Course # KEM332: Sampling & Sample Preparation

(5 cr, Lecturer: Norbert Maier)

2021/Part 13 Derivatization Techniques (GC-Applications)

Derivatization: Definition

Derivatization in context with sample preparation in analytical chemistry can be defined as the process of deliberately modifying the chemical structure of the analyte(s) of interest with the intention to enable, facilitate or enhance its isolation and/or quantification with a specific analytical technique.

Motivation for Performing Analyte Derivatization in Analytical Chemistry

- Facilitating Analyte Isolation

(Adaptation of polarity, lipophilicity, solubility, partition characteristics, adsorption characteristics, volatility).

- Stabilizing Analyte(s)

(Countering potential analyte degradation during sample preparation, e.g., by structural rearrangement, reaction with matric components, oxidation, polymerization etc).

- Adaptating of Analyte Properties for Chosen Separation Technique

(Volatility, Stability, Solubility, Retention Characteristics).

- Enhancing Analytic Specificity

(Introducing functional groups allowing for selective detection via UV, fluorescence, electrochemical and mass selection techniques; improving MS fragmentation; enabling separation of stereoisomers, such as diastereomers and in particular enantiomers).

- Optimization of Detection and Quantification Sensitivity

(Maximizing response for chosen detection technique).

Crucial Requirements for Derivatization Reactions

- Specificity

Derivatization reaction should form a single product (one analyte-one derivative)

- Efficiency

Analyte needs to be converted into derivative quantitatively.

- Speed

Derivatization reaction rate should be fast to ensure reasonable analytical throughput.

- Stability

No degradation of analytes and/or derivatives should occur during the reaction and upon storage of the products.

- Mildness

Derivatization reaction should occur at reasonable low temperature and pressures, ideally at ambient conditions.

- Simplicity

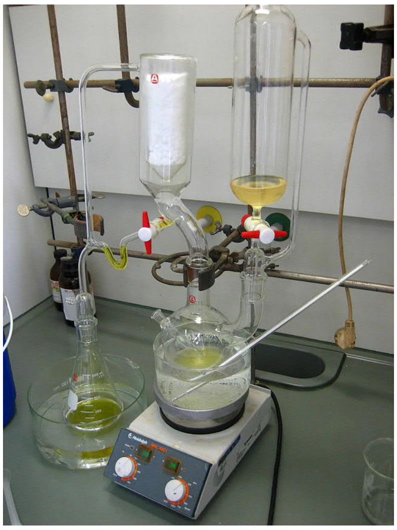
Derivatization reaction should not require complex equipment and elaborate manipulations.

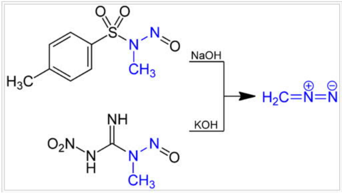
- Saftey

Derivatization reaction should occur under well-controlled and safe conditions.

Sampling & Sample Preparation, Course # KEM332, October/December 2021, Norbert Maier

Safety Concerns in Context with Derivatization Reagents & Reaction: Diazomethane





- Diazomethane is a highly efficient derivatization reagent frequently employed in form of ethereal solutions for the conversion of carboxylic acid to the corresponding methyl esters.
- Diazomethane may explode in contact with sharp edges, such as ground-glass joints, even scratches in glassware. The compound may also explodes when heated beyond 100 °C, upon exposure to intense light, alkali metals, or calcium sulfate.
- Diazomethane is toxic by inhalation or by contact with the skin or eyes. Symptoms include chest discomfort, headache, weakness and, in severe cases, collapse.

http://en.wikipedia.org/wiki/Diazomethane

Derivatization Techniques: Pre-Column Derivatization

- Pre-column derivatization involves the conversion of the analyte to the corresponding derivative prior to analysis.

- Often pre-column derivatization procedures require additional steps, such as extraction, removal of reaction byproducts, concentration, and solvent exchange.

- Both the separation method and the detection method need to be developed considering specifically the properties of the derivative.

Advantages
- Operational simplicity.
- Flexibility concerning optimization of reaction conditions for derivatization.
- Possibility to remove components that would damage instrumentation.

Disadvantages
- Method development of separation must be based on properties of derivative.
- Additional time-consuming sample manipulation steps prior to analysis; potential analyte losses.
- Higher efforts for method validation.
- More laborious automation.

Derivatization Techniques: Post-Column Derivatization

- Post-column derivatization involves the conversion of the analyte after having been separated on a chromatographic system prior to detection.
- No analyte manipulation needed prior to separation.
- Separation method must be developed considering specifically the properties of the analyte, while the detection method needs to be designed to address the properties of the derivative.

Advantages
- Method development for separation can fully exploit the properties of the analyte.
- Components associated with the derivatization reaction do not hit the chromatographic column.
- Ease of automation (once fully optimized).
- Multiple detection system might be used for maximizing information on analyte.

Disadvantages
- Involved method development (type of reagent, concentration, reaction time, temperature).
- Separation conditions (i.e. mobile phase conditions) must be compatible with derivatization approach.
- Need for dedicated instrumentation.
- High effort for method validation.
- More laborious automation.

Derivatization Techniques: In-Column Derivatization

- In-column derivatization (also in-liner or flash derivatization) involves the generation of the analyte derivatives directly within the injection/separation system by introducing simultaneously a mixture of the analyte and the corresponding derivatization reagent in an appropriate solvent.
- Essentially no additional sample manipulation prior to analysis required.
- Both the separation method and the detection method need to be developed considering specifically the properties of the derivative.

Advantages
- Operational simplicity.
- Speed.
- Ease of automation.

Disadvantages
- Considerable efforts to develop and optimize in-column derivatization procedure.
- All components involved in derivatization (derivative, byproducts, solvent, decomposition product) will pass through the separation system and detector; may damage instrumentation.
- Often forcing conditions needed (high temperature, highly reactive derivatization reagents).
- Limited range of derivatization techniques available.

Derivatization Techniques: Consideration for the Development of Derivatization Procedures

An Ideal Derivatization Procedure Should:

- ❑ Accomplish the desired chemical modification (on no others)
- ❑ Proceed quantitatively (or at least reproducibly).
- ❑ Produce products that are readily distinguishable and separable from starting materials.

Crucial Considerations for Developing a Derivatization Procedure

- Make sure the reaction is complete.
- Use more than one reaction temperature and time.
- Identify possible side reactions with additional functional groups on the compound or sample matrix.
- Determine the thermal/chemical stability of the derivative.
- Select reactions that proceed rapidly with simple and easy methodology.
- Use reagents and reactions that present no unusual hazards.
- The derivatized form of the analyte should be readily extractable.
- The derivative can be chromatographed accurately for trace analysis or separated from interfering compounds.

Derivatization for Gas Chromatography

Derivatization for Gas Chromatography: General Aspects

Gas chromatography (GC) is an highly efficient analytical technique for the separation and quantitation of volatile compounds that are thermally stable.

Gas chromatography is not applicable to non-volatile compounds or compound that would suffer decomposition under conventional high temperature separation conditions.

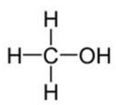
The range of compounds unsuitable (thermally instable) for GC includes many classes of analytes of biomedical and environmental interest, specifically compounds containing functional groups with active hydrogen atoms linked to heteroatoms (-COOH, -OH, -NH, and –SH).

Many of these analytes can be adapted for GC analysis by derivatization reactions, using silylation, acylation, alkylation, and esterification protocols and appropriate derivatization reagents.

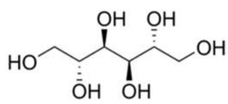
Analytes are derivatized prior to GC analysis to:

- Increase the volatility and decrease the polarity of the compound.
- Reduce thermal degradation of samples by increasing their thermal stability.
- Increase detector response by incorporating functional groups which lead to enhanced detector signals, e.g. CF_3 groups for electron capture detection (ECD).
- Improve selectivity, resolution and peak shapes.
- Enlarge the substrate spectrum.

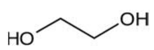
Derivatization for Gas Chromatography: Impact of Active OH-Groups on Volatility



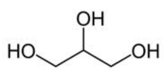
Methanol
bp.: 65°C



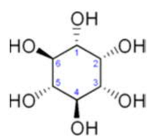
Mannitol
mp.: 168°C



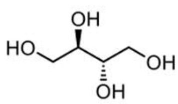
Ethylene Glycol
bp.: 198°C



Glycerol
bp.: 290°C



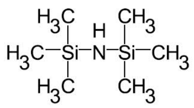
Inositol
mp.: 225°C



Erythrol
mp.: 121°C

Derivatization for Gas Chromatography: Silylation - Reagents

HMDS
Hexamethyldisilazane



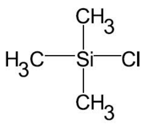
Molecular Formula: (CH3)3SiNHSi(CH3)3
CAS Number: 999-97-3
Formula Weight: 161.39
bp: 125 °C
Flash Point: 48 °F (8 °C)
d: 0.765
nD: 1.4079 at 20 °C
Appearance: Clear, colorless liquid
with characteristic odor,
moisture sensitive

Features/Benefits
● HMDS is inexpensive and has a relatively low boiling point (124-127 °C). It can be used without solvent but its silylating power can be increased by various (mostly acidic) catalysts. The only reaction byproduct, ammonia, can leave the reaction mixture as the reaction goes to completion.

Common Relative Functional Groups
● Alcohols, phenols, carboxylic acids, and amines.

Derivatization for Gas Chromatography: Silylation - Reagents

TMCS
Trimethylchlorosilane



Molecular Formula: ClSi(CH3)3
CAS Number: 75-77-4
Formula Weight: 108.64
bp: 57 °C
Flash Point: -18 °F (-27 °C)
d: 0.856
nD: 1.3870 at 20 °C
Appearance: Clear, colorless liquid
with a pungent odor,
moisture sensitive

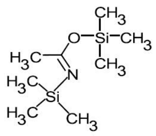
Features/Benefits
● TMCS increases the reactivity of other silylation reagents. Amides and many secondary amines and hindered hydroxyl groups, incompletely derivatized by BSTFA alone, can be derivatized by adding 1-20% TMCS to BSTFA.

Common Applications
Silylation catalyst, typically used with other silylating reagents.

Derivatization for Gas Chromatography: Silylation - Reagents

BSA

N,O-Bis(trimethylsilyl)acetamide



Molecular Formula: CH3C[=NSi(CH3)3]OSi(CH3)3
CAS Number: 10416-59-8
Formula Weight: 203.43
bp: 71-73°/35mm
Flash Point: 53°F (11°C)
d: 0.823
nD: 1.4170 at 20°C
Appearance: Clear, colorless liquid, moisture sensitive

Features/Benefits

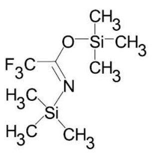
- Mild reaction conditions form highly stable products with most organic functional groups. TMS derivatives are thermally stable but more susceptible to hydrolysis than their parent compounds.
- Reactions are generally fast and quantitative.
- Will silylate unhindered hydroxyl groups.
- BSA and its byproducts are more volatile than many other silylating reagents, causing less chromatographic interference.
- BSA has good solvent properties and usually can function as an efficient silylating reagent without additional solvents (DMF, dimethyl formate, is the solvent most frequently used to improve silylation efficiency).

Common Reactive Functional Groups
Alcohols, phenols, carboxylic acids, amides and amines.

Derivatization for Gas Chromatography: Silylation - Reagents

BSTFA

N,O-Bis(trimethylsilyl)trifluoroacetamide



Molecular Formula: CF3C=NSi(CH3)3OSi(CH3)3
CAS Number: 25561-30-2
Formula Weight: 257.40
bp: 45-55°/14mm
Flash Point: 75°F (23°C)
d: 0.969
nD: 1.384 at 20°C
Appearance: Clear, colorless to very light yellow liquid, moisture sensitive

Features/Benefits

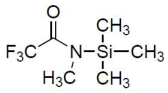
- Very versatile. Reacts with a range of polar organic compounds by replacing active hydrogens with a TMS group.
- TMS derivatives are thermally stable but more susceptible to hydrolysis than their parent compounds.
- Reacts rapidly and more completely than BSA.
- BSTFA and its by-products (trimethylsilyltrifluoroacetamide and trifluoroacetamide) are more volatile than many other silylating reagents, causing less chromatographic interference.
- TFA, a by-product of silylation with BSTFA, reduces detector (FID) fouling.
- Very soluble in most commonly used silylation solvents. Has a good solvent property and can function as a silylation reagent without additional solvents.

Common Reactive Functional Groups
Alcohols, phenols, carboxylic acids, carbohydrates, amides, and amines, and sulfonamides.

Derivatization for Gas Chromatography: Silylation - Reagents

MSTFA

***N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide**



Molecular Formula: CF3CON(CH3)Si(CH3)3
CAS Number: 24589-78-4
Formula Weight: 199.25
bp: 130-132 °C
Specific gravity: 0.075
nD: 1.38 at 20 °C
Appearance: Clear, colorless to pale yellow liquid

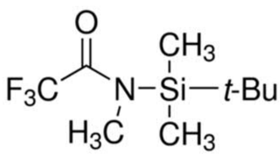
Features/Benefits
- More volatile than BSA or BSTFA but with similar silylation strength.
- Useful in the analysis of volatile trace materials.
- Used in preparation of volatile and thermally stable derivatives for GC and MS analysis.

Common Relative Functional Groups
- Amides, secondary amines, hydroxyls and hydrogen on polar compounds.

Derivatization for Gas Chromatography: Silylation - Reagents

N-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide
MTBSTFA
(containing 1% *tert*-Butyldimethylchlorosilane)

Features/Benefits
- More volatile than BSA or BSTFA but with similar silylation strength.
- Forms derivative thermally more stable and less sensitive to moisture than TMS derivatives.
- Useful in the analysis of volatile trace materials.
- Used in preparation of volatile and thermally stable derivatives for GC and MS analysis.

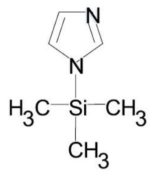


Common Relative Functional Groups
- Amino acids, amides, secondary amines, hydroxyls and hydrogen on polar compounds.

Derivatization for Gas Chromatography: Silylation - Reagents

TMSI

N-Trimethylsilylimidazole (or TMSIM)



Molecular Formula: (CH3)3SiNCH=NCH=CH
CAS Number: 18156-74-6
Formula Weight: 140.26
bp: 93-94°/14mm
Flash Point: 42°F (5°C)
d: 0.956
nD: 1.4750 at 20°C
Appearance: Clear, colorless to light yellow liquid, moisture sensitive

Features/Benefits

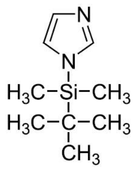
- Useful for derivatizing alcohols, hormones, fatty and other organic acids, phenols, prostaglandins, steroids, sulfonic acids, and thiols.
- Does not react with aliphatic amines. Less basic amines and amides may react with TMSI.
- Useful in multiderivatization schemes containing hydroxyl and amine groups.
- Derivatizes sugars in the presence of small amounts of water.
- TMS derivatives are thermally stable but more susceptible to hydrolysis than their parent compounds.

Common Reactive Functional Groups
Alcohols, fatty/organic acids, carbohydrates, carboxylic acids, sulfonic acids, phenols, and thiols.

Derivatization for Gas Chromatography: Silylation - Reagents

TBDMSIM

N-t-Butyldimethylsilylimidazole



Molecular Formula: C(CH3)3-Si(CH3)2–NCH=NCH=CH
CAS Number: 54925-64-3
Formula Weight: 182.34
bp: 53 °C/0.2mm
Flash Point: 211 °F (99 °C)
d: 0.939
nD: 1.480 at 20 °C
Appearance: Clear, pale yellow liquid, moisture sensitive

Features/Benefits

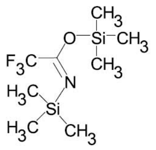
- t-Butyldimethylsilyl (TBDMS) derivatives are more stable to hydrolysis than the corresponding trimethylsilyl (TMS) ethers (the tert-butyldimethylsilyl group is larger than the TMS group).
- Stability of TBDMS-enol ethers is an advantage in the isolation of ketone enolates from aqueous solution.
- Does not release HCl.
- Useful for mass spectrometry (tends to provide high-mass ions).

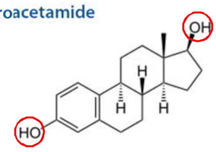
Common Reactive Functional Groups
Sterically unhindered alcohols, phenols.

Derivatization for Gas Chromatography
Silylation - Example: Estrogens

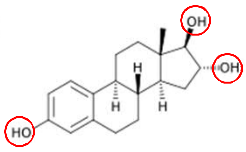
BSTFA

N,O-Bis(trimethylsilyl)trifluoroacetamide

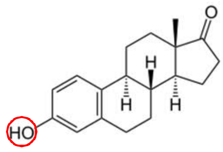




Estradiol



Estriol



Estrone

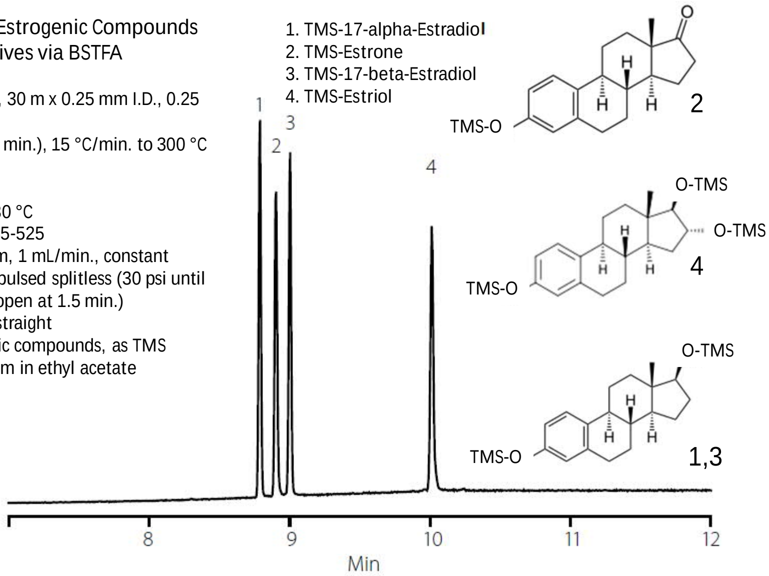
- The investigated estrogenic compounds (17-alpha-estradiol; estrone; 17 beta-estradiol; and estriol) show in underivatized forms little or no response by GC analysis.
- After introduction of TMS groups by reaction with BSTFA , these analytes show excellent peak shapes and greatly enhanced detector response.
- However, it is crucial to optimize and ensure that the derivatization reactions go to completion.
- Of the four estrogenic compounds, three were fully derivatized within 30 minutes at 75 °C.
- To achieve complete derivatization of estriol (sterically highly hindered hydroxy groups), a second derivatization cycle was performed at 75 °C reaction time increased to 45 minutes.
- After being allowed to sit overnight at room temperature, GC-MS analysis then confirmed that all three active hydrogens had been replaced with TMS groups.

Derivatization for Gas Chromatography:
Silylation - Applications: Estrogens

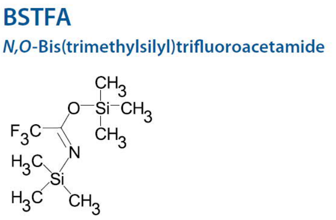
GC Analysis of Estrogenic Compounds as TMS Derivatives via BSTFA

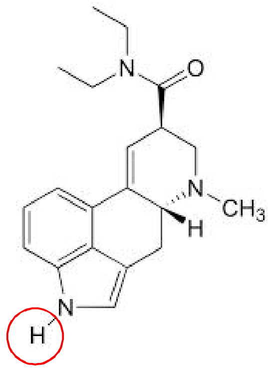
Column: SLB-5ms, 30 m x 0.25 mm I.D., 0.25 µm (28471-U)
Oven: 175 °C (0.5 min.), 15 °C/min. to 300 °C (5 min.)
Inj. Temp: 250 °C
MSD interface: 330 °C
Scan range: m/z 45-525
Carrier gas: helium, 1 mL/min., constant
Injection: 0.5 µL, pulsed splitless (30 psi until 0.5 min., splitter open at 1.5 min.)
Liner: 2 mm I.D., straight
Sample: Estrogenic compounds, as TMS derivatives, 10 ppm in ethyl acetate

1. TMS-17-alpha-Estradiol
2. TMS-Estrone
3. TMS-17-beta-Estradiol
4. TMS-Estriol



Derivatization for Gas Chromatography
Silylation - Example: Lysergic Acid Diethylamide



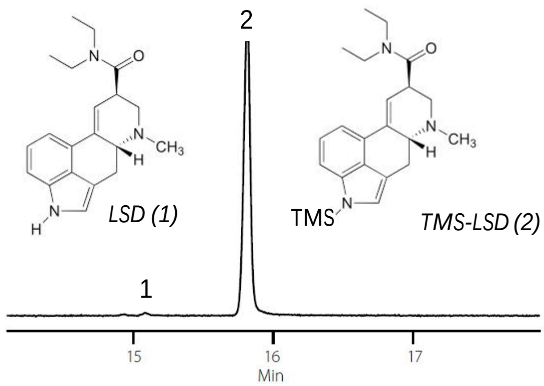


- Lysergic acid diethylamide (LSD) was derivatized with BSTFA at 68 °C.
- At this temperature only 60% derivatization was achieved in 5 hours.
- At 75 °C the reaction was approximately 95% complete after 3 hours, with both LSD and the corresponding TMS derivative being detectable.

Derivatization for Gas Chromatography
Silylation - Example: Lysergic Acid Diethylamide

GC Analysis of LSD as TMS Derivative via BSTFA

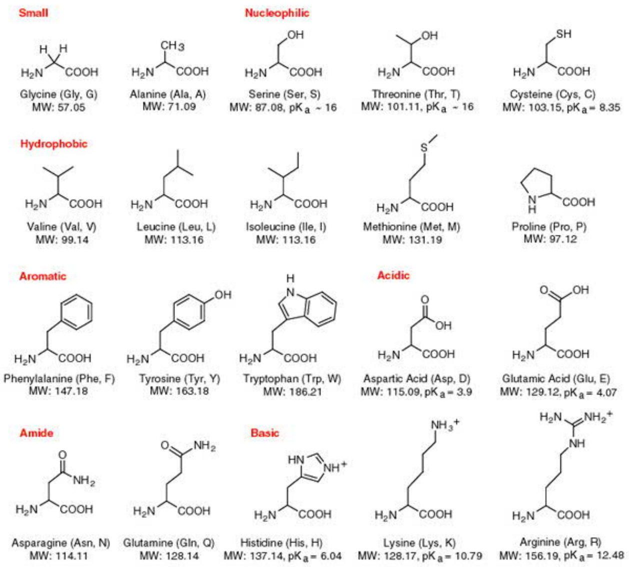
Column: SLB-5ms, 30 m x 0.25 mm I.D., 0.25 µm (28471-U)
Oven: 150 °C (1.5 min.), 20 °C/min. to 300 °C (20 min.)
Inj.Temp: 250 °C
MSD interface: 330 °C
Scan range: m/z 45-525
Carrier gas: helium, 0.7 mL/min. constant
Injection: 0.5 µL, pulsed splitless, 30 psi. (0.20 min.), purge on (1.5 min.), purge flow (50 mL/min.)
Liner: 2 mm I.D., straight
Sample: derivatized LSD using BSTFA



Derivatization for Gas Chromatography
Silylation - Example: Separation of Amino Acids as TBDMS Derivatives

- The polar nature of amino acids requires derivatization prior to GC analysis. Derivatization ensures a suitable level of volatility, improves thermal stability, suppresses non-specific interactions and thus improve overall chromatographic behavior.
- In amino acids, derivatization replaces all active hydrogens, both at the amino and carboxylic group and at the side chains, bearing OH, NH2, and SH functional groups with a nonpolar moieties.
- For this particular study, N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) was as use as the silylation reagent for the derivatization of amino acids.
- MTBSTFA forms tert-butyl dimethylsilyl (TBDMS) derivatives when reacted with polar functional groups containing an active hydrogen.
- As compared to TMS derivatives, the corresponding TBDMS derivatives are thermally more stable and less sensitive to moisture.

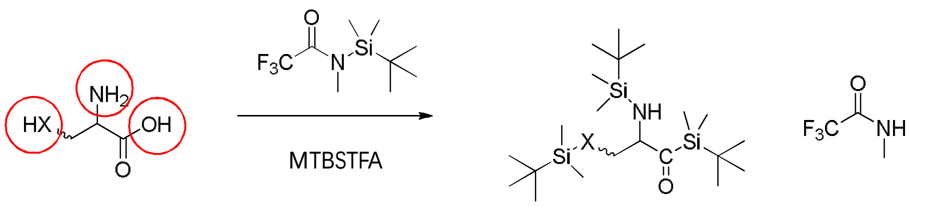
Derivatization for Gas Chromatography
Silylation - Example: Amino Acids



Amino Acids Properties

- amino group
- carboxylic group
- functional (polar) side chain
- zwitterionic
- Non-volatile
- high melting point
- decomposition on heating

Derivatization for Gas Chromatography
Silylation - Example: Separation of Amino Acids as TBDMS Derivatives



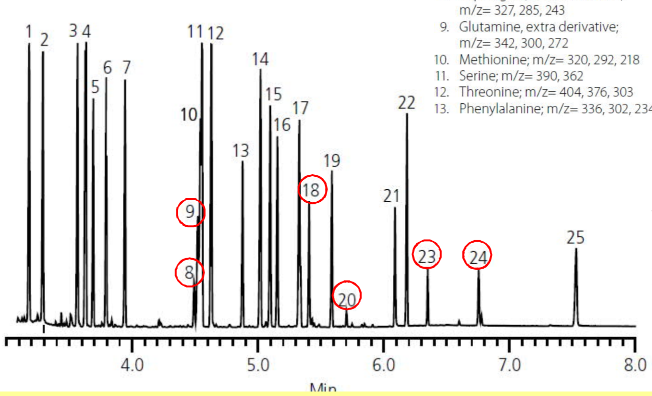
Zwitterionic Amino Acid

Volatile Lipophilic Amino Acid TBDMS Derivative

Trifluoroacetic Acid Methyl Amide (Volatile Byproduct)

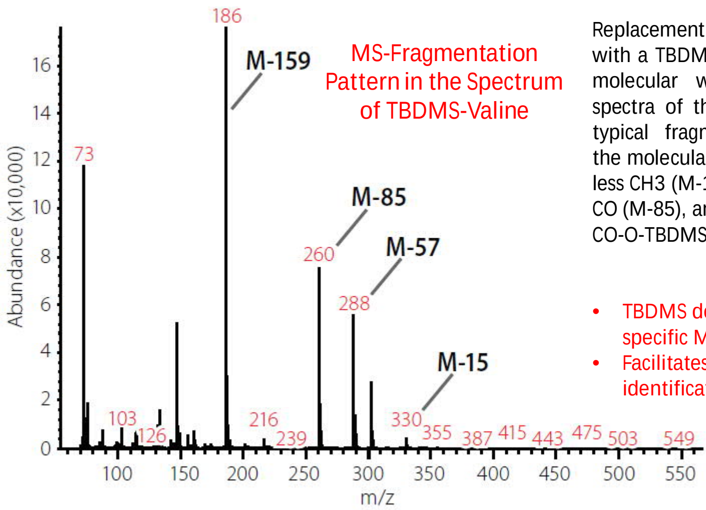
Derivatization for Gas Chromatography
Silylation - Example: Separation of Amino Acids as TBDMS Derivatives

1. Alanine; m/z=260, 232, 158
2. Glycine; m/z=246, 218
3. Valine; m/z= 288, 260, 186
4. artifact from derivatization
5. Leucine; m/z=302, 274, 200
6. Isoleucine; m/z=302, 274, 200
7. Proline; m/z= 286, 258, 184
8. Asparagine, extra derivative; m/z= 327, 285, 243
9. Glutamine, extra derivative; m/z= 342, 300, 272
10. Methionine; m/z= 320, 292, 218
11. Serine; m/z= 390, 362
12. Threonine; m/z= 404, 376, 303
13. Phenylalanine; m/z= 336, 302, 234
14. Aspartic acid; m/z= 418, 390, 316
15. Hydroxyproline; m/z= 416, 388, 314
16. Cysteine; m/z= 406, 378
17. Glutamic acid; m/z= 432, 330, 272
18. Asparagine; m/z= 417, 302
19. Lysine; m/z= 431, 329, 300
20. Glutamine; m/z= 431, 357, 329, 299
21. Histidine; m/z= 459, 440, 338, 196
22. Tyrosine; m/z= 466, 438, 364, 302
23. Tryptophan, extra derivative m/z= 417, 375, 347, 302, 273
24. Tryptophan; m/z= 489, 302, 244
25. Cystine; m/z=639, 589, 537, 348



Column: SLB-5ms, 20 m x 0.18 mm I.D., 0.18 µm (28564-U)
Oven: 100 °C (1 min.), 35 °C/min. to 290 °C (3 min.),
40 °C/min. to 360 °C
Inj. Temp: 250 °C
MSD interface: 325 °C
Scan range: m/z = 40-450
Carrier gas: helium, 1 mL/min., constant
Injection: 0.5 µL, splitless (1.0 min.)
Liner: 2 mm I.D., straight
Sample: TBDMS derivatives of amino acids, each approximately 23 µg/mL

Derivatization for Gas Chromatography Silylation - Example: Separation of Amino Acids as TBDMS Derivatives



Replacement of an active hydrogen with a TBDMS group adds 114 to the molecular weight. Electron impact spectra of these derivatives contains typical fragments corresponding to the molecular weight of the derivative less CH3 (M-15), C4H9 (M-57), C4H9 + CO (M-85), and CO-O-TBDMS (M-159).

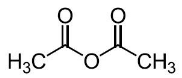
- TBDMS derivatives exhibit highly specific MS fragmentation pattern
- Facilitates compound identification.

Derivatization for Gas Chromatography: Acylation - Reagents

- Acylation is the conversion of compounds with active hydrogen such as –OH, -SH, and –NH into esters, thioesters and amides.
- Acylation is an important derivatization procedure given the fact that many important classes analytes bear amine and/or hydroxy groups.
- Acylation enhances overall GC performance due to enhancement of volatility, and improves peak shapes by reducing non-specific surface adsorption.
- Acylation can reduce the polarity of a molecule, making it more retentive in reversed-phase HPLC applications.
- Perfluoroacyl derivatives are both stable and highly volatile, and very popular for insertion of groups enabling electron capture detection (ECD); carbonyl groups adjacent to halogenated carbons enhance the response of ECD.
- Acylation favors the formation of fragmentation-directing derivatives for GC-MS analysis.
- GC Acylation reagents can be classified into two main groups: fluoro acid anhydrides and fluoracylimidazoles. Fluoracylimidazoles react readily with hydroxyl groups and primary and secondary amines to form acyl derivatives. The imidazoles produced as a by-product are relatively inert.

Derivatization for Gas Chromatography: Acylation - Reagents

Acetic Anhydride



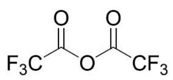
Molecular Formula: (CH3CO)2O
CAS Number: 108-24-7
Formula Weight: 102.09
d: 1.080-1.085
nD: 1.3901
Appearance: Clear, colorless liquid

Features/Benefits
- Acylation is an alternative to silylation, producing stable, volatile derivatives of alcohols, phenols, and amines for analysis by GC/FID.
- Acylated compounds are more stable than corresponding silylated compounds.

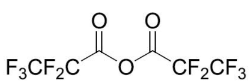
Common Reactive Functional Groups
Alcohols, phenols, carbohydrates and amines.

Derivatization for Gas Chromatography: Fluorous Acylation - Reagents

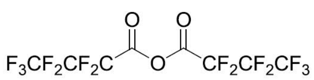
Trifluoroacetic acid anhydride (TFAA)



Pentafluoropropionic acid anhydride (PFPA)



Heptafluorobutyric acid anhydride (HFBA)

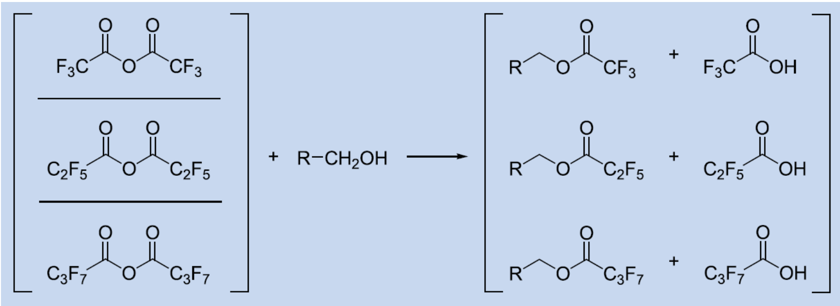


Low boiling, colorless liquid
Extremely sensitive to moisture

Features/Benefits
- Produce stable, volatile derivatives of alcohols, amines, and phenols for electron capture or flame ionization detection.
- Frequently used in confirmation testing for drugs of abuse by GC-MS. (TFAA is used to identify methamphetamine, PFPA is used to identify opiates and benzoylecgonine, HFBA is used to identify amphetamines and phencyclidine).

Common Reactive Functional Groups
Alcohols, amino acids, amides, amines, phenols, and steroids.

Derivatization for Gas Chromatography: Derivatization with Fluoro Acetanhydrides

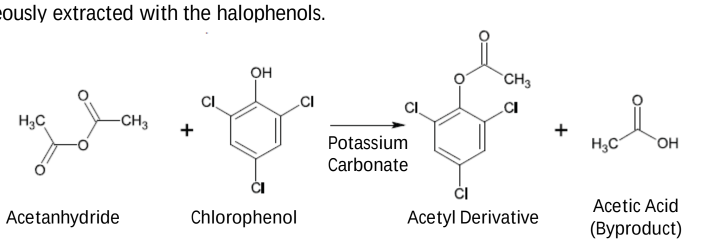


- Acylation reaction with fluoro acetanhydrides does produce the corresponding, highly acidic acids as a byproduct.
- These acid may attack instrumentation and chromatographic columns upon prolonged contact. It is recommended to remove these aggressive acids prior to analysis of the derivatives by liquid-liquid extraction.

Derivatization for Gas Chromatography: Example - Analysis of Chlorophenols as Acetyl Derivatives

"Cork taint" or a musty odor sometimes detected in wine results from 2,4,6-trichloroanisole (TCA). The source of TCA is thought to be the fungal methylation of chlorophenols present in the wine, with these compounds emanating from the cork or other sources such as biocides, fungicides, and exposure of processing equipment to antiseptic cleaning products containing halogenated phenols.

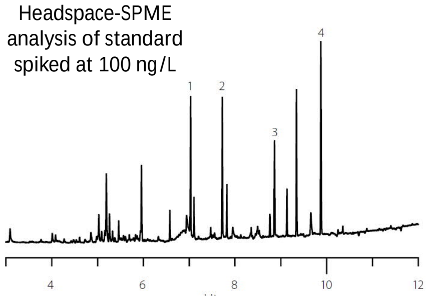
The chlorophenols were derivatized in matrix using acetic anhydride, and the acylated derivatives extracted via SPME from the headspace. TCA, lacking active hydrogens, is not derivatized, but simultaneously extracted with the halophenols.



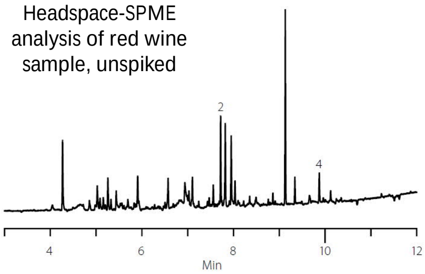
The halophenols were acylated with acetic anhydride prior to extraction. Acetic anhydride will hydrolyze in the presence of water, however the phenolic groups present on the analytes are more reactive, making it possible to conduct the derivatization directly in the aqueous matrix. Addition of K2CO3 drives the reaction by removing by neutralizing the acetic acid formed as an byproduct.

Derivatization for Gas Chromatography: Example - Analysis of Chlorophenols as Acetyl Derivatives

Headspace-SPME analysis of standard spiked at 100 ng/L



Headspace-SPME analysis of red wine sample, unspiked



1. 2,4,6-Trichloroanisole
2. 2,4,6-Trichlorophenol (acylated)
3. 2,3,4,6-Tetrachlorophenol (acylated)
4. Pentachlorophenol (acylated)

Sample: 1.5 mL sample + 600 µL 5% K2CO3 + 240 mg NaCl + 60 µL acetic anhydride;
SPME fiber: metal fiber coated with 100 µm PDMS (57928-U)
Extraction: headspace, 50 °C, 30 min., with stirring
Desorption temp.: 250 °C, 3 min.
Column: SLB-5ms; 30 m x 0.25 mm I.D. x 0.25 µm (28471-U)
Oven: 50 °C (1 min.), 25 °C/min. to 280 °C
Detector: ECD, 290 °C
Carrier gas: helium, 1.5 mL/min constant flow
Liner: 0.75 mm I.D. SPME

Spiked with 100 ng/L of:	Unspiked wine (ng/L)	Spiked wine (ng/L)	%Rec.
2,4,6-Trichloroanisole	ND	60.7	61
2,4,6-Trichlorophenol	22.7	96.3	74
2,3,4,6-Tetrachlorophenol	ND	55.7	56
Pentachlorophenol	3.3	33.5	30

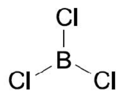
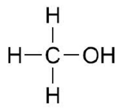
Derivatization for Gas Chromatography Application – Esterification and Alkylation

- Alkylation refers to the reaction of organic substances that contain a reactive hydrogen, such as R-COOH, R-OH, -SH, R-SH, R-NH-R, R-NH2, R-CONH2, and R-CONH-R' with an appropriate derivatizing agent.
- Generally, replacement of such a hydrogen with an alkyl group decreased polarity of the derivative as compared with the parent substance, facilitating analysis by chromatographic techniques.
- The decrease in polarity and intermolecular association is particularly important for analysis by gas chromatography and mass spectrometry. Mixtures of closely related compounds that show poor separation before derivatization may often be resolved by use of a proper derivative.
- Alkylation of weak acidic groups like alcohols requires strong basic catalysts (sodium methoxide, potassium methoxide). More acidic groups like phenols and carboxylic acids require less basic catalysts (boron trifluoride).
- The most common application of alkylation for analytical derivatization is the conversion of organic acids into esters, especially methyl esters. Note that, although TMS derivatives of carboxylic acids are easily formed, these derivatives suffer from limited stability. Alkyl esters afford largely enhanced stability, allowing their isolation and storage for extended periods of time.

Derivatization for Gas Chromatography: Esterification/Trans-Esterification

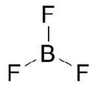
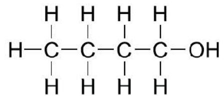
Boron trichloride-Methanol, 12% w/w

Boron trichloride Methanol

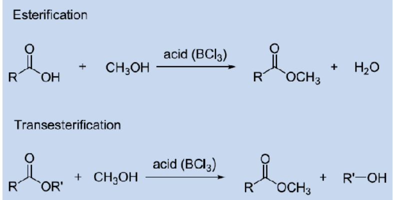



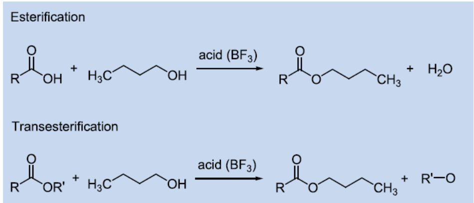
Boron trifluoride-Butanol, 10% w/w

Boron trifluoride n-Butanol

Applications/Benefits
- Used for derivatizing carboxylic acids and transesterifying esters.
- Particularly useful for derivatizing carboxylic acids in bacterial lipids and seed oils.
- Provides convenient, fast, quantitative esterification/transesterification.
- Clean reaction (no side reactions) with volatile by-products.
- BCl3-MeOH is more stable than BF3 -methanol at room temperature (BCl3 is not as volatile as BF3).



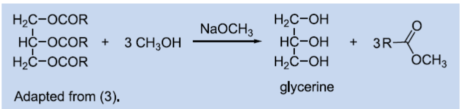


Derivatization for Gas Chromatography Esterification/Trans-Esterification

Methanolic Base, 0.5N

Metallic sodium in methanol

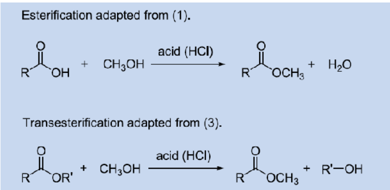
Applications/Benefits
- Transesterification of triglycerides, cholesteryl esters, phospholipids. Sequence of esterification is triglycerides, diglycerides, monoglycerides, methyl esters.
- Provides convenient, fast, quantitative derivatization.



Methanolic HCl, 0.5N and 3N

Hydrochloric acid in methanol

Applications/Benefits
- Derivatization of fatty acids, particularly volatile (short chain) fatty acids.
- Clean reaction (no side reactions) with volatile by-products.
- Provides convenient, fast, quantitative derivatization.

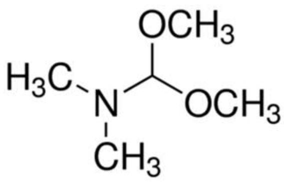


Derivatization for Liquid Chromatography
Esterification with Acetal Reagents

N,N-dimethylformamide dimethylacetal
(DMF-DMA)

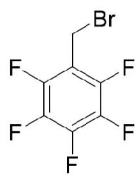
Application/Benefits

- Methylation with DMF-DMA can be applied for fatty acids to provide the corresponding methyl esters.
- DMF-DMA is a poor solvent, for this reason it is necessary to use a mixture of DMF-DMA with pyridine, THF, acetone (barbiturates) or another solvent.



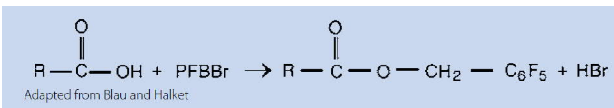
Derivatization for Gas Chromatography
Alkylating Esterification

Pentafluorobenzyl bromide (PFBBr)



Applications/Benefits

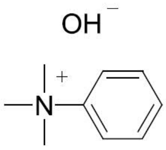
- Derivatization of halogenated derivatives of carboxylic acids, mercaptans, phenols, and sulfonamides fatty acids, particularly volatile (short chain) fatty acids.
- Clean reaction (no side reactions).
- Derivatives show enhanced response in ECD and favorable fragmentation pattern in MS detection.



Reaction performed in dry acetone in presence of potassium carbonate and a catalytic amount of crown ether.

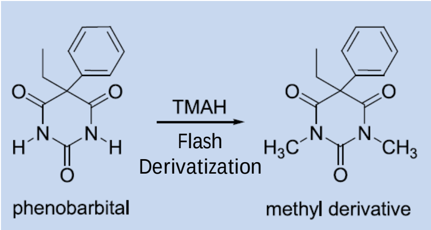
Derivatization for Gas Chromatography
Flash Alkylation

TMAH
Trimethylanilinium hydroxide



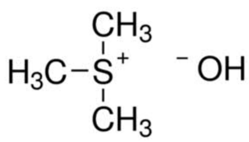
Elemental Formula: Mixture of trimethylphenylammonium iodine, silver oxide, methanol
bp: 65 °C at 760 mm Hg
d: 1.10
nD: 0.790 at 20 °C
Appearance: Clear, colorless liquid

Applications/Benefits
- Provides convenient, fast, quantitative derivatization of nitrogen-bearing molecules.
- Derivates are formed in-situ upon injecting a mixture of the analyte and the reagent into the heated GC liner ("flash-derivatization").
- Preferred reagent for derivatizing barbiturates.
- N,N-dimethylaniline and water are formed as byproducts; may interfere with analysis.



Derivatization for Gas Chromatography
Flash Alkylation

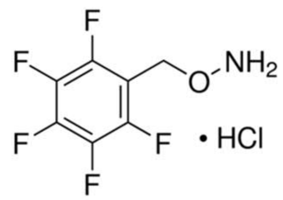
Trimethylsulfonium hydroxide (TMSH)
0.25 M solution in methanol



Application/Benefits
- Methylation of free acids, chlorophenoxycarboxylic acids, their salts and derivatives as well as for phenols and chlorophenols. One great advantage is simplification of the sample preparation.
- Lipids or triglycerides can be converted to the corresponding fatty acid methyl esters (FAMEs) by a simple trans-esterification. This reaction is very elegant and convenient, because it is just necessary to add the reagent (0.2 M in methanol) to the sample solution. Removal of excess reagent is not required, since in the injector of the gas chromatograph at 250 °C pyrolysis to volatile methanol and dimethylsulfide will occur (quite stinky and may also interfere with analysis).
- Due to the high reactivity, complete derivatization is often obtained at ambient temperature. Occasionally, heating (e. g. 10 min at 100 °C) in a closed sample vial may be necessary.

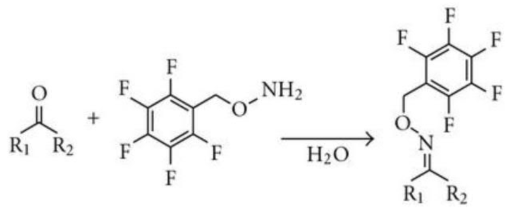
Derivatization for Gas Chromatography Condensation Keto-PFBHA Derivatives

O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA·HCl)

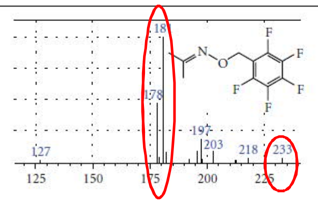
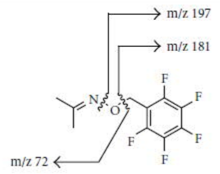
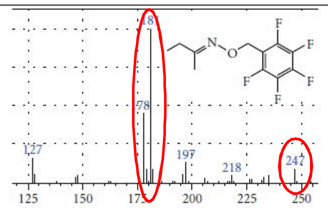
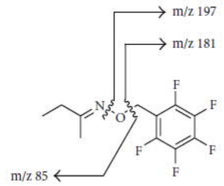


Applications/Benefits

- Provides convenient derivatization of keto compounds.
- Derivatives are stable in and extractable from aqueous solutions.
- Aldehydes react faster than ketones, which may need extended reaction time to achieve high derivatization yields.



Derivatization for Gas Chromatography: Specific MS Fragmentation of Ketone PFBHA Derivatives

Name	Structure formula and GC/NCI/MS spectra	Fragment ions from simple cleavages
Ace-PFBHA-oxime		
MEK-PFBHA-oxime		

Characteristic Allows Fragmentation for Structure Confirmation & Optional Quantitation

Derivatization for Gas Chromatography Recommended Derivatization Reagents

Selection guide for derivatization of important functional groups in GC

Function	Method	Derivative	Recommended reagents
Alcohols, Phenols R'OH	silylation	R'O – TMS	BSA, MSTFA, MSHFBA, TSIM, SILYL-2110, SILYL-21, SILYL-1139
	acylation	R'O – CO – R	TFAA, HFBA, MBTFA, MBHFBA
	alkylation	R'O – R	TMSH
sterically hindered	silylation	R'O – TMS	TSIM, BSTFA, SILYL-991
Amines primary, secondary	silylation	R' – NR" – TMS	BSA, MSTFA, MSHFBA, SILYL-991
	acylation	R' – NR" – CO – R	TFAA, HFBA, MBTFA, MBHFBA
hydrochlorides	silylation	R' – NR" – TMS	MSTFA
Amides	silylation	not stable	
	acylation	R' – CO – NH – CO – R	TFAA, MBTFA, HFBA, MBHFBA
Amino acids	silylation	R' – CH(NH – TMS) – CO – O – TMS	BSA, BSTFA, MSTFA, MSHFBA
	alkylation (a) + acylation (b)	R' – CH(NH – CO-R) – CO – O – R	a) MeOH/TMCS, TMSH b) TFAA, HFBA, MBTFA, MBHFBA
Carboxylic acids (fatty acids)	silylation	R' – CO – O – TMS susceptible to hydrolysis	BSA, MSTFA, MSHFBA, TMCS, TSIM, SILYL-2110, SILYL-21, SILYL-1139
	alkylation	R' – CO – O – R	DMF-DMA, MeOH / TMCS (1 M), TMSH
salts	silylation	R' – CO – O – TMS susceptible to hydrolysis	TMCS
Carbohydrates	silylation		MSTFA, TSIM, HMDS, SILYL-1139
	acylation		TFAA, MBTFA
Steroids	silylation		BSA, TSIM
	acylation		TFAA, MBTFA, HFBA, MBHFBA

Recommended Derivatization Reagents

Functional Method	Derivatization Group	Reagent
PHARMACEUTICALS, FORENSICS, AND DRUGS OF ABUSE		
Silylation	Alcohols	BSA
		BSA + TMCS
		BSTFA
		BSTFA + TMCS
		HMDS
	Amides	BSA
		BSTFA
		MTBSTFA
	Amines	BSA
		BSA + TMCS
		BSTFA
		BSTFA + TMCS

Recommended Derivatization Reagents

Functional Method	Derivatization Group	Reagent
PHARMACEUTICALS, FORENSICS, AND DRUGS OF ABUSE		
Silylation	Carboxylic Acid	BSA
		BSA + TMCS
		BSTFA
		BSTFA + TMCS
		HMDS
	Steroids	BSA
		BSTFA
		BSTFA + TMCS
		TMSI

Sampling & Sample Preparation, Course # KEM332, October/December 2021, Norbert Maier

Recommended Derivatization Reagents

Functional Method	Derivatization Group	Reagent
PHARMACEUTICALS, FORENSICS, AND DRUGS OF ABUSE (Contd.)		
Acylation	Amines	Acetic Anhydride
		HFBA
		PFPA
		TFAA
	Amides	HFBA
		PFPA
		TFAA

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Recommended Derivatization Reagents

Functional Method	Derivatization Group	Reagent
PHARMACEUTICALS, FORENSICS, AND DRUGS OF ABUSE (Contd.)		
Alkylation	Amines	DMF-DBA
		DMF-DEA
		DMF-DMA
		DMF-DPA
		PFBBr
		TMAH
	Amides	DMF-DBA
		DMF-DEA
		DMF-DMA
		DMF-DPA
		TMAH
	Steroids	BCl_3-Methanol
		DMF-DBA
		DMF-DEA
		DMF-DMA
		DMF-DPA
		TMAH

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Recommended Derivatization Reagents

Functional Method	Derivatization Group	Reagent
ENVIRONMENTAL		
Silylation	Steroids	BCl_3-Methanol
		DMF-DBA
		DMF-DEA
		DMF-DMA
		DMF-DPA
		TMAH
FOOD AND BEVERAGE		
Silylation	Carbohydrate	BSA
		BSA + TMCS
		BSTFA
		BSTFA + TMCS
BIOFUELS		
Silylation		MSTFA

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Course # 55238:
Sample Pretreatment Techniques
(5 cr, Lecturer: Norbert Maier)

2019/Part 14
Derivatization Techniques
(LC-Applications)

Derivatization for Liquid Chromatography: General Aspects

Liquid chromatography (LC) is an highly versatile analytical technique for the separation and quantitation of essentially all compounds of analytical interest, including low-molecular weight compounds (drugs, pesticides, toxins, food ingredients, fine chemicals etc.) to synthetic and natural polymers, spanning a wide range of polarity.

Although a wide variety of detection principles are currently available for LC applications (e.g., UV-, Fluorescence-, Electrochemical, and Mass Spectrometric Detection), some classes of compounds still pose challenges in terms of unfavorable retention characteristics and poor peak efficiency, insufficient stability and/or lack of selectivity.

In these particular cases, dedicated derivatization techniques may be considered to address these limitations.

Generally, analytes are derivatized prior to LC analysis to:
- Ensure detectability of the analyte(s) of interest.
- Improve analyte retention, peak shapes and/or resolution between critical peak pairs.
- Improve detection sensitivity and specificity.
- Chemically stabilize the analyte(s) of interest prior to analytical determination.
- Allow unbiased analysis of multi-analyte profiles in biomarker research & clinical diagnostics.
- Enable analytical distinction between compounds that would be otherwise inseparable (e.g. chemically closely related isomers, i.e. in particular, enantiomers.

Derivatization for Liquid Chromatography: Practical Aspects – Derivatization Reagents

- Choose appropriate reagents based on nature of analyte & matrix, analytical LC technology available and performance requirements of analytical method (LOD, LOQ, range, specificity).
- Choose best commercial quality of reagents available.
- Check quality of reagents ("trust-but-verify") using an appropriate analytical technique (gradient RP HPLC, UV and/or fluorescence spectroscopy, NMR).
- Perform test reaction with well-known analyte to check reagents and develop practical skills in derivatization with the chosen reagent.
- Employ always reagents from freshly opened containers; never use reagents that have been exposed to the lab atmosphere, air and/or moisture for prolonged periods of time. Given the reactive nature of the reagents, these batches may be partially or completely degraded, and will inevitably to poor and irreproducible results, or complete failure.
- All the above mentioned criteria also apply to all other compounds employed in the derivatization reaction, such as catalysts, auxiliary bases, buffer salts etc.

Derivatization for Liquid Chromatography: Practical Aspects – Derivatization Solvents

- Choose solvents capable of solubilizing the sample of interest and the components required for derivatization completely. Heterogeneous reaction solvent systems should be avoided as they frequently lead to irreproducible results.
- Choose best quality of solvent available. All impurities in the solvent may end up in your analytical test sample, leading to high background and potential interferences, compromising method performance.
- Check quality of solvent ("trust-but-verify") using an appropriate analytical technique (gradient RP, UV and/or fluorescence spectroscopy, NMR).
- Choose a solvent (solvent system) that is completely inert towards the components involved in the derivatization reaction (sample; reagent, catalysts, auxiliary bases etc). If possible, distill solvent prior to use.
- If any possible, choose solvent system that is suitable for direct injection into the analytical system to be employed.
- Choose solvent/solvent systems that have reasonably low boiling points in the case that solvent change is required prior to analysis.

Derivatization for Liquid Chromatography: Practical Aspects – Derivatization Conditions

- Use an appropriate concentration of analyte(s) and excess of reagents (typically analyte-to-reagent ratios 1:10 to 1:2000) to drive derivatization to completion in reasonable periods of time.
- Establish kinetics of derivatization reaction (use internal standards) employing blank or surrogate matrixes to learn about the impact of the matrix compounds on the progress and outcome of the derivatization reaction.
- Run reagent/solvent blanks.
- Destroy any active reagents that remains after completion of analyte derivatization employing an appropriate quenching agents, forming a product not interring with the detection and quantification of the analyte derivative of interest.
- Employ reaction conditions that allowing for the mildest possible derivatization protocol. Use the shortest reaction time possible. Avoid prolonged heating at high temperature, extremes of pH and ionic strength, and unnecessary high concentration of reagents, catalyst and auxiliaries.
- Perform all derivatization reaction in closed containers free of contaminations and consisting of proper materials (e.g. glass vials with tightly sealing teflon-lined screw caps).
- Process derivatized samples as soon as the reaction has been completed. Avoid prolonged storage of samples in the derivatization mixture.

Derivatization for Liquid Chromatography: Practical Aspects – Validation Issues

- Establish LOD, LOQ, linearity, range, precision and accuracy employing spiked blank samples.
- Establish robustness of derivatization reaction by studying to which extent the quantitative results do change upon minor variation of the reaction conditions (concentration of reagent; reaction time; temperature; presence of minor amounts of moisture; variation in the matrix composition).
- When using derivatization reaction over extent periods of time, always run intermediate quality control samples at different analyte spiking levels, to ensure appropriate performance.
- Whenever possible, compare the results emerging from the derivatization method with those obtained by alternative analytical methods, e.g. by analyzing samples of known analyte content available in the lab, or spiked samples, or certified reference materials.

Sampling & Sample Preparation, Course # KEM332, October/December 2021, Norbert Maier

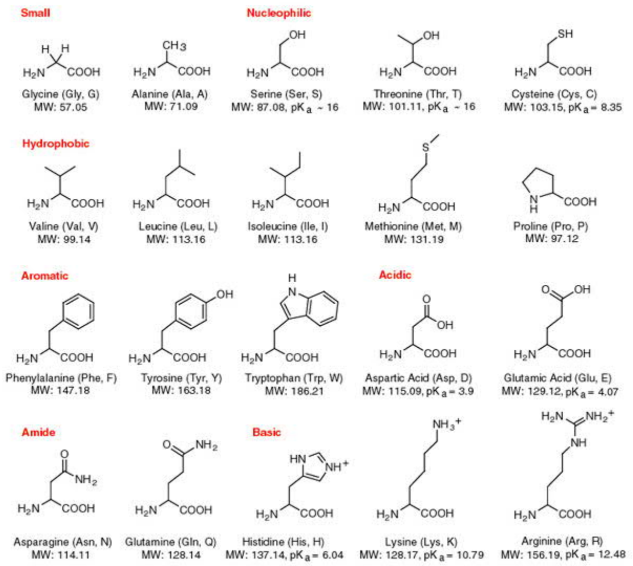
Derivatization for Liquid Chromatography: Ensuring Detectability of Compounds of Interest

Reagents available to confer to analytes properties suitable for

- UV Detection
- Fluorescence Detection
- Electrochemical Detection
- Sensitivity-enhanced MS Detection
- Separation of Enantiomers

Literature: http://www.sciencedirect.com/science/article/pii/B9780124158061000024

Ensuring Detectability of Analytes of Interest Challenges in LC Amino Acid Analysis

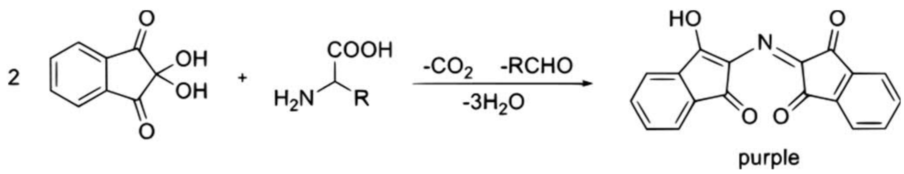


Amino Acids - Properties

- Amino group
- Carboxylic group
- Functional (polar) side chains
- Zwitterionic
- Low (no) RP retention
- Excessive NP retention
- Poor (no) chromophores

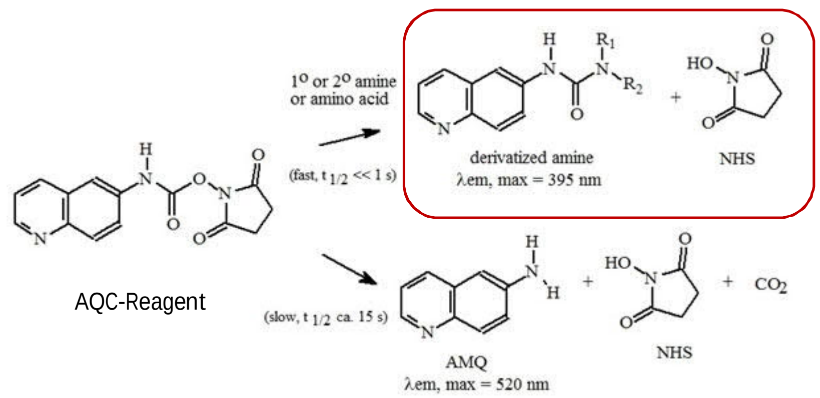
- Derivatization required

Ensuring Detectability of Analytes of Interest Amino Acid HPLC Analysis: Ninhydrin Derivatization Reaction



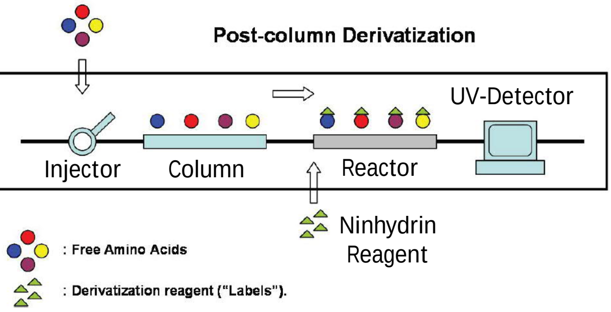
- Note that the reaction of ninhydrin with amino acids leads to complete degradation of the original analytes.
- Most of the biogenic amino acids would produce a single, identical product, i.e. information on the identity of he analytes would be lost.
- This fact renders precolumn derivatization with this reagent elusive.
- However, ninhydrin is an effective reagent to detect separated amino acid eluting individually from a chromatographic column.

Ensuring Detectability of Analytes of Interest Amino Acid UPLC Analysis: AQC Derivatization Reaction



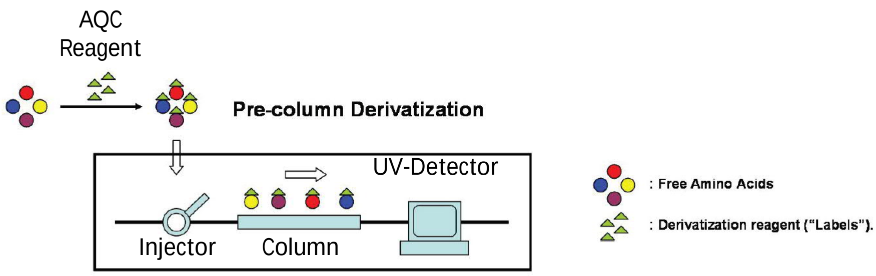
- AQC suffers fast hydrolysis under basic aqueous conditions, forming the corresponding amino quinoline (AMQ) and N-hydroxy succinimide (NHS) as byproducts.
- Successful derivatization is possible due to the favorable kinetics of the reaction of AQC with amines and amino acids.

Ensuring Detectability of Analytes of Interest Amino Acid HPLC Analysis: Ninhydrin/Postcolum Derivatization



- Amino acids from hydrolyzed protein are directly injected and separated on an ion exchange column.
- The eluting amino acids are mixed with ninhydrin reagent and transferred to the reactor unit.
- In the reactor unit, the amino acids are converted into the corresponding derivatives, which are subsequently detected in the downstream-positioned UV detector.

Ensuring Detectability of Analytes of Interest: Amino Acid UPLC Analysis: AQC/Precolumn Derivatization

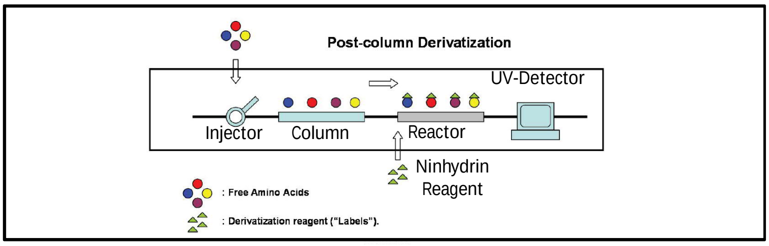


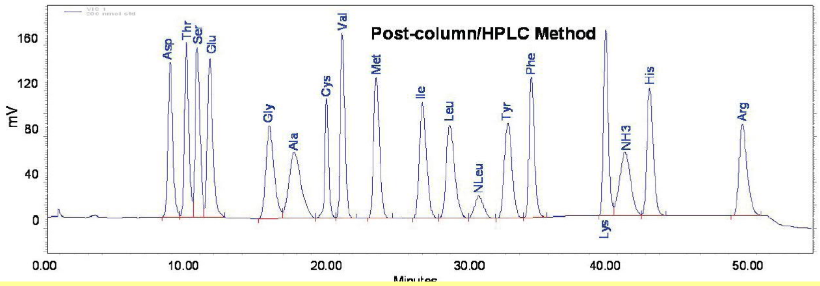
- Amino acids from hydrolyzed protein reacted with AQC reagent to yield the corresponding derivatives.
- The preformed derivatives are injected into the separation column and separated.
- The derivatives emerging from the column are detected by the the amino acids by the downstream-positioned UV detector.

Derivatization for Liquid Chromatography: Optimization of analyte retention, peak shapes and/or resolution between critical peak pairs

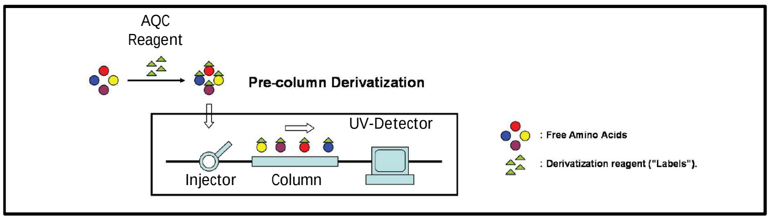
Method	Pre-column/UPLC	Post-column/HPLC
Instrument	Waters® ACQUITY UPLC™ System	Hitachi L-8800 amino acid analyzer
Column	Waters AccQ·Tag Ultra C18 1.7µm 2.1 x 100mm	Hitachi AAA special analysis column 4.6 x 60mm
Detection Wavelength	260nm	440nm (570nm for Pro)
Derivatization reagent	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC)	Ninhydrin
Run time	9.5min	84.8min
Injection Volume	1 µL	20 µL
Standard	Waters Amino Acid Standard	Thermo Scientific amino acid standard
Internal Standard	NorValine	NorLeucine
Sample	Bovine Serum Albumin (BSA) hydrolysate	

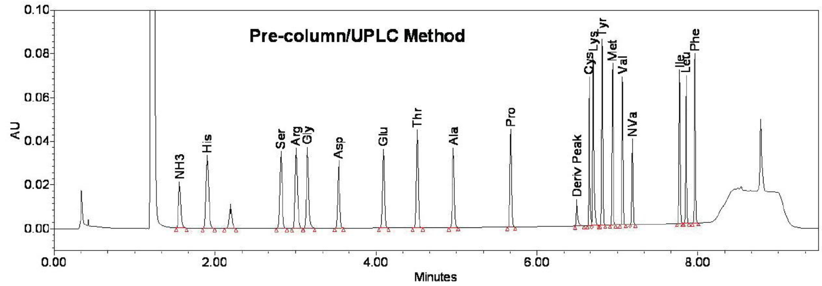
Ensuring Detectability of Analytes of Interest Amino Acid HPLC Analysis: Ninhydrin/Postcolum Derivatization



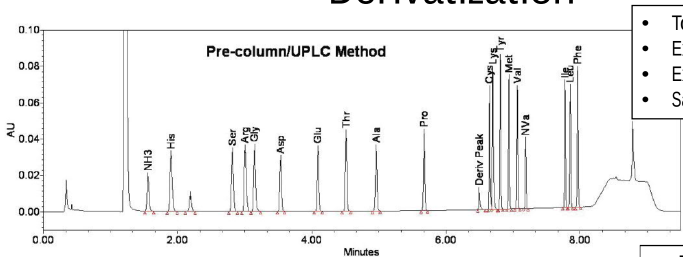


Ensuring Detectability of Compounds of Interest Amino Acid Analysis: AQC/Precolumn Derivatization

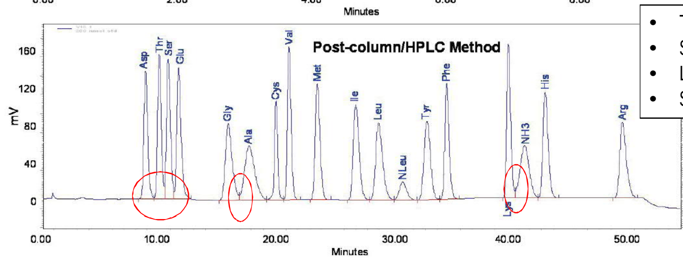




Optimization of Analyte Retention, Peak Shapes & Resolution between Critical Peak Pairs: AQC/Precolumn vs. Ninhydrin/Postcolumn Derivatization



- Total analysis time: 10 min
- Excellent peak resolution
- Excellent peak efficiency
- Sample volume: 1.0 microL



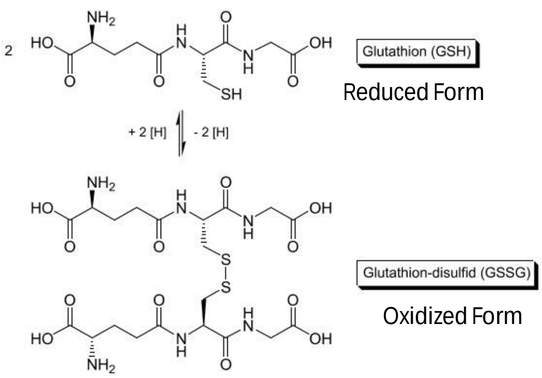
- Total analysis time: 60 min
- Several critical peak pairs
- Low peak efficiency
- Sample volume: 20 microL

Derivatization for Liquid Chromatography: Stabilizing Analytes of Interest Prior to Analysis The Glutathione Issue

Glutathione (GSH) is an essential metabolite for multiple fundamental antioxidant and detoxifying reactions of the cell, playing a key role in cell resistance to oxidative and nitrosative damage by providing reducing equivalents for the enzymes involved in the metabolism of reactive oxygen and nitrogen species.

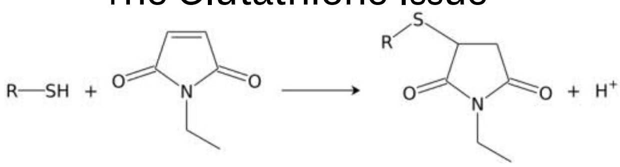
GSH depletion in the body may derive from decreased GSH production as a consequence of some pathophysiological conditions (e.g., cardiovascular diseases) as well as from drug treatments.

Measurement of GSH in blood has been considered an index of the whole organism oxidative status and a useful indicator of disease risk in humans because an alteration of the GSH level in whole blood should reflect that of other organs.



Analytical Biochemistry 415 (2011) 81–83

Derivatization for Liquid Chromatography: Stabilizing Analytes of Interest Prior to Analysis The Glutathione Issue

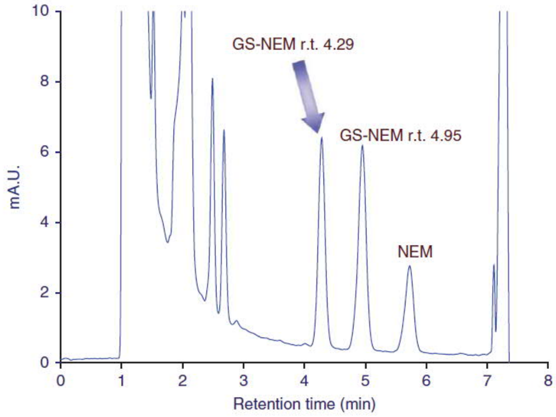


GSH NEM NEM-GSH

- The measurement of glutathione is a challenging task in that, during sample manipulation, a large percentage of this compound can be artificially oxidized.
- Stabilization of reduced glutathione from blood, along with introduction of a appropriate chrmphore for UV detection, is required for reliable quantification prior to high-performance liquid chromatography.
- Both of these tasks can be achieved by treatment of blood with N-ethylmaleimide (NEM), to which the thiol function of glutathione adds to form a “GS-NEM conjugate”, which protects the metabolite from oxidation during sample handling & storage.
- In this derivatized form, glutathione in blood samples is stable for 90 days, even if stored at 20°C.

Analytical Biochemistry 415 (2011) 81–83

Derivatization for Liquid Chromatography: Stabilizing Analytes of Interest Prior to Analysis N-Ethylmaleimide Glutathione Conjugate



- Representative chromatogram of the GS-NEM conjugate in blood samples. The GS-NEM conjugate was analyzed by reversed-phase HPLC with UV detection at 265 nm in supernatants obtained from NEM-treated blood after acidification.
- Note that the GS-NEM conjugate separation results in two peaks with the same area at 4.29 min and 4.95 min r.t.
- These two peaks form as a consequence of the generation of diastereomers, which are separable under achiral chromatographic conditions.
- The unreacted NEM gives a peak at an r.t. of 5.8 min.

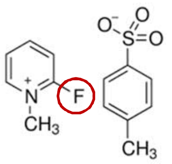
1660 | VOL.8 NO.9 | 2013 | nature protocols

Improvement of Detection Sensitivity and Specificity Mass-Sensitivity Enhancement by Derivatization

- In electrospray ionization (ESI) mass spectroscopy highly polar species often suffer from relatively poor ionization efficiency when compared with ionizable species with significant non-polar characteristics (e.g., surfactants).
- Introducing a nonpolar functionality in the form of a dansyl group into the polar structure has often the desirable effect of increasing ionization efficiency and sensitivity, especially when RP phase chromatographic conditions.
- Thus, derivatization with mass-sensistivity enhancing reagents often have the desirable effect of reducing detection limits for the analyte of interest.

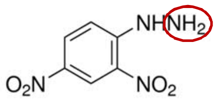
Anal. Methods, 2015, 7, 1000

Improvement of detection sensitivity and specificity
Typical Reagents for Mass-Sensitivity Enhancement



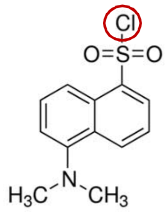
2-Fluoro-*N*-methylpyridinium *p*-toluenesulfonate

Alcohols



2,4-Dinitrophenylhydrazine

Ketones / Aldehydes

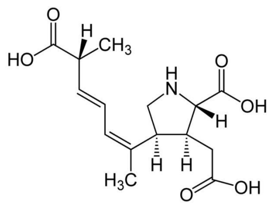


Dansyl Chloride

Amines / Phenols

Anal. Methods, 2015, 7, 1000

Improvement of Detection Sensitivity and Specificity
Effects of Shell Fish Toxin Domoic Acid on Wildlife

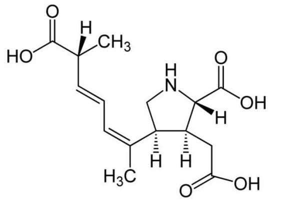


Domoic Acid

"In the spring of 2002 a crisis fell upon hundreds, if not thousands, of pregnant California sea lions, dolphins and pelicans along the Pacific coast of the United States. Domoic acid, a naturally occurring marine biotoxin, produced by marine diatoms which are members of the genus Pseudo-nitzschia, forced pregnant sea lions to beach themselves suffering major seizures, foaming from their mouths, the whites of their eyes bloody red and many of the sea lions, paralyzed from the toxin had blood excreting from their nipples."

http://whalerescueteam.org/rescue-stories/domoic-acid-poisoning-rescues/

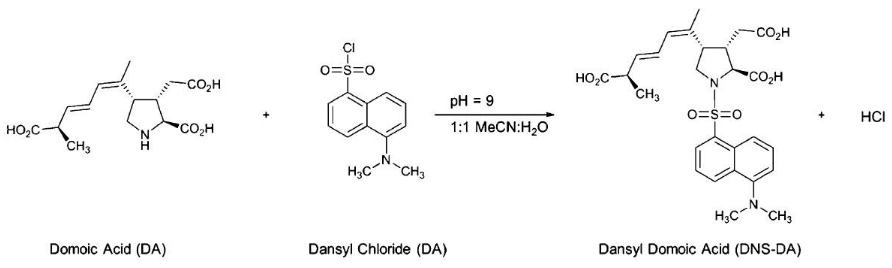
Improvement of Detection Sensitivity and Specificity
Effects of Shell Fish Toxin Domoic Acid on Wildlife



Domoic Acid

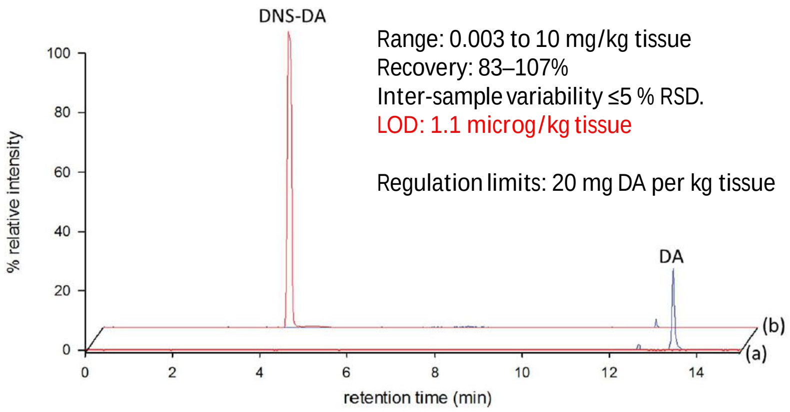
- Shell fish tissue was homogenized and extracted with methanol : water (1 : 1, v/v), by vortexing, followed by centrifugation.
- An aliquot of the supernatant was filtered and applied onto a strong anion exchange SPE cartridge conditioned with methanol followed by water and then by of methanol : water (1 : 1, v/v).
- The cartridge was washed with methanol : water (1 : 1, v/v) and then eluted with of 1 M formic acid in acetonitrile.
- The emerging eluate was evaporated to dryness under nitrogen, and then reconstituted in acetonitrile : water (1:1 v/v) for derivatization.

Improvement of Detection Sensitivity and Specificity
Derivatization of Domoic Acid with Dansyl Chloride



Anal. Methods, 2015, 7, 1000

Improvement of Detection Sensitivity and Specificity Derivatization of Domoic Acid with Dansyl Chloride



Range: 0.003 to 10 mg/kg tissue
Recovery: 83–107%
Inter-sample variability ≤5 % RSD.
LOD: 1.1 microg/kg tissue

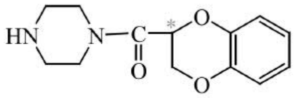
Regulation limits: 20 mg DA per kg tissue

HILIC-MS monitoring of domoic acid dansylation. Trace (a) shows domoic acid detection in a control sample without dansylation. Trace (b) shows the absence of DA and detection of DNS-domoic acid, respectively, after dansylation using a 5 min reaction time.

Derivatization for Liquid Chromatography: Achieving Separation of "Inseparable Analytes" Separation of Enantiomers via Chiral Derivatization

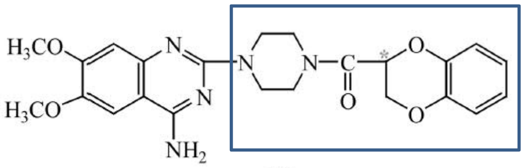
- Enantiomers (Optical Isomers) can not be separated with conventional separation techniques since they exhibit (in absence of a chiral environment) identical physico-chemical properties.
- However, when transformed into a pair of diastereomers by reaction with an enantiomerically pure "chiral derivatization agent" they yield a pair of diastereoisomers, which due have different physico-chemical properties, and may be resolved using e.g. RP chromatography.
- Separation of enantiomers is a crucial task in many fields, such as drug development, flavor and fragrance industry, biochemical research, clinical diagnostics etc.

Derivatization for Liquid Chromatography: Achieving Separation of Enantiomers via Chiral Derivatization – Doxazosin Key Intermediate



I

BFP



II

Doxazosin

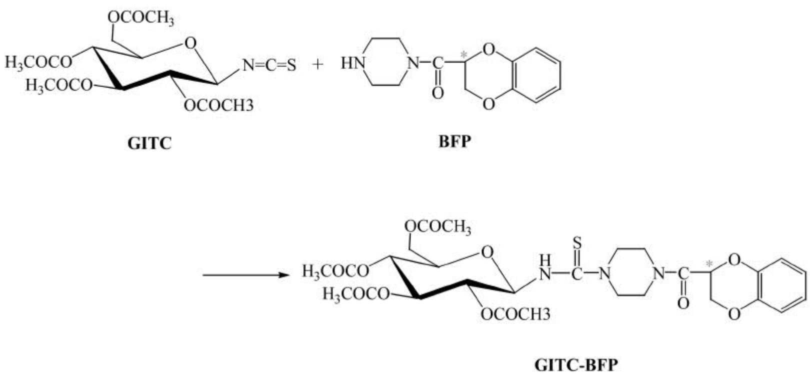
1-(1,4-Benzodioxane-2-formyl)piperazine (BFP) is an important pharmaceutical intermediate used in the preparation of doxazosin, an anti-hypertonic drug.

J. Sep. Sci. 2005, 28, 193–196

Derivatization for Liquid Chromatography: Achieving Separation of Enantiomers

Chiral Derivatization Agent

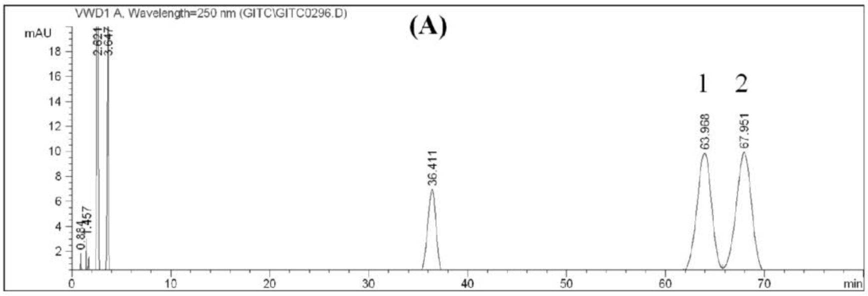
Pair of Enantiomers (inseparable)



Pair of Diastereomers (separable)

J. Sep. Sci. 2005, 28, 193–196

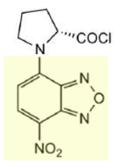
Derivatization for Liquid Chromatography: Achieving Separation of Enantiomers



Chromatograms of GITC derivatives under optimal conditions on the ODS column obtained by using a mobile phase consisting of 50:50 (v/v) methanol–0.02 mol/L potassium dihydrogenphosphate solution, flow rate 0.6 mL/min, detection wavelength 250 nm.

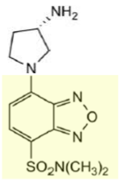
J. Sep. Sci. 2005, 28, 193–196

Derivatization for Liquid Chromatography: Chiral Derivatization Agents forming Fluorescent Diastereomeric Derivatives



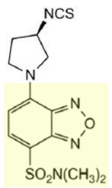
(R)-(+)-NBD-Pro-COCl

Chiral Derivatization Agent for Amines/Alcohols Forming Diastereomeric Amides/Esters



(R)-(-)-DBD-Apy

Chiral Derivatization Agent for Carboxylic Acids Forming Diastereomeric Amides



(R)-(-)-DBD-Py-NCS

Chiral Derivatization Agent for Amines Forming Diastereomeric Thioureas

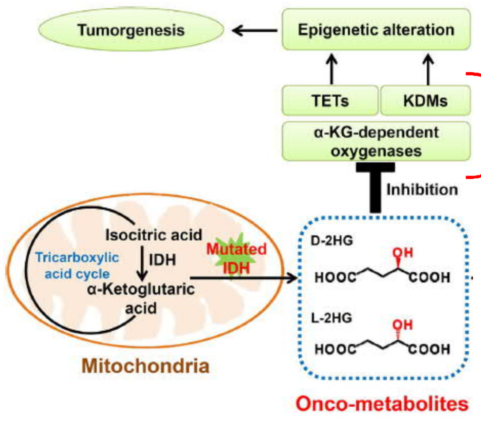
- ❑ Reagents contain as fluorogenic elements 7-nitro/sulfomamido-1,2,3-benzoxadiazole motifs.
- ❑ The derivatives exhibit strong fluorescence, allowing certain chiral analytes being resolved and quantified at the 10^{-15}M (aM) concentration level.

Application of Derivatization Concepts in Clinical Analysis

Sensitive Determination of Onco-Metabolites D- and L-2-Hydroxyglutarate by Chiral Derivatization Combined with HPLC-MS/MS Analysis

Scientific Reports | 5:15217 | DOI: 10.1038/srep15217

Determination of Onco-Metabolites D- and L-2-Hydroxyglutarate by Chiral Derivatization Combined with HPLC-MS/MS Analysis



Enzymes essential for repair of pathological DNA modifications

Mutations of isocitrate dehydrogenase (IDH) enzymes may lead to an accumulation of the enantiomers of 2-hydroxyglutarate (2HG) in tissues and biological fluids. 2HGs act as competitive substrate inhibitors of several α-ketoglutarate-dependent DNA repair enzymes, and may cause epigenetic alteration of the genome and ultimately cancer. Analysis of the 2HGs is therefore of interest in clinical cancer diagnostics.

Scientific Reports | 5:15217 | DOI: 10.1038/srep15217

Sampling & Sample Preparation, Course # KEM332, October/December 2021, Norbert Maier

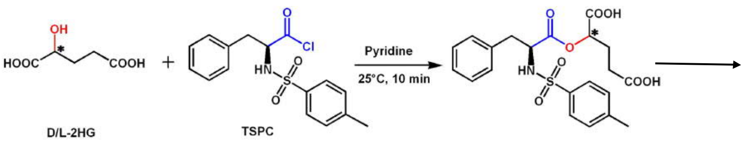
Determination of Onco-Metabolites D- and L-2-Hydroxyglutarate by Chiral Derivatization Combined with HPLC-MS/MS Analysis

Challenges in 2HG Analysis

- Low concentrations of 2HG metabolites in biological liquids/tissues - highly sensitive analytical method is needed.
- Information on the relative amounts of 2HG enantiomers are of relevance; however, enantiomers are not resolvable with conventional RP HPLC - enantioselective analytical method is needed.
- 2HG lacks chromophores/fluorophores – difficult to detect.
- 2HG shows poor ionization characteristics by ESI MS in parent form.
- 2HG is highly hydrophilic and has chelating properties – poor retention on conventional RP HPLC and broad peak shapes.

Scientific Reports | 5:15217 | DOI: 10.1038/srep15217

Determination of Onco-Metabolites D- and L-2-Hydroxyglutarate by Chiral Derivatization Combined with HPLC-MS/MS Analysis



Gradient RP HPLC MS/MS Analysis

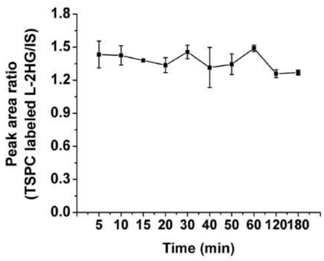
Analytical Benefits Achievable through TSPC-Derivatization:

- 2HGs are converted into more lipophilic molecules –> improved interactions with RP column leading to higher retention factors.
- Chelating OH-group of 2HG is blocked through derivatization –> improved peaks shapes.
- 2HG enantiomers are transformed through derivatization into separable diastereomers
- –> quantitation of individual 2HG enantiomers becomes possible.
- Introduction of chiral derivatization label dramatically improves detection sensitivity of 2HG and enriches its fragmentation pattern.

Scientific Reports | 5:15217 | DOI: 10.1038/srep15217

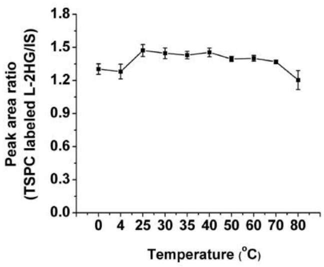
Determination of Onco-Metabolites D- and L-2-Hydroxyglutarate by Chiral Derivatization Combined with HPLC-MS/MS Analysis: Optimization of Derivatization Protocol

A Optimization of Reaction Time



L-2HG (2 nmol)@160 µL TSPC (2.5 mM in ACN) @2 µL pyridine. Incubated at 40 °C from 5 min to 3 h with shaking at 1,500 rpm.

B Optimization of Reaction Temperature

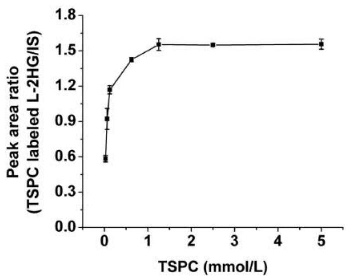


L-2HG (2 nmol)@160 µL TSPC (1.25 mM in ACN) @2 µL pyridine. Incubated at different temperatures (0 to 80 °C) for 10 min with shaking at 1,500 rpm.

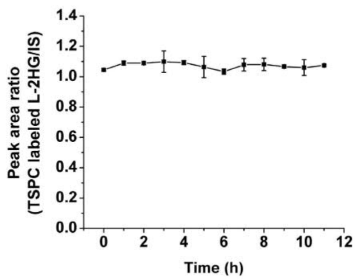
Scientific **Reports** | 5:15217 | DOI: 10.1038/srep15217h

Determination of Onco-Metabolites D- and L-2-Hydroxyglutarate by Chiral Derivatization Combined with HPLC-MS/MS Analysis: Optimization of Derivatization Protocol

C Optimization of Reagent Amount



D Stability of **TSPC-**Derivatives at 25°C



L-2HG (2 nmol)@160 µL TSPC (0.025 to 5.0 mM in ACN) @2 µL pyridine. Incubated at 40 °C for 10 min with shaking at 1,500 rpm.

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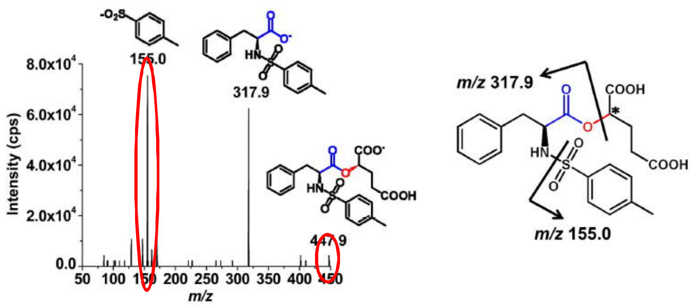
Determination of Onco-Metabolites D- and L-2-Hydroxyglutarate by Chiral Derivatization Combined with HPLC-MS/MS Analysis: Fragmentation Pattern



Fragmentation pattern of TPSC-2HG diastereomers: Note that the fragments emerging from the TPSC label are more intense than that of the parent derivative.

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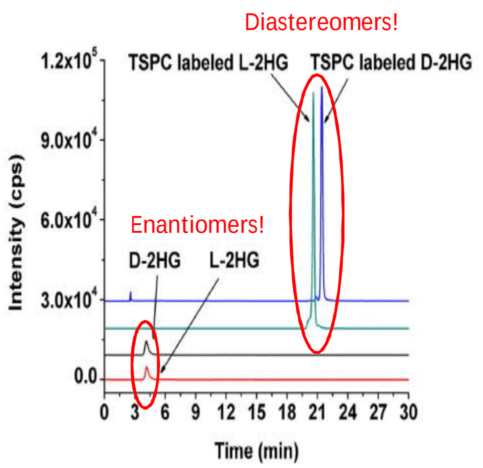
Sensitive Determination of Onco-Metabolites D- and L-2-Hydroxyglutarate by Chiral Derivatization Combined with HPLC-MS/MS Analysis



Extracted-Ion chromatograms of parent D-2HG / L-2HG metabolites and the respective TSPC-Derivatives

- **Major enhancement in LC-MS sensitivity for 2HG after derivatization.**
- **Derivatives allow successful discrimination between 2HG enantiomers.**

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Sensitive Determination of Onco-Metabolites D- and L-2-Hydroxyglutarate by Chiral Derivatization Combined with HPLC-MS/MS Analysis: Method Performance Characteristics

Analytical methods	LODs (fmol)		Increased folds of the detection sensitivity	
	D-2HG	L-2HG	D-2HG	L-2HG
Underivazitation	348.6	345.6	–	–
DATAN labeling	115.0	102.0	3.0	3.4
TSPC labeling	1.2	1.0	291	346

Limits of Detection (LODs) of D-2HG and L-2HG obtained by different methods. Derivatizations were performed under optimized conditions.

Note that the LODs for 2HG could be improved by a factor 300 (!!) through TSPC derivatization.

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Derivatization for Liquid Chromatography:

Supporting Literature:

Book Chapter: *Derivatization in Liquid Chromatography*, in "Liquid Chromatography-Application"

http://www.sciencedirect.com/science/article/pii/B9780124158061000024

Sampling & Sample Preparation, Course # KEM332, October/December 2021, Norbert Maier