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Chapter

Derivatization Methods in GC and GC/MS

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Abstract

The first part of this chapter presents the main objectives for performing derivatization of a sample to be analyzed by gas chromatography (GC) or gas chromatography/mass spectrometry (GC/MS). The derivatization is typically done to change the analyte properties for a better separation and also for enhancing the method sensitivity. In GC/MS, derivatization may improve the capability of compound identification. Examples illustrating such improvements are included. The second part describes several types of derivatization that are more frequently used in analytical practice. These include alkylation (e.g., methylation), formation of aryl derivatives, silylation (e.g., formation of trimethylsilyl derivatives), acylation (e.g., reactions with acyl chlorides or with chloroformates), and several other types of derivatizations. The chapter also presents typical derivatizations for analytes with specific functional groups and discusses artifact formation in certain derivatization reactions.

Keywords: gas chromatography, mass spectrometry, derivatization, alkylation, aryl derivatives, silylation, acylation

1. General comments

Two specific trends can be noticed in modern chemical analysis. One is the continuous demand for more sensitive and accurate analytical methods. The other is the desire for simpler methods that require as little as possible human intervention. One of the various procedures to make the analytical methods more sensitive and accurate is the use of specific chemical changes (e.g., derivatization) applied on the analytes or even on the whole sample. However, these changes frequently involve more human intervention than the direct use of advanced instrumentation. For this reason, the methods involving chemical changes such as derivatizations are not necessarily the first choice when selecting an analytical method. Nevertheless, in many cases, the benefits of derivatization are more important than the disadvantage of requiring human intervention, and for this reason, derivatization is still frequently used in the analytical practice. Also, modern GC, GC/MS (or GC/MS/MS) instrumentation may offer autosampling with the capability of adding reagents to the sample, as well as stirring, heating, and injecting the sample at specific time intervals in the GC system. This type of instrumentation may reduce significantly the human handling involved in derivatization.

Various chemical changes can be performed on an analyte in order to make it suitable for a specific method of analysis. The most common is derivatization, but

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other chemical changes can be utilized, for example, pyrolytic decomposition and, in the case of polymers, polymer fragmentation using reagents. The choice depends on the nature of the analyte, the sample matrix, the intended changes in the analyte properties, and the analytical method to be used.

The addition of a reagent on a sample may produce a chemical reaction only with the analytes without affecting the matrix. However, it is also possible that some matrix components are derivatized unintentionally. Usually, it is preferable to have only the analytes derivatized since in this way a better separation from the matrix is expected. Some derivatizations are used in the sample cleanup or concentration process. Also, the derivatization process may be combined with simultaneous extraction and concentration of the sample or may be followed by a second preparation step before the chromatographic analysis. More frequently, the derivatization is done to change the analyte properties for the core analytical procedure (GC, GC/MS, etc.).

Derivatization can be applied before the core chromatographic process or after it. Precolumn derivatization takes place before the separation and postcolumn derivatization after it. In GC precolumn derivatization is much more common and most derivatizations are performed "offline." There are however derivatizations that can be done "online," for example, in the injection port of the GC such as some methylations using tetramethyl ammonium hydroxide (TMAH). Postcolumn derivatizations are performed only for enhancing the detectability of the analytes. Typically, they must be done "online" and should be completed in the specific time frame needed by the analyte to reach the detector.

A wide variety of derivatization reagents and procedures are described in the literature, with the reagents carrying specific moieties that provide a desired property to the analytes, as well as with specific reactive groups that permit the reaction with the analyte. Multiple step derivatizations as well as derivatizations followed by a second one are known.

Derivatization is not always the first step in sample preparation. Sample preparation typically includes other operations, besides derivatization. Some of these steps are more complex such as sample cleanup or concentration and others more simple such as pH adjustments, addition of proton acceptors or donors, change of the medium (from one solvent to another), and addition of catalysts to enhance the derivatization, and these may be necessary for a successful derivatization.

Although derivatization is performed in order to make possible or to improve the results of a chemical analysis, there are also some disadvantages of using derivatization. Besides the potential need of more manpower for the analysis, the addition of more operations applied on the sample (including the analytes) can be a source of additional errors. In particular the involvement of a chemical reaction that may not be perfectly controlled can bring significant errors in the analytical results. To minimize the potential errors when using derivatization, specific aspects of the derivatization must be considered in its choice, such as the efficiency of the chemical reaction used in the derivatization, the stability of the derivatized analytes, the availability of reagents and necessary equipment, and the time necessary for performing the analysis. For a given analyte or group of analytes, the reaction with the derivatization reagent must be complete or at least close to complete, must take place in a length of time that is not prohibitive, and must have very little loss of the analyte with formation of artifacts or decomposition products. Only when such criteria are satisfied can a specific chosen derivatization be applied successfully.

The application of derivatization in chromatography is the subject of many studies. Numerous derivatizations have been reported in journals (e.g., *J. Chromatogr. A* and *B*, *J. Chromatogr. Sci*., *J. Sep. Sci*., *Chromatographia*, etc.), in various books [1–5], in application notes of instrument manufacturers, as well as on the web.

2. Derivatization for improving separation in gas chromatography

For GC analysis, the effect of derivatization can be beneficial in a variety of circumstances. Some of the most common uses of derivatization for improving the GC separation are the following:

(a) Derivatization that replaces active (polar) hydrogen atoms in the analyte to decrease its boiling point. The active hydrogens in a chemical compound typically enhance the capability to form hydrogen bonds and increase the compound polarity. For this reason, many compounds containing active (polar) hydrogens are not volatile, the volatility being necessary for using GC or GC/MS as a core analytical method. Derivatization can be used to replace active hydrogens from an analyte Y-H (or Y:H) in functional groups such as OH, COOH, SH, NH, and CONH. These reactions can be written in a simplified form as follows:

$$\text{Y-H} + \text{R} - \text{X} \rightarrow \text{Y-R} + \text{HX} \qquad (1)$$

In reaction (1), the reagent R-X contains an "active" group X and a group R that carries a desired property (e.g., lack of polarity for GC). Group R in the reagent can be a low molecular mass fragment such as CH_3 or C_2H_5, a short-chain fluorinated alkyl in alkylation reactions, $Si(CH_3)_3$ or other silyl groups in silylations, $COCH_3$ or short-chain fluorinated acyl groups in acylations, etc. An example of a chromatogram resulting from the GC/MS analysis of a silylated tobacco sample is given in **Figure 1**. Tobacco contains many hydroxy acids such as malic, trihydroxybutanoic, citric, quinic, glucuronic, and chlorogenic. Also, it contains monosaccharides (e.g., glucose, fructose), disaccharides (e.g., sucrose), and even trisaccharides. None of these compounds are volatile, having numerous active hydrogens. The replacement of these hydrogens with $Si(CH_3)_3$ by silylation renders these compounds volatile, and they can be analyzed by GC/MS as seen in **Figure 1**.

(b) Derivatization for enhancing the separation. Specific moieties added to an analyte may be necessary for enhancing the separation. This is frequently practiced for general GC separations and is also very useful for the separation of chiral molecules (see Section 4). The derivatized analytes may have significantly different properties from each other, for example,

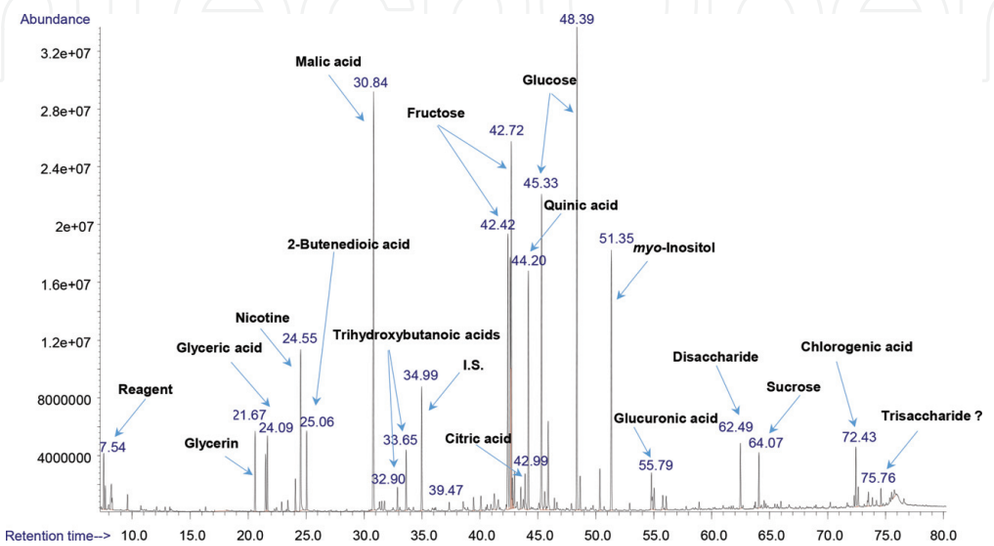


Figure 1.
GC/MS chromatogram of a silylated tobacco sample, with separation on a DB-5 MS column from Agilent (Agilent Technologies Inc., Wilmington, DE, USA) (Note: an internal standard I.S. was added to the sample).

regarding polarity and implicitly in their boiling point, allowing separations that are difficult to achieve otherwise. Also, derivatization may generate more significant differences between the analytes and the matrix components.

(c) Derivatization that replaces active hydrogens in the analyte to improve the behavior of the analyte in the chromatographic separation. The chromatographic column (e.g., a capillary column coated with a bonded stationary phase) may display additional capability to interact with polar molecules, besides the intended interactions due to its bonded phase. This may come, for example, from the silica wall of the column. Secondary interactions taking place with only a portion of the molecules of the analyte generate peak tailing. This is exemplified in **Figure 2** which shows a hypothetical case of two different types of interaction between the column and a specific molecular species.

(d) Derivatization for the improvement of stability of a compound. This stability may refer to thermal stability, a property which overlaps to a certain extent to what was described at point (a). However, even some volatile compounds may be further thermally stabilized by derivatization. Also, chemical stability can be enhanced by protecting specific groups in the analyte using derivatization. For example, thiols can be protected using derivatization against oxidation by the traces of oxygen in the heated injection port of the GC.

The choice of the appropriate derivatization is not always a simple task. The replacement of a hydrogen atom with a group of atoms may increase the molecular weight of the derivatized analyte. In such cases, it must be verified that the increase in the molecular weight by derivatization brings no or only a small increase in the boiling point of the analyte. Most of the time, low molecular weight substituents such as CH_3 or $Si(CH_3)_3$ are preferable for GC analysis to the active hydrogens for achieving the previously described goals. Large substituents may increase the boiling point too much and make the compound not acceptable for GC analysis.

Besides replacement of active hydrogens, other derivatization reactions can be utilized. For example, condensation reactions may decrease the boiling point and improve the thermal stability of an analyte. However, the generation of new active hydrogens must be avoided in condensation reactions or must be followed by a second derivatization.

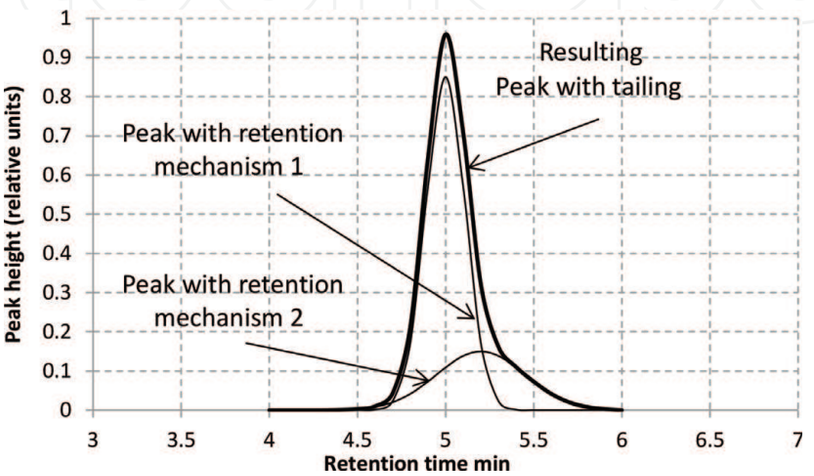


Figure 2.
Peak tailing due to multiple retention mechanisms.

3. Derivatization for chiral separation in gas chromatography

The compounds with structures that are mirror images to each other are indicated as enantiomers, and their molecules are not superimposable, having the property called chirality. Chirality is commonly caused by the existence in the molecule of at least one tetrahedral carbon atom substituted with groups that are different. However, chiral molecules may be generated with a phosphorus or a sulfur chiral atom. Not only chiral centers (such as an asymmetric carbon) generate enantiomers, but a chiral axis or a chiral plane can lead to enantiomers. The chirality in an enantiomer is specified using the symbols R and S based on specific rules. For the assignment of a symbol R or S to a chiral carbon, the substituents are arranged in a sequence a > b > c > d. For the four atoms directly attached to the asymmetric carbon, a higher atomic number outranks the lower, and a higher atomic mass outranks the lower mass. For the same atoms directly attached to the asymmetric carbon, the priorities are assigned at the first point of difference. After the sequence is established, the molecule is oriented in space with the group "d" of the lowest priority behind the asymmetric carbon. When viewed along the C—d bond (from C) and the three substituents a, b, and c are oriented clockwise, the compound contains an R asymmetric carbon, and it contains an S asymmetric carbon for counterclockwise arrangement.

More than one asymmetric carbon can be present in a molecule, as in the case of carbohydrates. The stereoisomers generated by more than one asymmetric carbon can be mirror image one to the other (enantiomers) or may have different steric arrangements being diastereoisomers. These types of molecules are schematically shown in **Figure 3**.

The (S,S)- and the (R,R)-compounds from **Figure 3** are enantiomers, while the (S,R)-compound is a diastereoisomer to both (S,S)- and to (R,R)-compounds (it is an enantiomer to the (R,S)-compound). The gas chromatographic separation of enantiomers can be done only using chromatographic columns having chiral stationary phases. The derivatization of enantiomers with non-chiral reagents generates molecules that remain enantiomers. This type of derivatization may improve the chromatographic separation from other molecules, but the derivatized compounds of remaining enantiomers cannot be separated except on chiral stationary phases. Sometimes, better separation can be obtained even between the enantiomers (on chiral chromatographic columns) after derivatization. One such example is the separation of (R)- and (S)-nornicotine derivatized with isobutyl chloroformate on a chiral Rt-BDEXsm column with separation improved compared to that of underivatized enantiomers [6]. The derivatization reaction is indicated below:

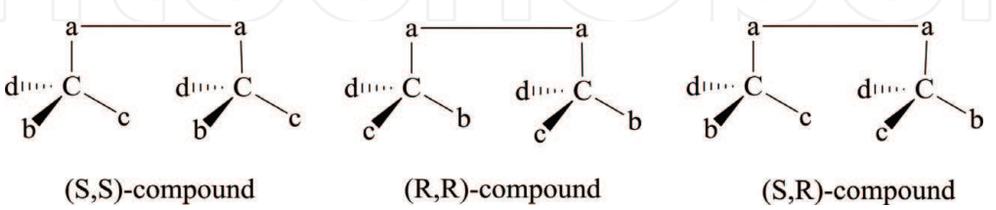
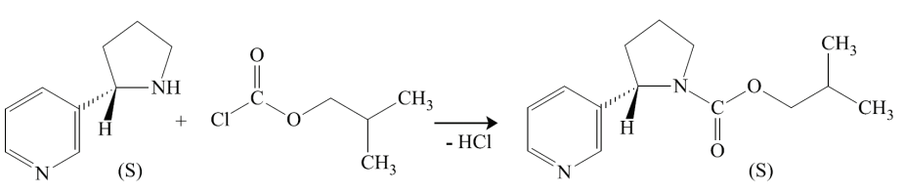


Figure 3.
Compounds with two chiral centers.

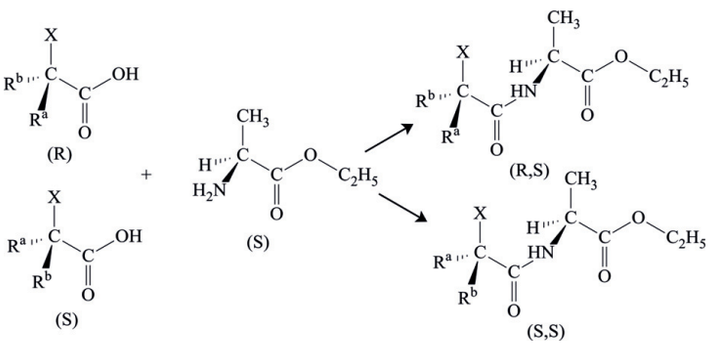


(2)

Diastereoisomers can be separated on chromatographic columns with non-chiral stationary phases which offer a much wider possibility to select the column. For this reason, an alternative procedure toward the separation of enantiomers is using derivatization with chiral reagents. This type of derivatization generates diastereoisomers which can be separated on non-chiral stationary phases.

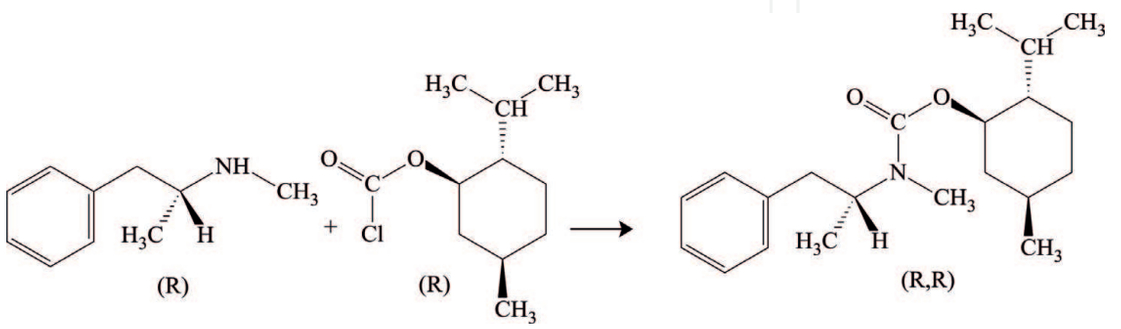
A discussion on the separation of enantiomers on chiral phases without derivatization is beyond the purpose of this chapter. Numerous publications are dedicated to this subject, including papers published in general chromatography journals or in dedicated journals (e.g., *Chirality*), books (see, e.g., [7]), and information on the web.

The separation after derivatization with a pure enantiomer reagent is based on formation of diastereoisomers that can be separated on regular stationary phases. Depending on the nature of the analyte and of the derivatization, different separation techniques can be applied. A variety of common columns are used for such GC separations. The choice of the column is again dependent on the analyte and the derivatization procedure. For example, α-substituted organic acids such as α-chloropropionic, α-bromocaproic, etc. can be derivatized with a specific enantiomer of an amino acid ester (e.g., ethyl 2-aminopropanoate) in the presence of a peptide coupling reagent (benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate or BOP) in a reaction of the type:



(3)

The derivatized acids that are now diastereoisomers (R,S) and (S,S) can be separated on a common capillary column (e.g., a DB-1701 column from Agilent). Another example of derivatization with a chiral reagent is that of methamphetamines with (R)-menthyl chloroformate. This derivatization allows the separation of over-the-counter (R)-methamphetamine from the illicit (S)-methamphetamine. The reaction of the (R)-enantiomer is indicated below [8]:



(4)

The separation of the (R,R) and (S,R) derivatives was possible on a non-chiral column for a GC/MS analysis.

4. Derivatization for improving gas chromatographic detection with other detectors than MS

Gas chromatography (not coupled with mass spectrometry, GC/MS being separately presented) used as an analytical technique can involve various detectors. The variety of such detectors is rather large, and several types include the following: thermal conductivity detector (TCD), flame ionization detector (FID), nitrogen-phosphorus detector (NPD), electron capture detector (ECD), flame photometric detector (FPD), photoionization detector (PID), electrolytic conductivity (Hall), sulfur chemiluminescence, nitrogen chemiluminescence, aroyl luminescence detector (ALD), atomic emission detector (AED), helium ionization detector (HID), vacuum ultraviolet (VUV) absorbance, infrared Doppler (IRD) absorption, FID with catalytic conversion of all analytes in CH_4 (e.g., Polyarc system [9]), etc. The derivatization with the purpose of improving detectability in GC is determined by the type of detector utilized. Most derivatizations are performed precolumn, even if they are applied only with the purpose of improving detection. However, it is important that the derivatization for improving detection does not deteriorate the separation. Preferably, both the detection and the chromatographic separation are improved by the same derivatization. Some specific postcolumn reactions applied to the analytes are part of certain types of detectors such as chemiluminescence detectors, atomic emission detectors (AED), and FID with catalytic conversion into CH_4. Some of these chemical changes in the analytes are not necessarily classified as derivatization reactions.

No specific derivatization is usually recommended to improve sensitivity when using nonselective detectors such as TCD and FID. However, in some cases when the detector is not sensitive to a specific analyte, such as formaldehyde or heavily halogenated compounds, derivatization can be used to enhance detection.

In case of NPD detector, derivatization with nitrogenous compounds can be done, which should give a higher sensitivity. However, this type of derivatization is not very common. An adverse result occurs for the NPD detectors when silylation is performed on the sample. Besides a possible reduction in the NPD response on silylated compounds containing nitrogen, a drastic decrease in the lifetime of the detector may occur, probably due to the excess of silylating reagent that commonly is injected with a derivatized sample and affects the alkali active element of the NPD.

The response of the photoionization detector (PID) depends on the ionization potential of the analyte, and compounds with higher ionization potential are not sensitive in PID, while those with lower ionization potential may have excellent sensitivity, as low as 10^{-12} mg of sample. A derivatization resulting in lowering the ionization potential of the analyte may be beneficial for PID detection. However, derivatization for enhancing PID response is not frequently used.

Some detectors such as electron capture detectors (ECD) may benefit very much from certain derivatization types. ECD (as well as negative chemical ionization mass spectrometry or NCI-MS) can be extremely sensitive, but they are selective to compounds that are able to form more stable negative ions. ECD, for example, can have sensitivity as low as 10^{-13} mg of analyte in the detector compared to the best sensitivity of FID that can be 10^{-8} to 10^{-11} mg of analyte. The efficiency of the process seems to be related to the ease of attaching an electron on the molecule. In ECD this process can be written as follows:

$$A + e^- \rightarrow A^- \tag{5}$$

With some exceptions, ECD response can be correlated with the electron affinity of the analyte [4]. In general, the halogen substituents increase the sensitivity in ECD

in the order I > Br > Cl > F. Multiple substitutions seem to have a cumulative effect. Besides halogens, nitro groups seem to have an effect similar to chlorine groups. For aromatic compounds, the substituents affect the sensitivity of the ECD according to their electron withdrawing capability. Strong electron withdrawing groups such as NO_2 increase the sensitivity of the detection, while electron donating groups reduce it.

A variety of substitution groups containing electronegative elements (halogens) or nitro groups can be attached to an analyte. The procedure to attach these groups is in most cases the typical substitution of an active hydrogen in the analyte Y-H with a group R from a reagent R-X that has the appropriate active X group. Some groups used for enhancing ECD (as well as NCI-MS) sensitivity following an alkylation or aryl derivatization reaction are shown in **Figure 4**, and several substitution groups introduced by acylation, chloroformylation, or sulfonation used for the same purpose are shown in **Figure 5**. Besides alkylation or aryl derivatization, other derivatization techniques used to replace an active hydrogen are applied to introduce into a molecule as a substituent containing halogens or nitro groups enhancing

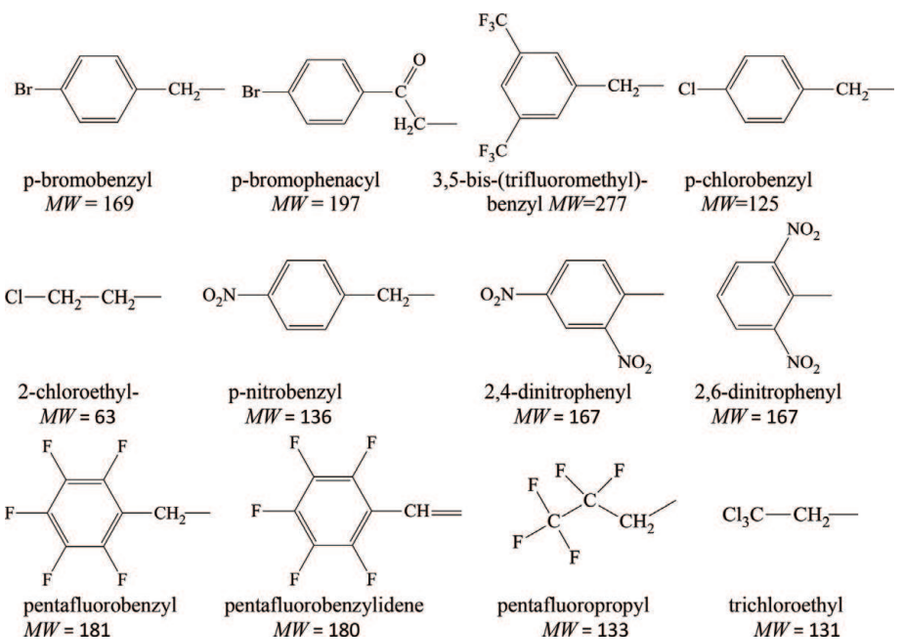


Figure 4.
Substitution groups used in alkylation and aryl derivatization for enhancing ECD (and NCI-MS) detectability (the masses are considered only for the most abundant isotope.).

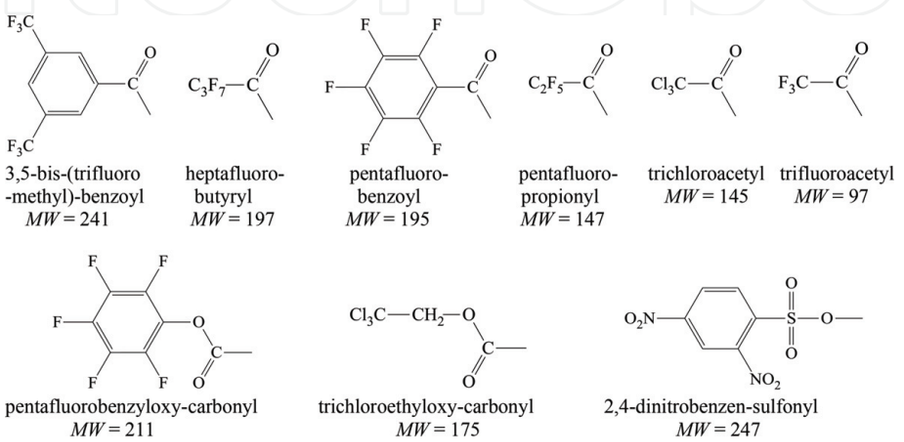


Figure 5.
Substitution groups used in acylation chloroformation and sulfonation for enhancing ECD (and NCI-MS) detectability.

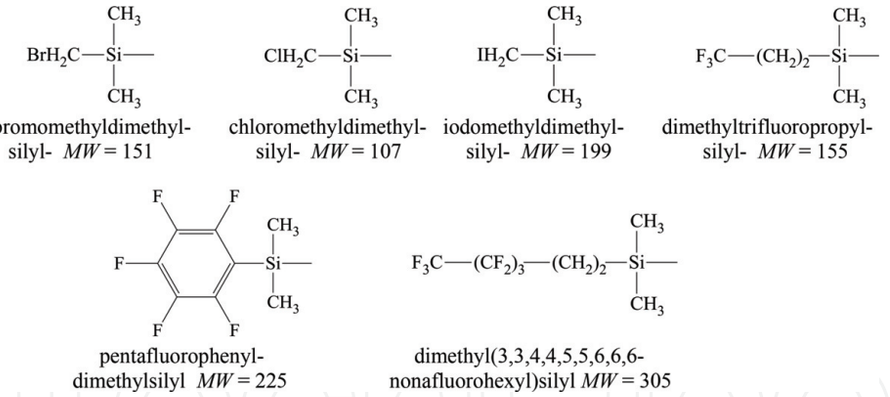


Figure 6.
Substitution groups used in silylation for enhancing ECD (and NCI-MS) detectability.

significantly the detectability of the derivatized analytes by ECD (as well as NCI-MS). Silylation, for example, can be used for this purpose when silyl groups used for derivatization contain halogens. Several silyl groups containing halogens that can be attached to an analyte by silylation with special reagents are given in **Figure 6** [4].

5. Derivatization for improving GC/MS qualitative and quantitative analysis

The most powerful tool used for compound identification purposes is very likely mass spectrometry (spectroscopy). This technique is capable to provide information from very low amounts of material such as that eluting from a chromatographic column and can be easily coupled with a gas chromatograph. Most analyses performed with MS detection (GC/MS or GC/MS/MS) are using EI+ ionization mode with electron impact at 70 eV. The electrons interact with the molecule A to eject an additional electron leaving a positively charged species (with an odd number of electrons) of the type $A^{\blacksquare+}$. The ions also receive energy during electron impact and the excess of energy induces fragmentation. For most molecules, this process can be written as follows:

$$A + e^- \rightarrow A^{\blacksquare+} + 2e^- \text{ and } A^{\blacksquare+} \rightarrow B_i^+ + C_i^{\blacksquare} \quad (6)$$

The fragments B_i^+ are commonly but not always with an even number of electrons. The formation of molecular ions takes place with a range of internal energies, and more than one fragmentation path is possible for a given molecule. Also, the fragments can suffer further fragmentations. In general, the most abundant fragment ion results from the fragmentations that form the most stable products (ion and neutral radical). The abundance of a fragment ion is affected by its stability. For this reason, the intensity of the response of a mass spectrometric detector can be very different for different molecular species, and the prediction of this intensity is difficult. As a result, the improvements in the sensitivity in EI + −type mass spectrometry (in GC/MS using EI+ ionization) are not usually sought (but not impossible) through derivatization.

Derivatization for enhancing sensitivity is, however, frequently applied in NCI-MS. In this technique, the electrons interact with the molecules of the CI gas which is lowering their energy but without forming ions. The ionization of analyte molecules takes place by interaction with the low-energy electrons or with already formed negative ions by electron capture, dissociative electron capture, ion pair formation, or ion molecule reaction. The ionization process with the formation of

negative ions is efficient only for molecules with positive electron affinities. For this reason, the sensitivity in NCI-MS is highly dependent on the electron affinity of the analyte, similarly to the sensitivity in ECD. For enhancing the electron affinity, the derivatization with reagents containing, for example, fluorinated moieties (indicated in **Figures 4–6**) is practiced. The sensitivity of the analytical methods where such derivatization is applicable can have very good sensitivity. For example, derivatization with heptafluorobutyric anhydride of aromatic amines that are present at low trace level in cigarette smoke leads to limit of detection (LOD) values as low as 0.05 ng/cig. for compounds such as 4-aminobiphenyl [10, 11].

The fragmentation pattern generated by EI+ ionization mode that generates a specific mass spectrum of a molecule is very likely the most utilized technique for the identification of the molecular species. For this identification, large libraries of mass spectra are available, and computer algorithms are used for automatic searches. The identification of compounds using mass spectroscopy is not a simple process even with the capabilities offered by the electronic searches in the mass spectral libraries. This is particularly true for analysis of complex mixtures or when the analyzed compound is present in traces. Some compounds do not have a very characteristic mass spectrum, or during the chromatographic process, the separation is not achieved, and it is difficult to make an identification due to the spectra overlapping. Also, numerous compounds may have a mass spectrum that matches more than one compound (with a good quality fit). In such cases, a derivatization with the purpose of obtaining a compound that forms more informative fragments in the mass spectrum can be very useful.

The fragments from derivatized compounds can be used for the identification of unknown compounds using library searches and even when the mass spectrum is not available in the libraries. As an example, the derivatization by silylation allowed the identification of a new pentacyclic triterpenoid present in several bioactive botanicals [12]. An unidentified compound with MW = 456.7 was detected by LC/MS/MS in a rosemary extract. The structure of the compound was elucidated after silylation of the plant material based on the comparison of mass spectrum of the unidentified compound with that of silylated betulinic acid. The new compound was identified as (3β)-3-hydroxy-lupa-18,20(29)-dien-28-oic acid (or betul-18-en-oic acid). The mass spectra of the two acids are shown in **Figure 7**.

The two mass units difference between different fragments from the mass spectra of the two compounds allowed the identification of the new compound structure. Neither free betulinic acid nor betul-18-en-oic acid are volatile, such that the use of GC/MS for identification was possible only after derivatization.

Another special procedure that may be utilized for compound identification based on mass spectra is the use of two parallel derivatizations, one of them being done with an isotope-labeled reagent. Common labeling isotopes are ^{2}H (deuterium, d), ^{13}C, ^{15}N, etc. One such isotopic labeling can be done, for example, using silylation with d_{18}-N,O-bis(trimethylsilyl)-trifluoroacetamide (d_{18}-BSTFA). Derivatization of an aliquot of sample with regular BSTFA and another with d_{18}-BSTFA provides a pairing chromatogram with peaks at retention times that have only small differences from the first but with spectra differing by a number of units. The comparison of the spectra for corresponding peaks (based on retention time) of a given compound allows the calculation of the number of silyl groups attached to that compound. In addition, the fragmentation in the spectra can be better interpreted allowing easier compound identification.

Derivatization in GC/MS analysis may have multiple other utilizations and benefits. For example, quantitative analysis frequently utilizes isotopically labeled internal standards. In an analysis with multiple analytes, addition of an isotopically labeled internal standard for each analyte may become a complex process. When a

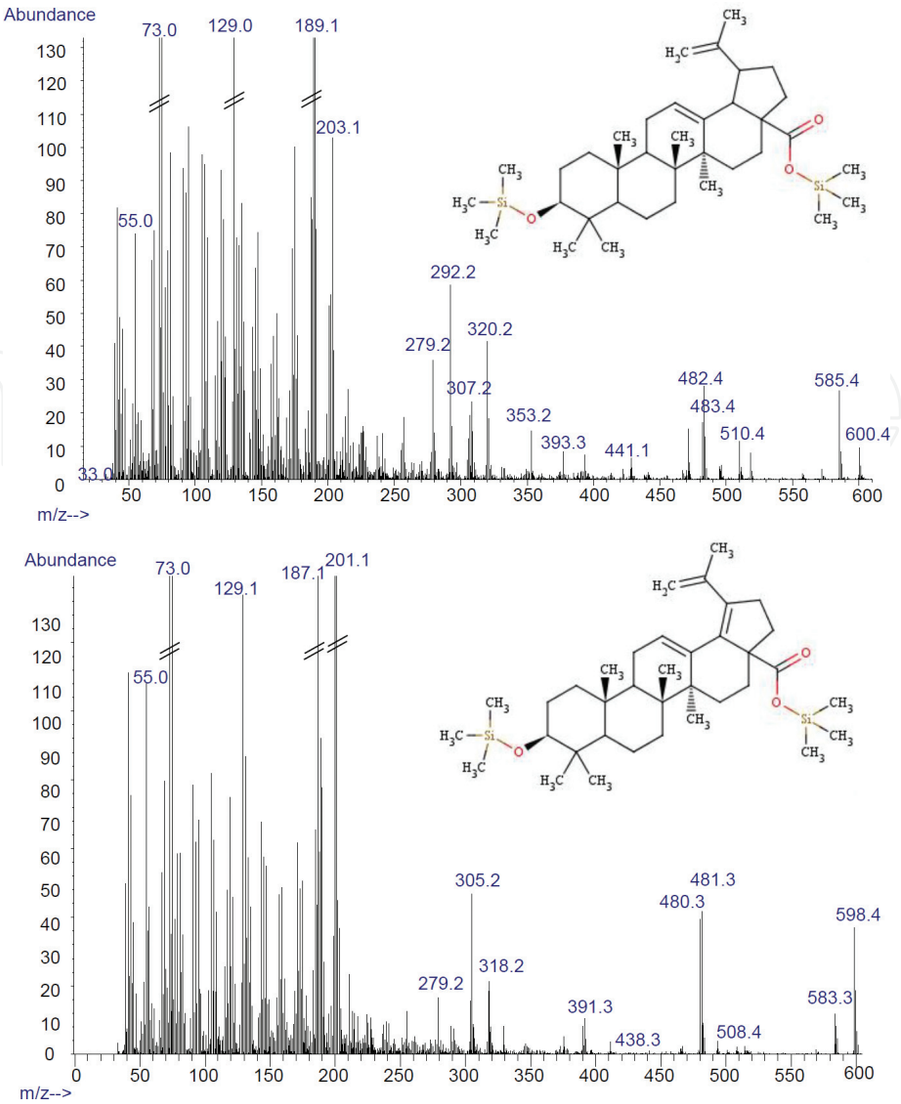


Figure 7.
Mass spectrum of silylated betulinic acid and that of silylated betul-18-en-oic acid.

derivatization is involved in the analysis, this can be done with a non-labeled reagent for the analytes in the sample, while the internal standards are obtained by derivatization of standards with the same reagent but isotopically labeled. Such technique has been proven to be very successful, for example, in the analysis of multiple amino acids (but using an LC/MS/MS procedure [13]).

6. General comments regarding the main types of chemical reactions used in derivatization

Derivatizations as chemical reactions can be classified as follows: (1) reactions with formation of alkyl or aryl derivatives, (2) silylation reactions, (3) reactions with formation of acyl derivatives, (4) reactions of addition to carbon-hetero multiple bonds, (5) reactions with formation of cyclic compounds, and (6) other reactions specific to a certain analysis. The selection of the derivatization reaction is typically done based on the desired property to be brought to the analyte and its possible reactivity. For this reason, the reagent is selected to have moieties that add the desired property to the analyte and also to have the capability to react with the specific functional group of the analyte. The matrix of the sample also has a role in

Properties	Increased acid character → / Increased nucleophile character ←				
Compound	Amine	Amide	Alcohol	Phenol	Acid
First derivatization preference	Acylation	Acylation	Silylation	Silylation	Alkylation
Second derivatization preference	Alkylation	Alkylation	Acylation	Acylation	Silylation

Table 1.
Derivatization preferences for compounds containing active hydrogens.

the choice of a specific derivatization procedure. Initial matrix of the sample is not always suitable for derivatization, and in some cases preliminary sample preparation is necessary to change this matrix. The change can be as simple as drying the initial sample but can also be rather complex [14]. **Table 1** gives a simplified view of preferences for the choice of a derivatization reagent for compounds containing active hydrogens [14].

Besides functionalities with active hydrogens, other functionalities can also be derivatized. Compounds containing carbonyls can be derivatized, for example, using condensation reactions. Some analytes may contain multiple functional groups such as the amino acids. Specific derivatization reactions can be selected for such cases.

7. Reactions with formation of alkyl or aryl derivatives

The formation of alkyl or aryl derivatives is applied to replace the active hydrogens from an analyte with an alkyl (R) or aryl (Ar) group. The replacement can be done in functionalities such as OH, COOH, SH, NH, or CONH. For example, the derivatization with short-chain alkyl bromides or iodides has numerous analytical applications for compounds such as steroids, amino acids, catecholamines, sulfonamides, phenols, barbiturates, organic acids, and mono- and oligosaccharides. A large number of reagents R-X are known, and in a simplified approach, it can be considered that R is carrying a specific property and X a specific reactivity, although the reactivity of a reagent is influenced by both R and X components of the molecule. The type of moiety R and that of reactive group X are guiding the selection process of selecting a reagent for a specific derivatization.

In most alkylation reactions, the analyte acts as a nucleophile (Y:, Y:H, Y:-) reacting in a substitution (SN) with the alkylating reagent R-X, which contains a leaving group X and an alkyl group R:

$$Y{:}H + R - X \rightarrow Y - R + X{:}H \tag{7}$$

Various reagents and conditions were utilized in the derivatizations for analytical purposes. As reagents R-X for alkylations, one of the most commonly used are the alkyl halides, especially alkyl iodides and alkyl bromides. Because some of the derivatizations can be slow and inefficient depending on the analyte and on the reagent, the reaction rate becomes an important parameter for the analytical applicability. The reaction with an alkyl halide for the preparation of methyl or ethyl substituents, for example, is frequently performed either with a specific methylation reagent, in the presence of a catalyst, or in some instances using a particular solvent. The enhancement of the alkylation efficiency can be achieved using several other procedures. For example, for the analytical alkylation of carboxylic

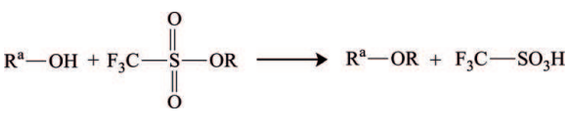
acids, specific cryptands such as crown ethers can be used to solvate the alkali metal portion of an organic acid salts, allowing the anion to be freer and increasing the rate of nucleophilic substitution. One other approach for enhancing the alkylation efficiency is the use of phase transfer alkylation. This approach is based on the formation of a compound easily extractable in an organic phase and on the displacement of the equilibrium in the direction of the formation of the desired product.

One different way of enhancing the alkylation efficiency is the use of different alkylating reagents besides short-chain alkyl bromides or iodides. One example of a halide that is particularly reactive is pentafluorobenzyl bromide. This reagent can be used for the derivatization of a variety of compounds containing active hydrogens. Another reactive halide is 2-bromoacetophenone (phenacyl bromide). This reagent is used mainly for the alkylation of compounds containing more acidic hydrogens such as carboxylic acids. Another example of methylation using a special reagent R-X is applied on carbohydrates [15]. This methylation uses methylsulfinylmethanide anion. The reagent is prepared from dry DMSO and NaH or KH in a reaction as follows:

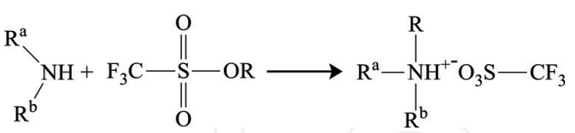
$$(CH_3)_2SO + NaH \rightarrow CH_3 - SOCH_2^-Na^+ + H_2 \quad (8)$$

A polyol or a monosaccharide dissolved in DMSO is easily methylated with methylsulfinyl-methanide anion.

Other alkylating reagents are known (different X in R-X), also reacting in a nucleophilic substitution. For example, dimethyl sulfate can be used for alkylations. Alkylfluoromethyl-sulfonates are even more reactive than sulfates, and the reaction may take place with the active hydrogen even from alcohols or amines as follows:



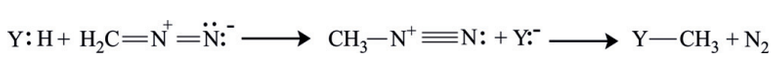
(9)



(10)

Even tertiary amines, such as pyridine, also react with this type of reagent forming quaternary ammonium salts. The alkylation with alkylfluorosulfonates can be catalyzed as other alkylation reactions for increasing the reaction rate. A catalyst that can be used in this reaction is $Hg(CN)_2$.

Diazomethane is another common alkylating (methylating) reagent. The alkylation using diazomethane is assumed to take place as follows:

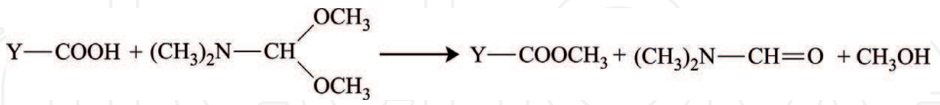


(11)

Diazomethane is a gaseous unstable substance, which cannot be stored for long periods of time. It is usually prepared in small quantities and used immediately with or without an intermediate step of dissolution in ether. The preparation can be done from different N-nitroso-N-alkyl compounds in a reaction with a base. A common preparation uses N-nitroso-N-alkyl-p-toluenesulfonamide (Diazald). Methylation with diazomethane may require addition of a Lewis acid catalyst such as BF_3. The

methylation of partly acetylated sugars and amino sugars using diazomethane and BF_3 in ether leads to the methylation of the free OH groups without the migration or substitution of the existent acyl groups.

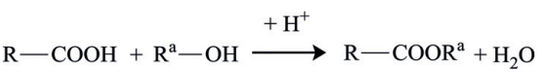
A common alkylation of acidic analytes such as carboxylic acids, phenols, and thiols is performed using another type of alkylating reagent, namely, N,N-dimethylformamide dialkyl acetals. N,N-Dimethylformamide dimethyl acetal (Methyl-8®) is commonly used for methylations. For a compound containing a COOH group, the reaction with this reagent takes place as follows:



(12)

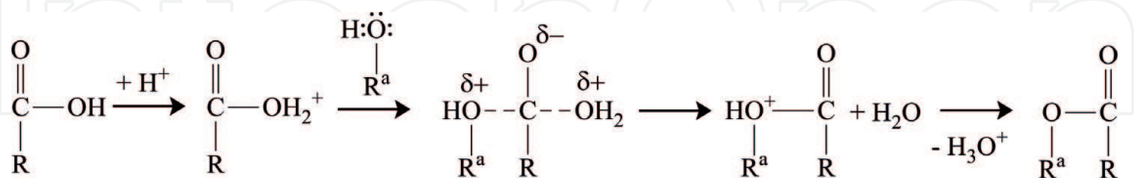
The compounds with acidic hydrogens can also be alkylated (methylated) using trimethyl orthoacetate, alkyl-p-tolyltriazenes (R—NH—N=N—C_6H_4—CH_3), and O-alkyl isoureas are also used for the formation of analytes containing acidic hydrogens, imino esters, etc.

Alcohols can also act as alkylating reagents in particular when the analyte contains a more acidic hydrogen. Catalyst such as HCl, BF_3, CF_3 COOH or a cation exchange resin in H^+ form is also frequently added to facilitate the reaction. The addition of HCl can be made as a water solution or as gaseous HCl that does not bring additional water to the reaction medium. The formation of alkyl or aryl derivatives of acids is a particularly important reaction known as esterification. Derivatization by esterification has been used with acids as the analyte and the alcohol as the reagent and also with the alcohol as the analyte and the acid the reagent. The esterification can be viewed either as the acid alkylation or as the acylation of the alcohol (see also the esterification mechanism). This reaction is typically catalyzed by strong acids and can be written as follows:



(13)

The mechanism of ester formation can be summarized by the following series of reactions:



(14)

The esterification efficiency can be improved by removing the water formed in this reaction. This can be done using a chemical reagent or distillation when the compounds of interest boil above 100°C. Among the materials able to eliminate water are desiccants such as anhydrous $MgSO_4$, molecular sieves, or substances that react with water such as CaC_2, $(CH_3)_2C(OCH_3)_2$ (2,2-dimethoxypropane), and even an appropriately chosen acid anhydride that reacts faster with water than with the reacting alcohol. The derivatization also may be performed in the presence of $SOCl_2$ (thionyl chloride), which reacts with the water assisting in its removal, and when present in excess, may react with the alcohols forming alkyl chlorides or with the acids forming acyl chlorides. Chloride is a better leaving group in a nucleophilic

alkylation reaction, and the efficiency of alkylation increases. Acids also can be esterified using a mixture of an alcohol and an acyl halide.

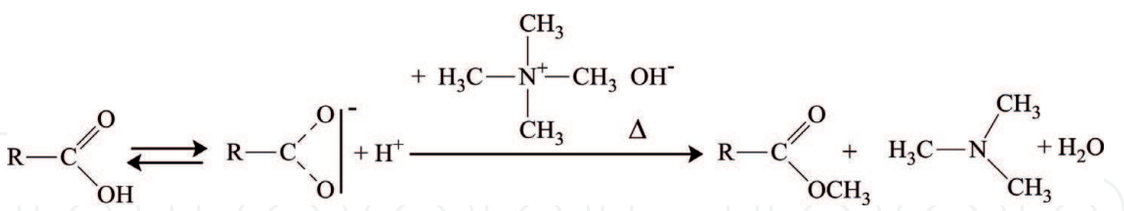
One procedure for the formation of esters with less active organic acids applies the addition of dicyclohexylcarbodiimide (DCCI) in the derivatization process, to facilitate esterification. The reaction can be performed by adding to the acids that need to be analyzed the appropriate alcohol and DCCI usually in a solvent such as pyridine. Dicyclohexylurea, which is formed in the reaction, is not soluble in pyridine and can be separated. Besides DCCI, other carbodiimides can be used in the reaction of acids and alcohols. Among these are carbonyldiimidazole (CDI), 6-chloro-1-p-chlorobenzensulfonyloxybenzotriazole (CCBBT), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDAC), etc. Also, 2-chloro-1-methylpyridinium iodide, 2,4,6-triisopropylbenzenesulfonyl chloride, trialkyloxonium fluoroborate, etc. can be used to facilitate esterification.

Transesterification is another technique applicable for obtaining certain alkyl derivatives of acids (or acyl derivatives of alcohols). The reaction can be written as follows:

(15)

Transesterification can be catalyzed by acids (or Lewis acids) such as HCl, BF_3, and H_2SO_4 or by bases such as CH_3OK, CH_3ONa, or C_4H_9ONa. The basic catalysts are commonly used for the methanolysis of triglycerides, followed by the analysis of the fatty acid methyl esters using GC or GC/MS [16].

A special alkylation can be achieved online during the heating in the injection port of a gas chromatograph using tertraalkylammonium hydroxides or alkylarylammonium hydroxides. Tetramethylammonium hydroxide (TMAH) is the most common reagents of this type. The reaction takes place as follows (Δ indicates heating):



(16)

Numerous other reactive compounds may be used for replacing active hydrogens in specific compounds. For example, epoxides, aziridines, and episulfides react easily with compounds with active hydrogens. Formation of a second group containing an active hydrogen may preclude the use of such reagents for analytical purposes.

Besides the desired derivatives, certain unexpected compounds that can be considered artifacts for the particular analysis can also be formed in alkylation reactions. The artifacts may be formed from unexpected interactions of the reagent with the analyte or may be a result of undesired effects of the catalysts or medium used for derivatization. In some cases, the control of the alkylation process may be difficult. Longer or shorter reaction times or intervals between derivatization and analysis may lead to errors, even when an internal standard is used for quantitation.

One common case of artifact formation occurs during the reaction with compounds containing O-acyl or N-acyl groups, such as previously acylated

carbohydrates, glycolipids, or glycoproteins, in particular when the reaction is done with short-chain alkyl bromides or iodides. When the OH groups of different sugars or NH_2 groups of amino sugars were already protected with acyl groups, it was noted that, depending on the catalyst and the chosen medium, these acyl groups can be replaced by alkyl groups, or they may migrate from one position (such as C1) to other positions.

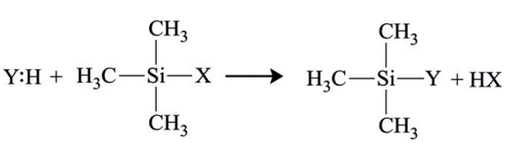
Oxidation is another common side reaction when using Ag_2O as a catalyst. The oxidation effect of Ag_2O can be seen on free sugars as well as when attempting to permethylate peptides. Sulfhydryl groups are particularly sensitive to oxidation with Ag_2O as a catalyst. The use of methylsulfinyl carbanion as a methylating reagent may also produce undesired side reactions with certain esters generating methylsulfinylketones. Also, strong alkylating reagents may produce undesired artifacts by unexpected alkylations.

The derivatization with the purpose of obtaining aryl derivatives is similar in many respects to the alkylation reaction. The reaction takes place with compounds containing active hydrogens. Simple aryl halides are generally resistant to be attacked by nucleophiles and do not react similar to alkyl halides. This low reactivity can be significantly increased by changes in the structure of aryl halide or in the reaction conditions. The nucleophilic displacement can become very rapid when the aryl halide is substituted with electron attracting groups such as NO_2.

8. Silylation reactions

Silylation is the chemical reaction of replacing a reactive hydrogen atom in OH, COOH, SH, NH, CONH, POH, SOH, or enolisable carbonyl with a silyl group, most frequently with trimethylsilyl (TMS). A large number of analytical methods involve silylation applied to alcohols including carbohydrates [17], phenols [18], amines, sterols [19], etc. The purpose of silylation in chromatography is mainly to reduce the polarity of the analyte, increase its stability, and improve the GC behavior. The differences in the mass spectra of the silylated compounds as compared to the initial analyte may also be an advantage for detectability. However, the mass spectra of many silylated compounds may not be available in common mass spectral libraries. Also, the silylated compounds plus the commonly present excess of silylating reagent may deteriorate some types of stationary phases such as that of Carbowax (polyethylene glycol)-type columns, and for this reason, their separation cannot be done on such columns.

Silylation can be performed on specific analytes or directly on complex samples such as a plant material (see, e.g., [12]). The silylating agent and the solvent can play the double role of extractant and silylating reagent. Many publications describe the use of silylation reactions for analytical purposes (e.g., [1, 5, 20]). The reaction of an analyte Y:H with the formation of a TMS derivative can be written as follows:



(17)

The molecular weight for TMS is 73.047 calculated considering in the elemental composition of only the masses of the most abundant isotope. Numerous reagents have been synthesized to be used in silylations. Various aprotic solvents can be used as medium for silylation. The analysis can be focused on one analyte or on a mixture of analytes. The main factors contributing to the increase of the efficiency and the rate of the silylation reaction are the silyl donor ability of the reagent and the ease of

silylation of different functional groups in the analyte. The solvent (or mixture of solvents) used as a medium and the compounds present or added in the silylation medium may also play a role for silylation efficiency. The reagent excess is sometimes important for displacing the equilibrium in the desired direction, and usually an excess up to ten times larger than stoichiometrically needed is used for silylation. Temperature also increases reaction rate, as expected, and heating of the sample with the reagents at temperatures around 70°C for 15 to 30 min is common. Some reagents used for trimethylsilylation are shown in **Figure 8** [14].

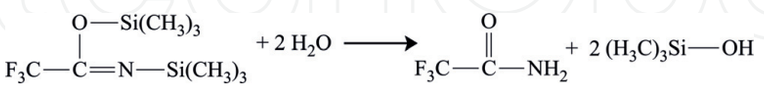
The approximate order of the increasing silyl donor ability for the reagents shown in **Figure 8** is HMDS < TMCS < MSA < TMSA < TMSDEA < TMSDMA < MSTFA < BSA < BSTFA < TMSI. This order may be different on particular substrates where other reagents or reagent mixtures may be more reactive.

Silylation reagents can be used pure or in mixtures of two or even three reagents. The reagent mixtures may provide a more efficient silylation for specific compounds. For example, silylation of 3,4-dimethoxyphenylethylamine with BSA leads to the substitution of only one active hydrogen in the NH_2 group, while the silylation with BSA in the presence of 5% TMCS produces silylation of both hydrogens in the NH_2 [21]. A common silylating mixture is BSTFA with 1% TMCS.

One of the determining factors regarding the silylation efficiency is the nature of the molecule Y:H that is being silylated (the analyte) and plays a crucial role in the choice of the derivatization conditions. It was noticed experimentally that the decreasing ease of silylation follows approximately the order shown in **Table 2**.

In general, the silylation of OH and COOH groups takes place with better results than that of NH_2, CONH, or NH groups. Excellent results are obtained, for example, for the analysis of phenols after silylation [19]. A chromatogram of a solution containing a mixture of phenols at concentrations between 2.0 and 2.5 μg/mL in DMF, derivatized with BSTFA, separated on a BPX-5 chromatographic column (SGE Anal. Sci.), followed by MS analysis in single-ion monitoring (SIM) mode is shown in **Figure 9**. Details regarding the analyzed phenols are given in **Table 3**.

Besides organic active hydrogens, several inorganic compounds with active hydrogens can also react with silylating reagents. Among these are H_2O, H_2O_2, and strong inorganic acids. Also, some salts of the acids may be silylated. The reaction of silylating reagents with water imposes that water should be at the low level in the matrix or the solution of the analytes. The reaction with water takes place as follows:



(18)

In many solvents used as medium for derivatization, the trimethylsilanol formed in the reaction with water is separated as a distinct layer of solvent. The formation of two layers impedes a proper sampling of the derivatized material in the GC/MS instrument. In addition to that, the presence of an excess of water suppresses the derivatization of other compounds. The silylation is not recommended on samples with a water content higher than about 10%.

The silylation reaction is commonly performed in a solvent that does not have active hydrogens. The most commonly used solvents as a medium for silylation are dimethylformamide (DMF), pyridine, and acetonitrile. The main role of the solvent is to dissolve the analyte and the reagents. The by-product HX of silylation shown in reaction (17) can be an acid, a base, or a neutral compound. As examples, for TMCS the by-product is HCl, for HMDS the

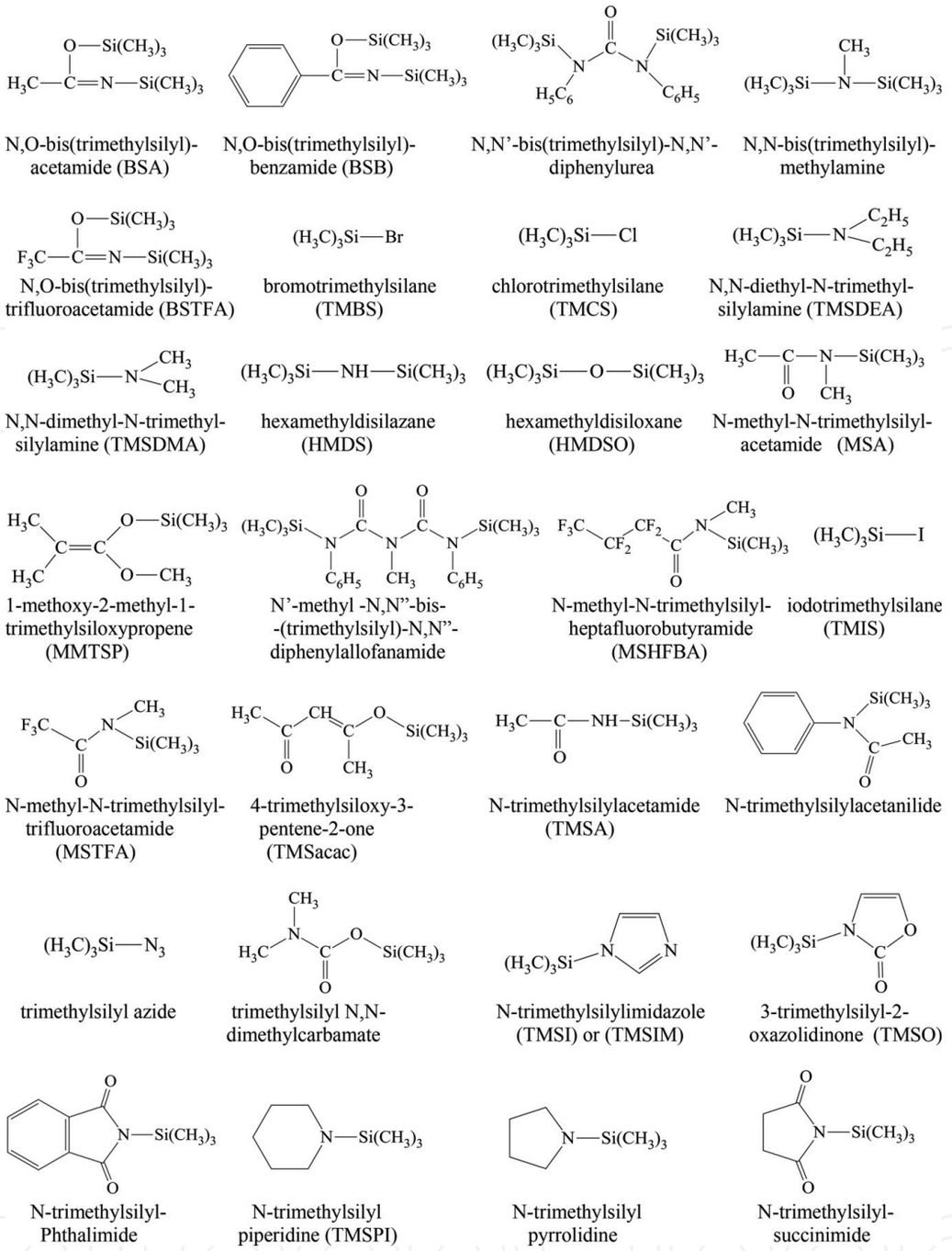


Figure 8.
Some reagents used for trimethylsilylation.

by-product is NH_3, for BSTFA the by-product is N-TMS-trifluoroacetamide, and for TMSI the by-product is imidazole. When the silylation reagent generates an acid as a by-product of the reaction, this may interfere with the silylation. For this reason, silylation can be promoted by any acid acceptor used as solvent or present in the solvent. Among such solvents are pyridine, triethylamine, and to a lower extent DMF. They can be used as both solvents and acid acceptors. Mixtures of solvents are commonly used for both enhancing solubility and promoting silylation. For example, formamide in the presence of pyridine may react with an acidic by-product generating CO and an ammonium salt. The addition of basic compounds to the silylation reaction may also influence the

	Compound	Functional group	Decreasing reactivity
(1)	Primary alcohol	OH	↓
(2)	Secondary alcohol	OH	
(3)	Tertiary alcohol	OH	
(4)	Phenol	OH	
(5)	Thiophenol	SH	
(6)	Aliphatic acid	COOH	
(7)	Aromatic acid	COOH	
(8)	Primary amine	NH_2	
(9)	Thiol	SH	
(10)	Amide	$CONH_2$	
(11)	N-TMS amide	$CONH\text{-}Si(CH_3)_3$	
(12)	Secondary amine	NH	
(13)	Indole	NH	

Table 2.
Several functional groups that can be silylated (listed in the approximate order of decreasing ease of silylation).

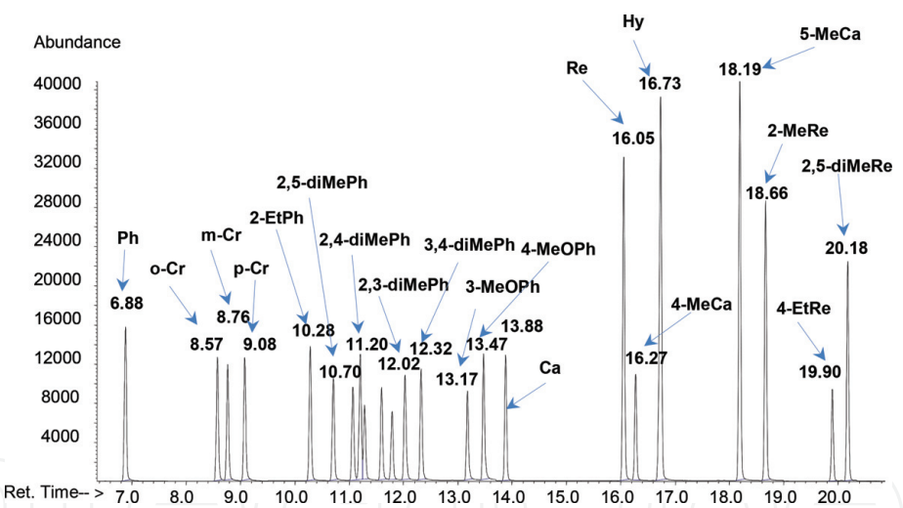
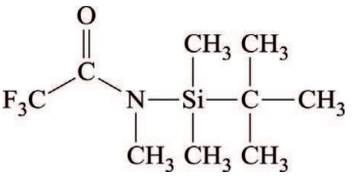


Figure 9.
Chromatogram of a set of phenol standards in DMF with the concentrations between 2.0 and 2.5 μg/mL derivatized with BSTFA, separated on a BPX-5 chromatographic column followed by MS analysis.

efficiency of the silylation. Also, some compounds may act as catalysts for silylation.

Although the TMS derivatives are by far the most commonly used in the derivatization for analytical purposes, other substituents in the silyl group can be used as reagents. Several such groups are indicated in **Figure 10**. The groups can be present in a variety of reagents connected to leaving groups "X-" such as Cl-, imidazolyl, $F_3C\text{-}(CO)\text{-}N(CH_3)$-, etc. For example, a common reagent containing *tert*-butyldimethylsilyl group is N-methyl-N-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA), which has the following structure:



The use of different groups than TMS may serve different purposes. For example, a fluorinated or brominated group may enhance significantly the detection sensitivity when using ECD or NCI-MS. Also, the stability toward hydrolysis of compounds silylated with different groups than TMS may be higher, and such silylation can be advantageous. This is, for example, the case of *tert*-butyldimethylsilyl group that is typically more stable to hydrolysis than trimethylsilyl.

As an example, silylation of amino acids with MTBSTFA is commonly used [22, 23], and it is preferred to the silylation generating TMS derivatives. The chromatogram of a set of amino acid standards with the concentration of 0.05 μmol/mL derivatized with MTBSTFA and separated on a DB-5MS chromatographic column (from Agilent) followed by MS analysis is shown in **Figure 11**. Details regarding the analyzed amino acids are given in **Table 4**.

In most situations, silylation generates only the desired derivatives. However, there are cases when the expected silylated compound is not formed, and either the silylation is not complete, or some compounds such as aldehydes, ketones, or esters with no obvious active hydrogen generate silylated compounds. Incomplete

No.	Compound	Ret. time	m/z	Abrrev.	No.	Compound	Ret. time	m/z	Abrrev.
(1)	Phenol	6.88	166	Ph	(14)	3,4-Dimethylphenol	12.32	194	3,4-diMePh
(2)	o-Cresol	8.57	180	o-Cr	(15)	3-Methoxyphenol	13.17	196	3-MeOPh
(3)	m-Cresol	8.76	180	m-Cr	(16)	4-Methoxyphenol	13.47	196	4-MeOPh
(4)	p-Cresol	9.08	180	p-Cr	(17)	Catechol	13.88	254	Ca
(5)	2-Ethylphenol	10.28	194	2-EtPh	(18)	Resorcinol	16.05	254	Re
(6)	2,5-Dimethylphenol	10.70	194	2,5-diMePh	(19)	4-Methylcatechol	16.27	268	4-MeCa
(7)	3,5-Dimethylphenol	11.07	194	3,5-diMePh	(20)	Hydroquinone	16.73	254	Hy
(8)	2,4-Dimethylphenol	11.20	194	2,4 diMePh	(21)	3-Methylcatechol	16.71	268	3-MeCa
(9)	2-Methoxyphenol	11.28	196	2-MeOPh	(22)	5-Methylresorcinol	18.19	268	5-MeCa
(10)	4-Ethylphenol	11.59	194	4-EtPh	(23)	2-Methylresorcinol	18.66	268	2-MeRe
(11)	4-Chlorophenol	11.71	185	4-ClPh	(24)	4-Ethylresorcinol	19.90	282	4-EtRe
(12)	2,6-Dimethylphenol	11.79	194	2,6-diMePh	(25)	2,5-Dimethylresorcinol	20.18	282	2,5-diMeRe
(13)	2,3-Dimethylphenol	12.02	194	2,3-dimePh					

Table 3.
Details regarding the analyzed phenols with the chromatogram shown in Figure 9.

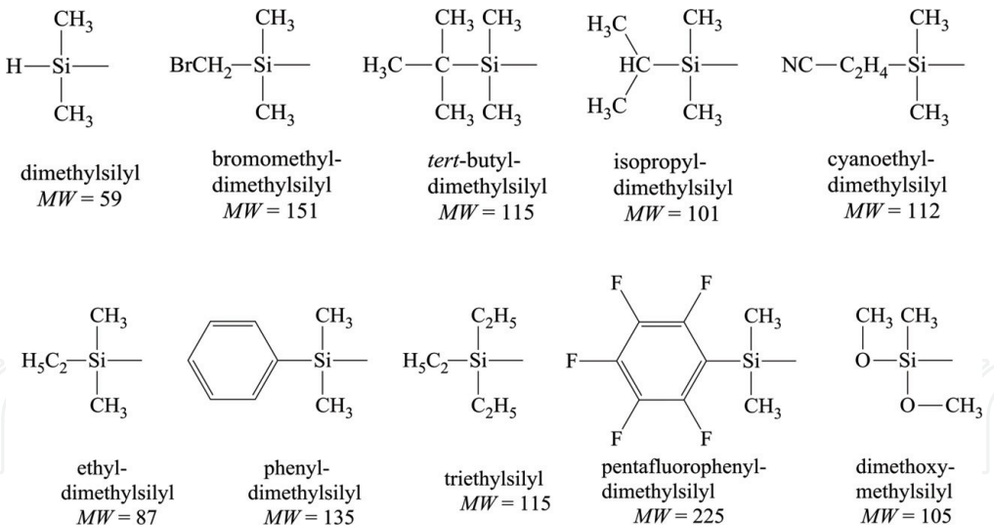


Figure 10.
Examples of silyl groups different from TMS used in silylation reagents.

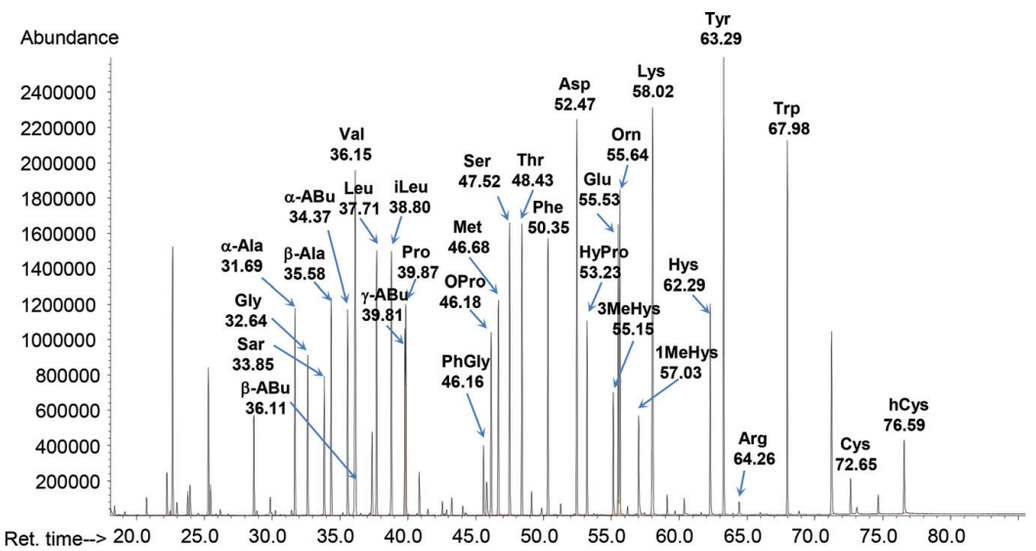


Figure 11.
Chromatogram of a set of amino acid standards with the concentration of 0.05 μmol/mL derivatized with MTBSTFA separated on a DB-5MS chromatographic column.

silylation is usually the result of inappropriate reaction conditions. However, when compounds with multiple functionalities are silylated, it is possible to generate a variety of derivatized compounds, regardless of the intention to obtain fully silylated or partly silylated compounds.

In some cases, artifacts are formed due to the modification of the analyte under the influence of the reagents during derivatization. For example, when the silylation is done in basic or acidic conditions, the analytes that are sensitive to acidic or basic media may suffer unexpected transformations. The most frequent artifacts with compounds not containing obvious active hydrogens occur with aldehydes. Some aldehydes are able to undergo two types of chemical reactions with formation of OH groups, namely, enolization and acetal formation in the presence of water. The OH groups formed in this manner react with different silylating reagents and give the corresponding silylated products. Although the enolization or the acetal formation is negligible for the initial aldehyde, the reactions may be significantly displaced toward the formation of the silylated compounds of the enol or of the acetal. Artifacts can also be generated when the reaction is allowed to continue for an

Peak No.	Amino acid	Abbrev.	MW	Formula + x TBDMS	MW + x TBDMS	Charact. ion	Ret. time
(1)	α-Alanine	α-Ala	89.09	$C_{15}H_{35}NO_2Si_2$	317	260	31.69
(2)	Glycine	Gly	75.07	$C_{14}H_{33}NO_2Si_2$	303	246	32.63
(3)	Sarcosine	Sar	89.09	$C_{15}H_{35}NO_2Si_2$	317	260	33.85
(4)	α-Amino-n-butyric acid	α-ABu	103.10	$C_{16}H_{37}NO_2Si_2$	331	274	34.36
(5)	β-Alanine	β-Ala	89.09	$C_{15}H_{35}NO_2Si_2$	317	260	35.58
(6)	Urea		60.06	$C_{13}H_{32}N_2OSi_2$	288	231	36.01
(7)	β-Aminoisobutyric acid	β-ABu	103.10	$C_{16}H_{37}NO_2Si_2$	331	274	36.11
(8)	Valine	Val	117.15	$C_{17}H_{39}NO_2Si_2$	345	186	36.15
(9)	Leucine	Leu	131.17	$C_{18}H_{41}NO_2Si_2$	359	200	37.71
(10)	Norleucine		131.17	$C_{18}H_{41}NO_2Si_2$	359	200	38.8
(11)	Isoleucine	iLeu	131.17	$C_{18}H_{41}NO_2Si_2$	359	200	38.8
(12)	γ-Aminobutyric acid	γ-ABu	103.10	$C_{16}H_{37}NO_2Si_2$	331	274	39.79
(13)	Proline	Pro	115.13	$C_{17}H_{37}NO_2Si_2$	343	184	39.87
(14)	2-Phenylglycine	PhGly	151.17	$C_{20}H_{37}NO_2Si_2$	379	220	46.16
(15)	5-Oxoproline	oPro	129.13	$C_{17}H_{35}NO_3Si_2$	357	300	46.18
(16)	Methionine	Met	149.20	$C_{17}H_{39}NO_2SSi_2$	377	320	46.68
(17)	Serine	Ser	105.09	$C_{21}H_{49}NO_3Si_3$	447	390	47.52
(18)	Threonine	Thr	119.12	$C_{22}H_{51}NO_3Si_3$	461	404	48.43
(19)	Phenylalanine	Phe	165.19	$C_{21}H_{39}NO_2Si_2$	393	336	50.35
(20)	Aspartic acid	Asp	133.10	$C_{22}H_{49}NO_4Si_3$	475	418	52.47
(21)	Hydroxyproline	HyPro	131.13	$C_{23}H_{51}NO_3Si_3$	473	314	53.23
(22)	3-Methyl-L-histidine	3MeHys	169.20	$C_{19}H_{39}N_3O_2Si_2$	397	340	55.15
(23)	Glutamic acid	Glu	147.13	$C_{23}H_{51}NO_4Si_3$	489	432	55.53
(24)	Ornithine	Orn	132.20	$C_{23}H_{54}N_2O_2Si_3$	474	286	55.64
(25)	1-Methyl-L-histidine	1MeHys	169.20	$C_{19}H_{39}N_3O_2Si_2$	397	302	57.03
(26)	Lysine	Lys	146.19	$C_{24}H_{56}N_2O_2Si_3$	488	300	58.02
(27)	α-Aminoadipic acid		161.20	$C_{24}H_{53}NO_4Si_3$	503	446	58.06
(28)	Histidine	Hys	155.16	$C_{24}H_{51}N_3O_2Si_3$	497	440	62.29
(29)	Tyrosine	Tyr	181.19	$C_{27}H_{53}NO_3Si_3$	523	302	63.29
(30)	Arginine	Arg	174.20	$C_{24}H_{56}N_4O_2Si_3$	516	144	64.26
(31)	Tryptophan	Trp	204.22	$C_{29}H_{54}N_2O_2Si_3$	546	244	67.98
(32)	Cystine	Cys	240.30	$C_{28}H_{64}N_2O_4S_2Si_4$	668	348	72.65
(33)	Homocystine	hCys	268.30	$C_{32}H_{72}N_2O_4S_2Si_4$	724	362	76.59

Table 4.
Details regarding the analyzed amino acids with the chromatogram shown in Figure 11.

extended period of time. Other uncommon reactions with a specific silylation reagent and analyte may occur. An example of an uncommon reaction is the ring opening of flavanones.

9. Acylation reactions

The formation of acyl derivatives is applied for replacing the active hydrogens from an analyte in functionalities such as OH, SH, NH [11, 24], CONH, etc. The acylation is also used for reducing polarity and improving the behavior of the analytes in the chromatographic column. Acylation may confer a better volatility of the analytes, although not as marked as for silylation or methylation. Only the derivatization with acetyl groups or with fluorinated acyl groups (not heavier than heptafluorobutyryl) improves volatility, while other heavier acyl groups are not suitable for this purpose. Acetylation, for example, can be used for compounds such as monosaccharides and amino acids to allow GC analysis. The detectability improvement on the other hand is a very common purpose for acylation. Acylation with fluorinated compounds is frequently used for enhancing detectability in GC with ECD or NCI-MS detection. Other uses of acylation include the enhancement of separation of chiral compounds, etc.

Most acylation reactions are nucleophilic substitutions where the analyte is a nucleophile (Y:, Y:H, Y:-) reacting with the acylating reagent RCO-X that contains a leaving group X and an acyl group RCO: as shown in the following reaction:

(19)

Some common acyl groups present in acylation reagents are indicated in **Table 5**.

As shown in **Table 5**, the acyl groups in the reagent can be attached to various "X" groups. One such group is OH and among the acylating reagents are some free acids. When nucleophile is an alcohol, the reaction is known as esterification and has been discussed in Section 7. The acylation with acids can be applied besides alcohols to certain thiols, phenols, amines, etc. and can be written as follows:

$$Y:H + R - COOH \rightarrow R - COY + H_2O \tag{20}$$

The reaction can be displaced toward the formation of the acyl derivatives by eliminating the water using compounds such as anhydrous $MgSO_4$, molecular sieve, or substances that react with water such as CaC_2, or $(CH_3)_2C(OCH_3)_2$. Dicyclohexylcarbodiimide (DCCI) also is used for modifying the yield of the desired product. The reaction with reagents containing a carboxylic acid reactive group also can be done in the presence of 2,4,6-trichlorobenzoyl chloride or with various sulfonyl chlorides such as 2,4,6-triisopropyl-benzenesulfonyl chloride or 2,4,6-trimethyl-benzenesulfonyl chloride. The reaction of amines with acids can be displaced toward the formation of the amides using a peptide coupling reagent such as benzotriazol-1-yl-oxy-tris(dimethyl-amino)-phosphonium hexafluorophosphate (BOP), diethyl cyanophosphonate, O-benzotriazol-1-yl-N,N,N′,N′-bis (tetramethylene)uronium hexafluorophosphate, 2,2′-dipyridyl disulfide + triphenylphosphine, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDAC), etc.

Common acylating reagents are acyl halides such as chlorides or bromides, which are reactive compounds suitable for acylation. The reaction of an acyl chloride with an amine, for example, takes place as follows:

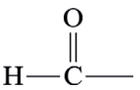
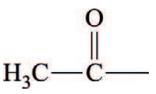
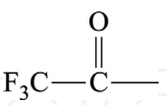
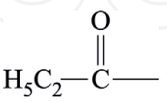
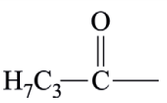
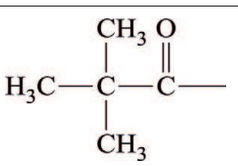
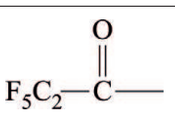
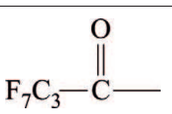
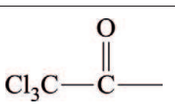
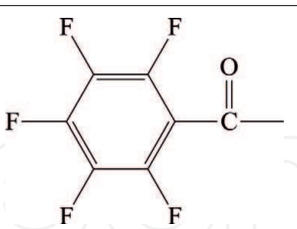
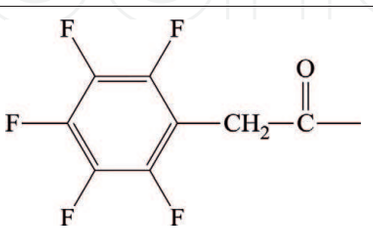
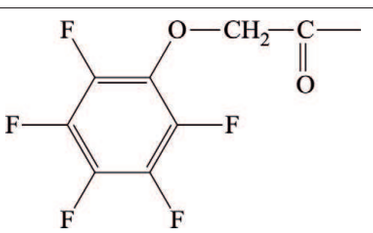
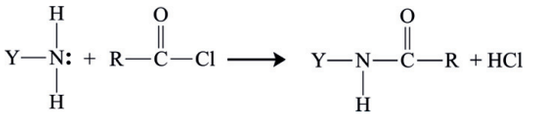
Group	Group structure	Mass of the group	Example of reagents	Common analytes
Formyl		29	Formic acid	Steroids
Acetyl		43	Acetyl chloride, acetic anhydride	Alcohols
Trifluoroacetyl		97	N-Methyl-bis (trifluoroacetamide), bis(trifluoroacetamide), trifluoroacetic acid (TFA)	Alcohols
Propionyl		57	Propionic anhydride	Alcohols
Butyryl		71	Butyric anhydride	Alcohols
2,2-Dimethyl-propionyl-(pivaloyl)		85	Pivaloyl chloride, pivalic anhydride	Amino acids
Pentafluoro-propionyl		147	Pentafluoropropionic anhydride (PFPA)	Alcohols, amines, amino acids
Heptafluorobutyryl		197	Heptafluorobutyric anhydride (HFBA), heptafluorobuty rylimidazole	Alcohols, amines, amino acids
Trichloroacetyl		145	Trichloroacetic anhydride	Alcohols
Pentafluorobenzoyl		195	Pentafluorobenzoyl chloride, pentafluorobenzoyl-imidazole	Alcohols, amides
(Pentafluorophenyl)-acetyl		209	(Pentafluorophenyl)-acetyl chloride	Alcohols, amides
(Pentafluorophenoxy)-acetyl		225	(Pentafluorophenoxy)-acetyl chloride	Alcohols

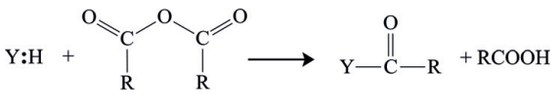
Table 5.
Some common groups present in acylating reagents used in derivatizations for GC analysis [14].



(21)

Since the reactivity of amides is lower than that of amines, the second hydrogen in the amine is more difficult to replace. Also, steric hindrance may negatively influence the reaction. The generation of a strong acid such as HCl is a disadvantage in the reaction with acyl halides, and usually the acid should be removed either by adding basic compounds such as Na_2CO_3 or $MgCO_3$ or using pyridine as the reaction medium. The high reactivity of acyl halides is used for the acylation of compounds with less reactive hydrogens. Certain carbonyl cyanides react similarly to acyl chlorides.

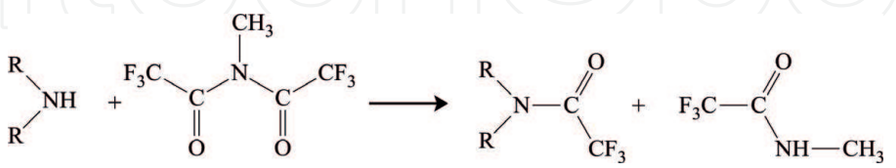
The disadvantage of generating a strong inorganic acid in the acylation with acyl halides also can be avoided by having, instead of the acyl halide, an anhydride. The reaction of Y:H with an anhydride takes place as follows:



(22)

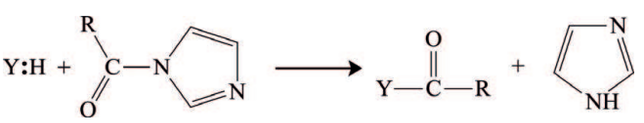
The acid resulting together with the acylated compound is not a strong acid such as HCl. The anhydrides of trifluoroacetic acid (TFA), pentafluoropropionic anhydride (PFPA), and heptafluorobutyric (HFBA) acids are commonly used for derivatization of alcohols, phenols, amines, etc., with the purpose of enhancing detectability (by ECD or NCI-MS) and also for improving the chromatographic behavior (higher volatility, better thermal stability, better separation). The volatility of fluorinated compounds allows the GC applications. The reactivity of the perfluorinated anhydrides increases in the order HFBA < PFPA < TFA. However, the differences are not significant. Once formed, the heptafluorobutyrates are more stable to hydrolysis than the trifluoroacetates. An inert solvent such as CH_2Cl_2, ether, ethyl acetate, acetone, tetrahydrofuran or in CH_3CN, etc. can be used as a medium for the reaction with perfluoroanhydrides. For the neutralization of the acids formed during derivatization, the basic compounds such as triethylamine, pyridine, or even solid $NaHCO_3$ can be utilized.

In order to avoid the formation of water or of a strong acid in the acylation reaction, certain amides such as N-methyl-bis(trifluoroacetamide), bis(trifluoroacetamide), or 2,2,2-trifluoro-N-methyl-N-(2,2,2-trifluoroacetyl)acetamide (MBTFA) can be used as reagents. Acylation of amines takes place at room temperature. Solvents such as CH_3CN, pyridine, DMSO, or THF can be used as a reaction medium:



(23)

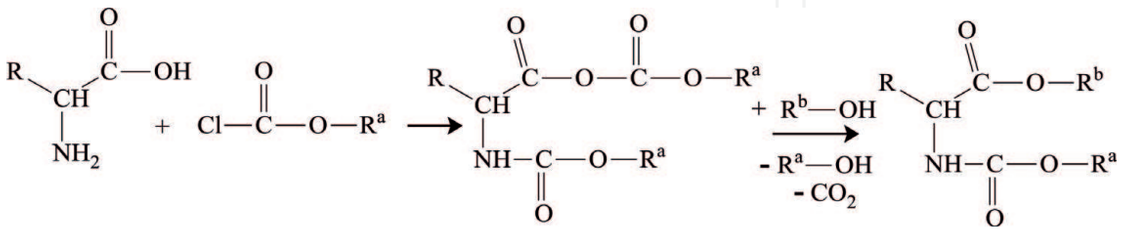
One other procedure successfully applied to obtain acyl derivatives is the use of acyl imidazoles as reagents. This class of compounds reacts with analytes containing alcohol, primary and secondary amino groups, or thiols. The reaction generates as a by-product imidazole:



(24)

Succinimidyl esters also can be used for acylation purposes. Amines and the amino group in amino acids also can be acylated using urethane-protected α-amino acid-N-carboxyanhydrides or oxycarbonyl-amino acid-N-carboxyanhydrides. Alkylketenes and their dimers may be used for acylation.

A special type of acylation is that using chloroformates. Carbonic acid, $O{=}C(OH)_2$, can form amides, esters, halides, etc., due to the presence of two OH groups bonded to the CO group. Carbonic acid ester halides, also called chloroformates or chloroformate esters, with the formula R—O—C(=O)—X, where R is an alkyl or aryl group and X is F, Cl, Br, or I, can react with various compounds containing active hydrogens, such as acids [25], amines, alcohols, thiols, and amino acids. Amino acids, for example, in the presence of an alcohol in water form carbamate esters (urethanes) reacting as follows [26]:



(25)

The formation in reaction (25) of the alcohol R^a–OH may lead to traces of a resulting compound with both substituted radicals being R^a. For this reason it is typically recommended to perform the reaction in the presence of an alcohol having the same radical as the chloroformate reagent ($R^a = R^b$). Chloroformates containing in the alkyl or aryl group halogen substituents are particularly reactive. Even tertiary amines can react with specific chloroformates, such as pentafluorobenzoyl chloroformate or with trichloroethyl chloroformate, by displacing an alkyl group connected to the nitrogen atom and forming the carbamate ester.

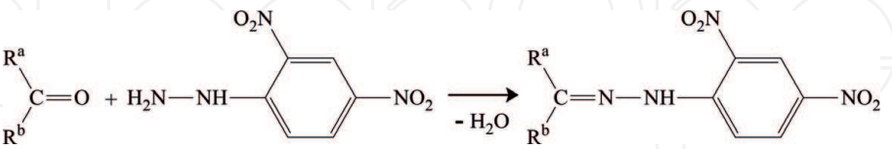
Similar in many respects to that of acyl derivatives R–CO–X are the reactions of sulfonyl derivatives $R–SO_2–X$. Sulfonyl halides are in general less reactive than halides of carboxylic acids. The reaction of a sulfonyl derivative may take place with alcohols, phenols, amines, etc. The reactivity toward the sulfonyl sulfur is $RNH_2 > CH_3COOR > H_2O > ROH$.

High reactivity toward active hydrogens in alcohols, amines, etc. can also be achieved using reagents with other functionalities. These functionalities include isocyanates, isothiocyanates, carbonyl azides, etc. These reactions can be seen as a replacement of an active hydrogen with a CO-R group or CS-R group as it occurs in other acylations.

10. Other derivatization reactions

A variety of other derivatization reactions are reported in the literature (see, e.g., [1]) and used for GC and GC/MS analyses. Among these are the addition to hetero multiple bonds in functional groups such as C=O, C=S, C=N, or C≡N. Many such reactions are additions to multiple bonds. Such reactions are, for example, the additions to the C=O groups in aldehydes and ketones. Reagents containing active hydrogens in groups such as NH_2, OH, H_2N-NH-, etc. can react, for example, with aldehydes and ketones. Alcohols, for example, form hemiacetals or acetals with

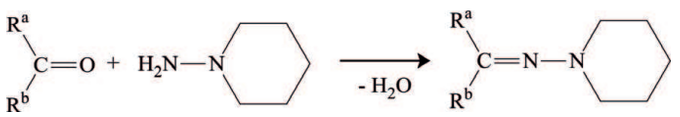
aldehydes and ketals with ketones, and although most of such compounds are not stable enough to be suitable for derivatization, cyclic acetals and ketals may be stable and used for analytical purposes. A common reaction of carbonyl compounds is with amines. The initial addition reaction usually continues with water elimination forming a substituted imine or a Schiff base. Similar to the reaction of amines is the reaction with substituted hydroxylamines or hydrazines. A typical reaction of derivatization of carbonyl compounds is that using dinitrophenylhydrazine (DNPH). The derivatized compound can be analyzed either by LC [27] or by GC/MS [28]. The reaction takes place as follows:



(26)

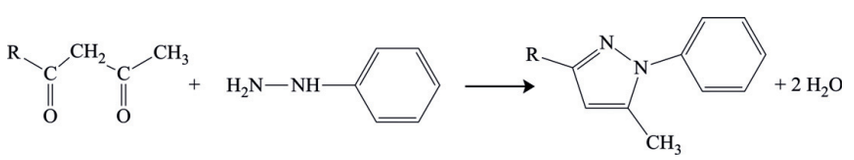
The groups R^a and R^b can be H or alkyl or various other substituents.

Another reagent that can be used for ketone derivatization is N-aminopiperidine in the presence of catalytic amounts of acetic acid. The resulting substituted hydrazone can be used in GC analysis:



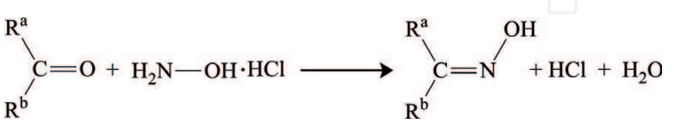
(27)

β-Diketones may react differently with hydrazines generating pyrazole derivatives as shown below:



(28)

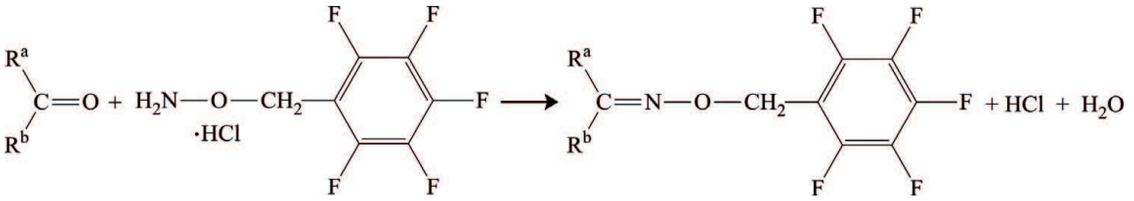
Several other classes of compounds similar to hydrazines react with the carbonyl compounds. Among these are hydrazones (NH_2—N=CR_2), hydrazides (NH_2NH-COR), and semicarbazide (NH_2NH-$CONH_2$). Hydroxylamines also react with carbonyl compounds forming oximes. Hydroxylamine itself, hydroxylamine hydrochloride (STOX® reagent), or derivatives such as H_2N-OSO_3H in a solvent like pyridine can be used in this reaction:



(29)

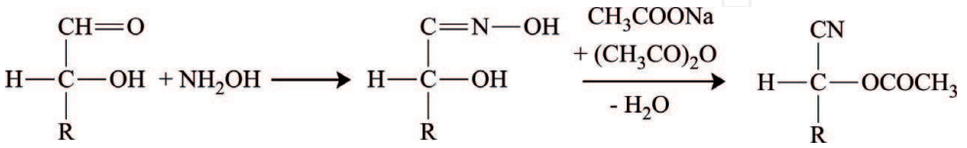
When the reaction is performed with hydroxylamine, the generated oxime contains an active hydrogen. This can be further derivatized, for example, by silylation in a reaction with a common silylation reagent.

For derivatization purposes other reagents can be used, such as substituted hydroxylamines like methoxyamine hydrochloride NH_2OCH_3•HCl (MOX® reagent) and O-(pentafluorobenzyl)-hydroxylamine hydrochloride (FLOROX® reagent). The reaction of a ketone or aldehyde with FLOROX is shown below:



(30)

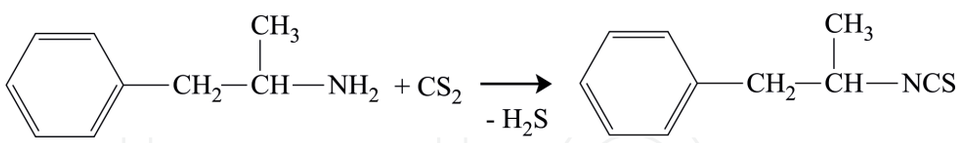
The oximes existing in *sin-* and *anti-* forms can produce double peaks in the GC chromatographic separations. To avoid this effect, oximes can be converted into nitriles when treated with acetic anhydride in the presence of CH_3COONa. This reaction was used in the derivatization of carbohydrates when a simultaneous acetylation takes place (Wohl degradation). The reaction can be written as follows:



(31)

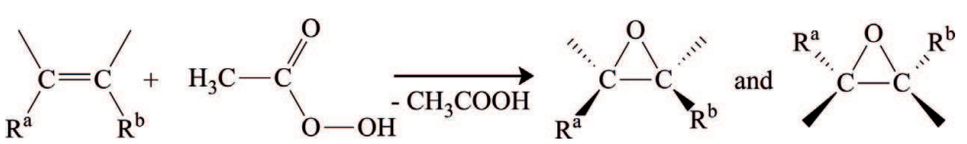
The transformation of the oximes into nitriles generates one single compound from the two (syn- and anti-) isomers and can be used to simplify the chromatograms of sugars derivatized as oximes.

Alcohols, amines, and thiols also can react at other hetero multiple bonds leading to analytical applications. This addition may occur at the isocyanates (—N=C=O), —C=O group in an amide, at a nitrile, at CS_2, or at other groups. One example is the addition under special conditions of alcohols to dimethylformamide. The resulting acetals are very reactive and are used themselves as reagents, as shown previously for N,N-dimethylformamide dimethyl acetal (see reaction 12). Another example is the reaction of CS_2 with alcohols in the presence of a base, leading to the formation of xanthates. Amines also react with CS_2, and the formed isothiocyanate can be analyzed using GC analysis. The reaction takes place as follows:



(32)

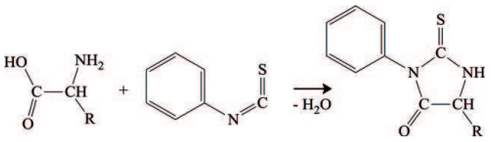
Formation of new cycles from noncyclic compounds or replacement of old cycles with new ones that are more stable or have a desired property is also exploited in sample processing using derivatization. Epoxides, for example, can be formed in the reaction of a compound with a carbon–carbon double bond and a peroxy acid. Among the peroxy acids more frequently used for the formation of epoxides are peracetic, performic, perbenzoic, trifluoroperacetic, and 3,5-dinitroperoxybenzoic acids. However, in this reaction a mixture of enantiomers is formed, as shown below for a *cis* olefin:



(33)

The separation of the epoxides may be easier to achieve than that of olefins, and this type of derivatization has been utilized, for example, for better separation of various *cis* and *trans* unsaturated fatty esters.

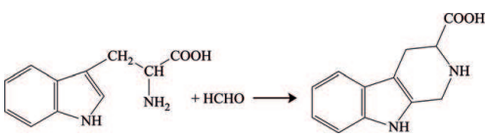
Another reaction with formation of new cycles is that of amino acids with phenyl isothiocyanate leading to a thiohydantoin derivative:



(34)

This reaction has been successfully used for the analysis of amino acids in proteins [29, 30]. *p*-Bromophenyl isothiocyanate has been used in a similar reaction.

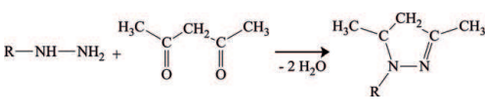
A variety of aromatic cycles can be formed in reactions involving bifunctional compounds. Addition reactions to hetero multiple bonds in bifunctional molecules frequently lead to cyclic compounds. For example, formaldehyde can react with tryptophan or tryptamine generating a β-carboline derivative as follows:



(35)

The new compound can be analyzed by GC, usually after further derivatization by silylation of the carboxyl group.

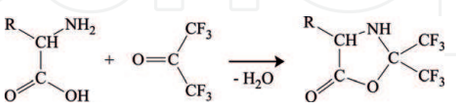
A typical reaction leading to pyrazoles is the reaction of hydrazines with diketones such as 2,4-pentandione (acetylacetone). For example, the reaction between hydrazine or methylhydrazine and acetylacetone takes place as follows:



(36)

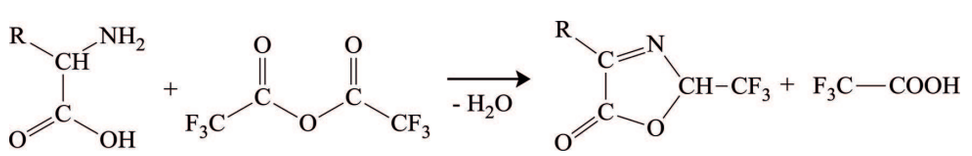
The resulting compound can be analyzed using a GC separation.

Activated carbonyl groups such as those in hexafluoroacetone are known to react with difunctional compounds. The reaction may take place with an amino acid as follows:



(37)

Amino acids can react with an activated anhydride such as trifluoroacetic anhydride (TFAA):



(38)

The reaction takes place by heating the amino acids with an excess of TFAA. The reaction mixture is then dissolved in ethyl acetate and analyzed by GC.

Numerous other types of derivatization reactions were used for making the analytes suitable for GC and GC/MS analyses. These include formation of various cyclic types of compounds such as azines, siliconides, boronates, etc., that are thermally stable and do not have polar hydrogens such that GC or GC/MS analysis is possible. In addition to reagents that add specific moieties to the analytes, oxidation and reduction were sometimes used for the analyte modification (see, e.g., [4]).

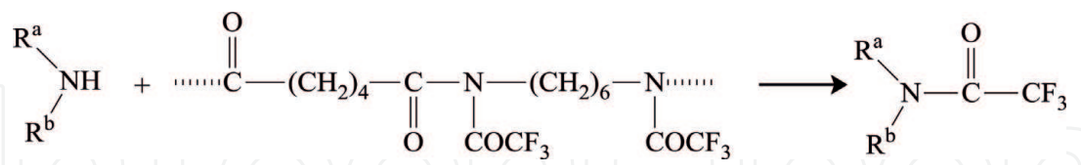
11. Derivatization reactions involving solid-phase reagents or derivatization on a solid support

Solid-phase reagents are polymeric materials with specific groups that are reactive and can be transferred to the analyte molecule producing derivatization. For an analyte of the form Y:H, the reaction with a solid-phase reagent can be written as follows:

$$\text{Y:H} + \text{R-Polymer} \rightarrow \text{R} - \text{Y} + \text{H-Polymer} \tag{39}$$

Solid-phase reagents must work analogously to the corresponding small-molecule reagents containing the group R (a tag). Reagents that are insoluble in certain solvents at high concentrations can often provide a high ratio of analyte/substrate in a polymeric microenvironment that yields a high kinetic rate for the heterogeneous reaction.

A variety of materials can be used as solid support, such as specifically bound reagents on a silica support (used, e.g., for online derivatization in HPLC analysis), ion exchange resins, as well as other supports [31]. One example of solid-phase support that can produce derivatization is trifluoroacetyl nylon 6,6. This solid-phase reagent can be obtained from poly(hexamethylene adipamide) (nylon 6,6) and trifluoroacetyl anhydride. This solid-phase reagent can be used in amine derivatization in a reaction as follows:



(40)

This derivatization of the amine is done by mixing the solid-phase reagent with a solution of amine solution in CH_3CN. Following derivatization, the solid-phase reagent is separated by centrifugation, and the solution is concentrated by evaporating part of the solvent and analyzed by GC (an amine internal standard must be used in this procedure). However, some such derivatizations require a long time of interaction between the solid-phase reagent and the analytes and found only limited applications.

(Another) alternative of derivatization of specific analytes is using the reaction between the reagent and the analyte both adsorbed on a solid support. This type of derivatization has been used, for example, in connection with a solid-phase microextraction (SPME) technique [32]. In this technique a reagent is initially adsorbed in the SPME fiber, followed by exposure to the analytes. The derivatized analytes are further desorbed in the injection port of the GC and analyzed using a detector such as MS. For example, formaldehyde from air can be analyzed using a

polydimethylsiloxane (PDMS) fiber containing *o*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride as a reagent. After exposing the fiber to the air sample contaminated with formaldehyde, the derivatization agent reacts with formaldehyde absorbed onto the coating forming an oxime. The oxime is thermally desorbed in a GC injector port and analyzed by GC with ECD [33].

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