in ethyl acetate prior to injection.

2.3.1.6 4-Carbethoxyhexafluorobutyryl chloride (4-CB)

4-Carbethoxyhexafluorobutyryl chloride (4-CB) forms stable products with secondary amines, such as methamphetamine, allowing the removal of excess agent by adding protic solvents. 4-Carbethoxyhexafluorobutyryl chloride decreases the net charge of the peptides and increased hydrophobicity (Klette, 2005). The following equation 25 shows the derivatization of an amine using 4-CB reagent.

$$ClOCC_3F_6OCOC_2H_5 + R\text{-}NH_2 \rightarrow RNHOCC_3F_6OCOC_2H_5 + HCL$$
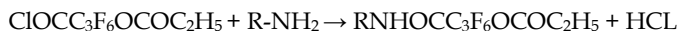
Equation 25: Amine derivatization using 4-Carbethoxyhexafluorobutyryl chloride (4-CB) reagent.

The procedure for derivatization follows the one used by Dasgupta et al., (1997). In the procedure, 50 µL of 4-CB is added to dried sample (dried by passing of a gentle stream of nitrogen gas to remove the organic phase) followed by incubation at 80 °C for 20 minutes. After derivatization the excess 4-CB is evaporated and the derivatized sample is reconstituted in 50 µL ethyl acetate of which 1 - 2 µL is injected to GC for analysis.

3. GC Chiral Derivatization

Chiral derivatization involves reaction of an enatiomeric molecule with an enatiomerically pure chiral derivatizing agent (CDA) to form two diastereomeric derivatives that can be separated in this case using GC. A solution in which both enantiomers of a compound are present in equal amounts is called a racemic mixture. Diastereomers are stereoisomers (they have two or more stereo centers) that are not related as object and mirror image and are therefore not enantiomers. In other word, unlike enatiomers which are mirror images of each other and non-sumperimposable, diastereomers are not mirror images of each other and non-superimposable. Diastereomers can have different physical properties and reactivity.

Any molecule having asymmetric carbon is called as chiral molecule. Chirality of analyte molecules requires special consideration in their analysis and separation techniques. Scientists and other regulatory authorities are in the demand of data on concentrations and toxicity of the chiral pollutants, and therefore chiral derivatization is becoming an essential, urgent and demanding field. However the derivatization procedures are tedious and time consuming due to the different reaction rates of the individual enantiomers (Schurig, 2001).

Generally, there are two ways of separating enantiomers by chromatography:

i. Separation on an optically active stationary phase
ii. Preparation of diastereomeric derivatives that can be separated on a non chiral stationary phase.

The second option (ii above) requires derivatization of the analyte molecule. The presence of a suitable functional group in a pollutant is a condition for a successful derivatization of a chiral molecule. The indirect chromatographic analysis of racemic mixtures can be achieved by derivatization with a chiral derivatizing agent resulting into the formation of diastereoisomeric complex/salt (Hassan et al., 2004). From the resulting chromatograms, calculations are made to determine the enantiomeric concentration of the analyte. The

diastereoisomers having different physical and chemical properties can be separated from each other by an achiral chromatographic method. Nowadays, the chromatographic methods are the most popular for enantiomeric analysis of environmental pollutants (Hassan et al 2004).

3.1 Gas chromatography chiral derivatization reagents

Gas Chromatography analysis of enantiomeric compounds on nonracemic or achiral stationary phases requires the use of enantiopure derivatization reagents (Hassan et al, 2004). Enantiopure compounds refer to samples that contain molecules having one chirality within the limits of detection. These reagents generally target one specific functional group to produce diastereomers of each of the enantiomeric analytes in GC to produce chromatograms. Some of the most common Gass Chromatography chiral derivatization reagents are: (-) menthylchloroformate (MCF), (S)-(–)-N-(Trifluoroacetyl)-prolylchlorides (TPC), (–)-α-Methoxy-α-trifluoromethylphenylacetic acid (MTPA). Examples of the use of these derivatizing agents were employed in analysis of major drugs of forensic interest, and for optically active alcohols (Tagliaro et al., (1998).

3.1.1 N-trifluoroacetyl-L-prolyl chloride (TPC)

The reagent is used for optically active amines, most notably amphetamines as represented in the following reaction (equation 26) where N-Trifluoroacetyl-L-prolyl chloride couples with amines to form diastereomers which can be separated on GC columns as it increases the sample volatility.
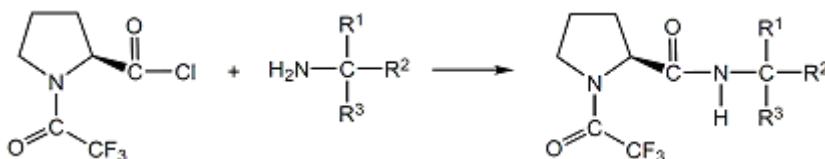
Equation 26: N-Trifluoroacetyl-L-prolyl chloride derivatization of amines

The standard TPC derivatization procedure is as follows; following sample clean up and extraction procedure, 1.0 mL of the TPC reagent is added to the clean sample. The mixture is allowed to stand for 5 min before the addition of 20 μL of triethylamine to take up excess unreacted TPC. After 15 min of intermittent shaking, 1.0 mL of 6 N HC1 is used to remove the ammonium salt. The mixture is finally washed with 1 mL of distilled water and then dried over anhydrous magnesium sulfate before dilution and analyzed by GC.

3.1.2 (S)-(–)-N-(Trifluoroacetyl)-prolylchloride (l-TPC)

(S)- (–)-N-(Trifluoroacetyl)-prolylchloride (l-TPC) is widely used for amine drugs analysis. Qiao Feng Tao & Su Zeng (2002) analyzed the enantiomers of chiral amine drugs by using stereo selective methods to separate enantiomers on an achiral capillary gas chromatography by pre-column chiral derivatization with S-(_)-N-(fluoroacyl)-prolyl chloride. In the study, it was noted that the stereo selectivity and sensitivity can be improved by chiral derivatization. The method has been used to determine S-(+) - methamphetamine in human forensic samples
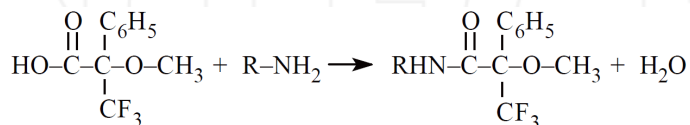
and to analyze enantiomers of amphetamine and fenfluramine in rat liver microsomes. Two analytical procedures used by Qiao Fang Tao and Su Zing (2002) for aqueous and organic phase derivatization methods are described below.

Aqueous phase derivatization method: Urine or liver homogenate was added to a 10 ml Teflon-lined, screw-capped test tube. The pH of the sample was adjusted to 9 by 10 M NaOH, then 0.5 ml of chilled 1 M sodium bicarbonate/sodium carbonate buffer (pH 9.0). After mixing, 40 µL of S-(_)-TFAPC was added, and the tube was vortex mixed for 30 min at room temperature. The resulting mixture was saturated with NaCl and adjusted to pH 9, followed by addition of 0.5 ml of chilled 1 M sodium bicarbonate/sodium carbonate buffer (pH 9.0) and extracted with 4 ml of ethyl acetate by gently shaking for 15 min. After the phases separate, the organic layer was washed with 2 ml of deionized water. The organic layer was evaporated to dryness under a gentle stream of air at 40 °C. The residue was cooled to room temperature and reconstituted with ethyl acetate. An aliquot of 1 µL of analyte was analyzed by GC/MS.

Organic phase derivatization method: A 1.0 ml sample of microsomal mixture spiked with chiral amine was piped into a 15 ml screw capped test tube. The pH of the sample was adjusted to 12 – 13 with 40% NaOH. The mixtures were extracted with 2 ml of chloroform by rotatory shaking for 1 min. After centrifugation at 4000 rpm for 10 min, the aqueous phase was removed by aspiration. The remaining organic phase was dried by anhydrous sodium sulfate, and then transferred to another clean screw-capped test tube. A 10 µL sample of triethylamine as a catalytic agent and 40 µL S-(_)-TFAPC were added into the test tube and mixed. The tube was capped and allowed to react for 15 min at room temperature by gentle shaking. The chloroform layer was washed with 2 ml distilled water and evaporated to dryness at under a water bath and a gentle stream of air 50° C. The residues were allowed to cool to room temperature and reconstituted with 40 µL ethyl acetate prior to injection into the GC/FID system (Qiao Feng Tao & Su Zeng, 2002).

3.1.3 (–)-α-Methoxy-α-trifluoromethylphenylacetic acid (MTPA)

(–)-α-Methoxy-α-trifluoromethylphenylacetic acid is also mostly used in drug analysis. In a study by S-M Wang et al., (2005) pharmaceutical drugs were analyzed using compounds that were derivatized with (–)-α-Methoxy-α-trifluoromethylphenylacetic acid (MTPA) as the derivatizing reagent. These types of derivatization reactions are represented by equation 27. In the reaction the hydroxyl group on the MTPA molecule is lost in the derivative formation.

$$\underset{\underset{CF_3}{|}}{HO-\overset{\overset{O}{\|}}{C}-\overset{\overset{C_6H_5}{|}}{C}-O-CH_3} + R-NH_2 \longrightarrow \underset{\underset{CF_3}{|}}{RHN-\overset{\overset{O}{\|}}{C}-\overset{\overset{C_6H_5}{|}}{C}-O-CH_3} + H_2O$$

Equation 27: Derivatization reaction between (–)-α-Methoxy-α-trifluoromethylphenylacetic acid and an amine group of an analyte molecule.

The derivatization using MTPA can be achieved as follows: after extraction, clean up and drying the sample under nitrogen, the residue is added 50 µL N, N-dicyclohexycarbodiimide and 100 µL MTPA. The reaction mixture is then thoroughly mixed, then incubated at 70 °C for 20 min before GC analysis.

4. Summary

The choice of the derivatization technique for analysis of compounds will depend on the available reagent and reaction types that can produce derivatives that give desirable results in GC. The derivatives must be suitable, detectable and efficient for GC analysis. The evaluation of the functional group of the analyte, the GC detector and even the by products of the reaction among others considerations will guide the choice of derivatization technique.

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 in an analyte enhances the electron capture detector (ECD) response. For mass spectroscopic detector, acyl derivatives tend to direct the fragmentation patterns of compounds and therefore, providing useful information on the structure of molecules.

For acid analytes, the first choice for derivatization is esterification. Acids are reactive compounds and also more polar to be separated well by gas chromatography. The underivatized acids will tend to tail because of the adsorption and non-specific interaction with the column. Esterification is used to derivatize carboxylic acids and other acidic functional groups. The reaction involves the condensation of the carboxyl group of an acid and the hydroxyl group of an alcohol, with the elimination of water. Consequently, the polarity of the molecule is reduced.

Nearly all functional groups which present a problem in gas chromatographic separation (hydroxyl, carboxylic acid, amine, thiol, phosphate) can be derivatized by silylation reagents. The derivatives of the silylation reactions are generally less polar, more volatile and more thermally stable. The introduction of a silyl group(s) can also serve to enhance mass spectrometric properties of derivatives, by producing either more favorable diagnostic fragmentation patterns of use in structure investigations, or characteristic ions of use in trace analyses in other related techniques.

In considering the functional group or compound type, dimethylformamide-dialkylacetals (DMF-DEA) or similar type of reagents are recommended for sterically hindered aldehydes, amines, carboxylic acids, and phenols. Shorter chain reagents like DMF- DEA will produce more volatile derivatives than longer chain reagents. The by products of the reaction will also determine the choice of reagent. In this view, perfluoroacylimidazole is a better choice rather than perfluoroacid anhydrides. Perfluoroacid anhydrides produce acidic byproducts which must be removed from the reaction mixture before the derivatives are injected onto the GC column. With perfluoroacylimidazole there are no acid byproducts.

The complexity surrounding chiral derivatization is generally because of different reaction rates of the individual enantiomers. Derivatization with a chiral derivatizing agent resulting into the formation of diastereoisomeric complexes is an option in GC analysis, and has been applied to analyze various analytes (Hassan et al., 2004 & Schurig, 2001). Table 1 gives a summary guide of derivatization technique and reagent selection based on the functional group of the analyte molecule.

Functional group	Reaction type	Derivatization reagent
Alcohols and Phenols	Silylation	Bis(trimethylsilyl)–acetamide, Bistrimethylsilyltrifluoroacetamide, N-methyl-N-t-butyldimethylsilyltrifluoroacetamide
	Acylation	Heptafluorobutyrylimidazole, Pentafluoropropionic Anhydride, Trifluoroacetic anhydride, N-Methyl-bis(trifluoroacetamide)
	Alkylation	Dimethylformamide, Pentafluorobenzyl bromide
Carboxylic acids	Silylation	Bis(trimethylsilyl)–acetamide, Bistrimethylsilyltrifluoroacetamide, Trimethylsilylimidazole, N-methyl-N-t-butyldimethylsilyltrifluoroacetamide
	Acylation	Pentafluoropropanol / pentafluoropropionic anhydride
	Alkylation	Dimethylformamide, Tetrabutylammonium hydroxide
Active hydrogens	Silylation	Bis(trimethylsilyl)–acetamide, Bistrimethylsilyltrifluoroacetamide / Trimethylchlorosilane, Hydrox-Sil, N-methyl-trimethylsilyltrifluoroacetamide,
	Acylation	Pentafluoropropanol / pentafluoropropionic anhydride
	Alkylation	Dimethylformamide, Tetrabutylammonium hydroxide
Carbohydrates and Sugars	Silylation	Hexamethyldisilzane, Trimethylsilylimidazole
Amides	Silylation	Bis(trimethylsilyl)–acetamide, N, O-bis-(trimethylsilyl)-trifluoroacetamide
	Acylation	Heptafluorobutyrylimidazole
	Alkylation	Dimethylformamide
Amines	Silylation	Bistrimethylsilyltrifluoroacetamide, N-methyl-N-t-butyldimethylsilyltrifluoroacetamide
	Acylation	Trifluoroacetic anhydride, Pentafluorobenzoyl chloride, Heptafluorobutyrylimidazole
	Alkylation	Dimethylformamide (Diacetals)
Amino acids	Silylation	Bistrimethylsilyltrifluoroacetamide, Trimethylsilylimidazole
	Acylation	Heptafluorobutyrylimidazole
	Alkylation	Dimethylformamide, Tetrabutylammonium hydroxide
Catecholamines	Silylation	Trimethylsilylimidazole
	Acylation	Pentafluoropropionic Anhydride, Heptafluorobutyrylimidazole,
Inorganic anions	Silylation	Bistrimethylsilyltrifluoroacetamide, N-methyl-N-t-butyldimethylsilyltrifluoroacetamide
Nitrosamine	Silylation	Bistrimethylsilyltrifluoroacetamide
	Acylation	HFBA, Pentafluoropropionic anhydride, Trifluoroacetic anhydride
	Alkylation	Dimethylformamide, Pentafluorobenzyl bromide
Sulfonamides	Acylation	Trifluoroacetoic & Heptafluorobutyric Anhydride, Pentafluorobenzyl bromide
Sulfides	silylation	Trimethylsilylimidazole

Table 1. A summary guide for the derivatization techniques and reagents selection based on sample types.

5. References

Allmyr M, McLachlan MS, Sandborgh-Englund G, Adolfsson-Erici M. (2006). Determination of Triclosan as its pentafluorobenzoyl ester in human plasma and milk using electron capture negative ionization mass spectrometry. Anal Chem. 2006; 78: 6542–6.

Bao Ming-Liang, Pantani Francesco, Griffini Osvaldo, Burrini Daniela, Santianni Daniela, Barbieri Katia (1998). Determination of carbonyl compounds in water by derivatization–solid-phase micro extraction and gas chromatographic analysis. Journal of Chromatography A. 809 (1998) 75–87

Blau, K., King, G. (1997). Handbook of Derivatives for Chromatography; Heyden & Sons Ltd.; London, 1979

Butts W.C. (1972). Two-column gas chromatography of trimethylsilyl derivatives of Biochemically significant compounds.. Analytical Biochemistry. Volume 46, Issue 1, March 1972, Pages 187-199

Chen, B.-G., Wang, S.-M. and Liu, R. H. (2007). GC-MS analysis of multiply derivatized opioids in urine. Journal of mass spectrometry JMS (2007). Volume: 42, Issue: 8, Pages: 1012-1023

Chien, C.-J.; Charles, M. J.; Sexton, K. G.; Jeffries, H. E. (1998). Analysis of airborne carboxylic acids and phenols as their pentafluorobenzyl derivatives: gas Chromatography/Ion Trap Mass Spectrometry with a novel chemical ionization reagent, PFBOH, Environmental Science And Technology. 1998, 32, 299-309.

Christie, W.W. (1993): preparation of ester derivatives of fatty acids for chromatographic analysis. Advances in Lipid Methodology - *Two*, pp. 69-111 (1993) (Ed. W.W. Christie, Oily Press, Dundee),

Danielson, Neil D., Gallagher Patricia A., and Bao James J. (2000). Chemical Reagents and Derivatization Procedures in Drug Analysis. Encyclopedia of Analytical Chemistry. John Wiley & Sons Ltd, Chichester, 2000. R.A. Meyers (Ed.). pp. 7042–7076.

Dasgupta, Amitava, Blackwell Walter and Burns Elizabeth (1997). Gas Chromatographic-mass spectrometric identification and quantitization of urinary phenols after derivatization with 4-carbethoxyhexafluorobutyryl chloride, a novel derivative. Journal of Chromatography B: Biomedical Sciences and Applications Volume 689, Issue 2, 21 February 1997, Pages 415-421

Destaillats, H.; Spaulding, R. S.; Charles, M. J. (2002). Ambient air measurement of acrolein and other carbonyls at the Oakland-San Francisco Bay Bridge toll plaza. Environmental Science and Technology 2002, 36, 2227-2235.

Donike, M. (1973). Acylation with bis (acylamides). N-methyl-bus (trifluoroacetamide) and bis (trifluoroacetamide), two new reagents for trifluoroacetylation. Journal of Chromatography A. Volume 78, Issue 2, 25 April 1973, Pages 273-279.

Galceran, M. T.; Moyano, E.; Poza, J. M. (1995). Pentafluorobenzyl derivatives for the gas-chromatographic determination of hydroxy-polycyclic aromatic hydrocarbons in urban aerosols. Journal of Chromatography A 1995, 710, 139-147.

Gehrke, Charles W. (1968). Quantitative gas-liquid chromatography of amino acids in proteins and biological substances: Macro, semimicro, and micro methods. Analytical Biochemistry Laboratories (Columbia, Mo). 1st edition. 101-289-264

Hannestad, U.; Lundblad, A. (1997). Accurate and Precise Isotope Dilution Mass Spectrometry Method for Determining Glucose in Whole Blood. Clinical Chemistry, 1997, 43, 5, 794-800.

Hassan, Y. Aboul-Enein & Imran Ali (2004): Analysis of the chiral pollutants by Chromatography, Toxicological & Environmental Chemistry, 86:1, 1-22

Isoherranen, Nina and Soback Stefan (2000). Determination of gentamicin after Trimethylsilylimidazole and trifluoroacetic anhydride derivatization using gas Chromatography and negative ion chemical ionization ion trap mass spectrometry. Analyst, 2000, 125, 1573–1576

Jang Myoseon and Kamens Richards, M. (2001). Characterization of Secondary Aerosol from the Photooxidation of Toluene in the Presence of NOx and 1-Propene Environ. Sci. Technol. 2001, 35, 3626-3639

Kataoka, Hiroyuki (2005). Gas chromatography of amines as various derivatives. Quantitization of amino acids and amines by chromatography - methods and protocols. Journal of Chromatography Library. Volume 70, 2005, Pages 364-404.

Kawahara, F.K. (1968). Micro determination of pentafluorobenzyl ester derivatives of Organic acids by means of electron capture gas chromatography.

Klette, L Kevin., Jamerson H. Matthew, Morris-Kukoski L. Cynthia, Kettle R. Aaron, and Snyder J. Jacob (2005). Rapid Simultaneous Determination of Amphetamine, Methamphetamine, 3, 4-Methylenedioxyamphetamine, 3, 4-Methylenedioxymethamphetamine, and 3, 4-Methylenedioxyethyla. Journal of Analytical Toxicology. Volume Number: 29: Issue 7: 669–674

Knapp, Daniel R. (1979). Handbook of Analytical Derivatization Reaction; pages 10, 10; Wiley & Sons; New York, 1979.

Kühnel, E., Laffan Dr, D.D.P., Lloyd-Jones, G.C., Martínez del Campo, T., Shepperson, I.R. Slaughter,J.L.,(2007). Mechanism of Methyl Esterification of Carboxylic Acids by Trimethylsilyldiazomethane. Angewandte Chemie International Edition. Volume 46, Issue 37, pages 7075–7078, September 17, 2007.

Kuo, H.W., Ding, W.H. (2004). Trace determination of bisphenol A and phytoestrogens in infant formula powders by gas chromatography-mass spectrometry. J. Chromatogr. A, 1027, 67.

Lelowx, M.S. De jong E.G and Meas RA.A. (1989). Improved screening method for beta-blockers in urine using solid- phase extraction and capillary gas chromatography— mass spectrometry. Journal of Chromatography. 488 (1989). 357-.367.

Lin, D.-L. Wang, S.-M. Wu, C.-H. Chen, B.-G. Liu, R.H. (2008). Chemical Derivatization for the Analysis of Drugs by GC- MS — A Conceptual Review. Journal of Food and Drug Analysis, Vol. 16, No. 1, 2008, Pages 1-1

Matsuhisa, M.; Yamasaki, Y.; Shiba, Y.; Nakahara, I.; Kuroda, A.; Tomita, T. (2000). Important Role of the Hepatic Vagus Nerve in Glucose Uptake and Production by the Liver Metabolism, 2000, 49, No 1 (January), 11-16.

Naritisin, D.B. and Markey, S.P. (1996). Assessment of DNA oxidative damage by Quantification of thymidine glycol residues using gas chromatography/electron Capture negative ionization mass spectrometry. Anal. Biochem. 241:35-41.

Orata, F. Quinete, N. Wilken, R.D. (2009). Long Chain Perfluorinated Alkyl Acids Derivatization and Identification in Biota and Abiota Matrices Using Gas

Chromatography. Bulletin of Environmental Contamination and Toxicology 2009 Vol. 83 No. 5 pp. 630-635

Palmer, R.B, Kim N.H. and Dasgupta A (2000). Simultaneous determination of fenfluramine and phentermine in urine using gas chromatography-mass spectrometry with pentafluoropropionic anhydride derivatisation. Ther. Drug Monit. 22 (2000). 418-422.

Pierce, Alan E. (1968). Silylation of Organic Compounds. Pierce Chemical Company, 1968. Pierce, (2004) .GC Derivatization. .Applications Handbook & Catalog.

Qiao Feng Tao and Su Zeng (2002). Analysis of enantiomers of chiral phenethylamine drugs by capillary gas chromatography/mass spectrometry/flame–ionization detection and pre-column chiral derivatization. J. Biochem. Biophys. Methods 54 (2002) 103–113.

Rao, X.; Kobayashi, R.; White-Morris, R.; Spaulding, R.; Frazey, P.; Charles, M. J. (2001). J GC/ITMS measurement of carbonyls and multifunctional carbonyls in PM2.5 particles emitted from motor vehicles.Journal of AOAC International 2001, 84, 699-705.

Ribeiro Bárbara, Guedes de Pinho Paula, Andrade B. Paula, Baptista Paula, Valentão Patrícia. (2009). Fatty acid composition of wild edible mushrooms species: A comparative study. Micro chemical Journal 93 (2009) 29–35 Regis (1999). Chromatography Catalog, pages 89, 90

Saraji, M. and Mirmahdieh, S. (2009). Single-drop micro extraction Followed by in-syringe derivatization and GC-MS detection for the determination of parabens in water and cosmetic products. J. Sep. Sci. 2009, 32, 988 – 995

Schauer, J. J.; Kleeman, M. J.; Cass, G. R.; Simoneit, B. R. T. (2002). Environ. Science and Technology 2002, 36, 1169-1180.

Schurig V (2001). Chiral separation of amino acids is of great importance in biology, Pharmaceutics and agriculture. This has been performed traditionally with Chromatographic methods such as gas chromatography (GC). J. Chromatogr. A 906 (2001) 275.

Scott, R. P.W. (2003). Gas chromatography. book 2 Chrom-Ed Book Series. http://www.library4science.com/eula.html. 2

S-M Wang, Lewis j. Russell, Canfield Dennis, Li Tien-Lai, Chen Chang-Yu, Liu H. Ray (2005). Enantiomeric determination of ephedrines and norephedrines by chiral derivatization gas chromatography–mass spectrometry approaches. *J. Chromatogr. B 825 (2005) 88–95*

Sobolevsky, T.G. Alexander I.R. Miller, B. Oriedo, V., Chernetsova, E.S., Revelsky, I. A. (2003). Comparison of silylation and esterification/acylation procedures in GC-MS analysis of amino acids. Journal of Separation Science. Volume 26, Issue 17, pages 1474–1478, November 2003

Spaulding, R. S.; Schade, G. W.; Goldstein, A. H.; Charles, M. J. Journal of Geophysical Research, [Atmospheres] 2003, 108, ACH7/1-ACH7/17.

Szyrwińska, K. Kołodziejczak A., Rykowska I., Wasiak W., and Lulek J. (2007). Derivatization and Gas chromatography– low-resolution Mass Spectrometry of Bisphenol A. Acta Chromatographica, No. 18, 2007

Tagliaro Franco, Smith Frederick P, Tedeschi Luciano, Castagna Franca, Dobosz Marina, Boschi Ilaria and Pascali Vincenzo (1998). Toxicological and Forensic Applications Chapter 21. Journal of Chromatography Library Volume 60, 1998, Pages 917-961

Thenot, J.-P., Horning, E.C. (1972). Amino acid *N*-dimethyllaminomethylene alkyl esters. New derivatives for GC and GC-MS studies. Anal. Lett.5 (8): 519-29.

Thenot, J. P, Stafford M. and Horning M. G. (1972). Fatty acid esterification with N, N-Dimethylformamide dialkyl acetals for GC analysis. Anal. Lett. 5(4): 217-23.

Zaikin, V.G., Halket, J.M (2003). Derivatization in mass spectrometry--2. Acylation. Eur J Mass Spectrum. 2003; 9(5):421-34.

Zenkevich, Igor, G. (2009). Acids: Derivatization for GC Analysis Encyclopedia of Chromatography, Third Edition 2009, DOI: 10.1081/E-ECHR3-120045222
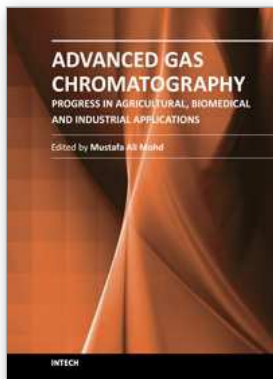
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