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Optimization and analytical applications of chemical vapour generation of anionic species at trace level by aqueous phase alkylation with triethyloxonium salts

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Abstract

Alkylating properties of trialkyloxonium salts have recently been exploited for analytical purposes in the determination of anionic/nucleophilic species at trace levels in aqueous solution. The procedure entails generating alkylated volatile derivatives, to be analyzed by Headspace/Gas Chromatography-Mass Spectrometry (HS/GC-MS).

Several features can play an important role in this type of reactions and in this work they have been systematically investigated for the optimization of analytical methods devoted to the determination of I^-, Br^-, SCN^-, CN^-, S^{2-} by HS/GC-MS.

In order to maximize reaction yields and recovery of the volatile products, the role of some parameters has been examined: pH of the reaction medium, time and temperature of the process, concentration of alkylating agent and amount of a non-reactive salt Na_2SO_4 to induce salting out effect.

The present study allowed us to define several sets of experimental conditions for the optimized determination of different anionic species. Detection limits obtained were: iodide 0.05 ng/mL, bromide 0.51 ng/mL, cyanide 48.97 ng/mL, thiocyanate 2.24 ng/mL and sulphide 0.12 ng/mL. They have been improved by factors 5 (Br^-, CN^-), 24 (I^-), 125 (SCN^-), up to 3083 (S^{2-}) with respect to those previously reported in the literature. The optimized conditions have been employed for the implementation of an analytical method for the determination of SCN^- in human saliva.

Introduction and aim of the thesis

Derivatization reactions, in analytical chemistry, are widely used for the qualitative and the quantitative analysis of trace and ultra-trace levels. By using trialkyloxonium salts we can obtain volatile alkyl derivates; that's resulting in an increased of the analytes volatility, decreasing its polarity. This approach, known in literature as *Chemical Vapour Generation* (CVG), can convert a non volatile analyte – usually an ion in aqueous solution – in a volatile derivate which can be separated from its matrix as a gas or a vapour. CVG is thus, first of all, a separation technique. Separations are of primary importance in analytical chemistry and usually they also present some additional advantages as the possibility of eliminating or limiting the interferences arising from the sample matrix, and of developing preconcentration strategies which may dramatically improve the limits of detection.

Chemical Vapour Generation techniques have been developed by using aqueous phase reactions of tetrahydridoborate salts ($NaBH_4$ and KBH_4) and sodium tetralkylborates ($NaBR_4$; R= Et, Ph, Isopropyl) for the determination and speciation of elements at trace and ultratrace levels. These reagents allows to obtain volatile hydrides and alkyl derivatives, respectively, for a series of elements (Ge, Sn, Pb, As, Sb, Bi, Se, Te,Hg, Cd, Cu) which are present in solution from inorganic or simple organometallic substrates. Both the reagents, $[BH_4]^-$ and $[BR_4]^-$, behave similarly with the analytical substrates: the element, which is present in the analytical substrate, is activated towards nucleophilic attack by an appropriate choice of reaction conditions (pH, type of acid or buffer, addition

of selected complexing agents etc) and the reactions can be seen as the transfer of H^- or R^- from boron to the element of the analytical substrate.

The potentialities of CVG have been recently expanded with the use of aqueous phase derivatization reaction with trialkyloxonium salts ($R_3O^+X^-$). Among various chemical agents able to derivatize anionic/nucleophilic species, trialkyloxonium salts are very efficient for the alkylation of several anionic species, such as halides (F^-, Cl^-, Br^-, I^-) nitrate, nitrite, sulfide, cyanide and thiocyanate. These salts give fast alkylation reactions and lead to the formation of volatile alkyl derivates. The final structure derives from the attack of a carbocation (R^+) to the substrate present in solution.

In this work the possibility of improving the analytical performances of aqueous phase CVG with $Et_3O^+BF_4^-$ has been investigate to determine some of the above mentioned anionic species. The study systematically investigated the definitions of possible reaction scheme of the primary reaction as well as those of competitive and interferring reactions.

The optimized method has been applied to the analysis of a biological matrix, in particular to the detection and quantification of SCN^- in samples from human saliva. Concentration levels of SCN^- are correlated to the presence of specific pathologies in human body. In particular, the amount of thiocyanate detected in each sample allows to distinguish smokers from non-smokers.

Chapter 1

State of the art

1.1 Chemical Vapour Generation (CVG)

The generation of volatile species, often coupled with atomic or mass spectrometry, is a procedure of derivatization which allows to determine some elements at trace levels, in simple and complex matrices. Among the different derivatization procedures, chemical derivatization are the most widespread. *Chemical Vapour Generation* allows to obtain volatile species that can be removed from the matrix and analyzed; this allows, in most cases, to reduce the matrix effect and carry out both qualitative and quantitative analysis. Some metals, such as Au[1], Ag, Ni, Co[2] and Hg[3] can be analyzed by CVG. Hg^{2+}, for example, can be reduced, by $SnCl_2$ as reductant, to $Hg^{(0)}$. Mercury vapours generated are separated from the solution by stripping with an inert gas and introduced into a system of atomization/detection. With the *Chemical Vapour Generation* it is possible to perform derivatization reactions in simple, cheap and fast conditions, in aqueous phase. Moreover, thanks to the separation of the analyte from the matrix, the matrix effect is downed, and improved detection limits are obtained. By CVG analytes can be determined in complex matrices, such as biological matrices, without sample dilution or pretreatment.

Generation of volatile hydrides: The generation of volatile hydrides, by reaction of ionic inorganic compounds containing elements such as As, Sb, Bi, Se, Te, Ge, Sn, Pb, Hg, and Cd with $NaBH_4$ in aqueous phase, is a consolidated method for the determination and speciation of elements at trace and ultra-trace levels with atomic spectrometry. This method has also been extended to the determination of In, Tl, Cu, Au, Ni, Ag, Zn, and other transition metals, although the results are not yet consolidated from the analytical point of view. *Table 1.1* can be considered a good summary.

Generation of volatile halides: This is an emerging technique for its simplicity and the absence of side reactions: in most cases the chloride of the analyte of interest, generated in an environment of 6M HCl[4] is distilled.

Table 1.1 CVG in analytical chemistry

Reactive	***Reaction***	***Elements***	***Products***
$SnCl_2$	Reduction	Hg	$Hg^{(0)}$
BH_4^-	Formation of hydrides and atomic species	As, Sb, Bi Ge, Sn, Pb [In, Tl] Se, Te Cd, Hg	AsH_3, SbH_3, BiH_3 GeH_4, SnH_4, PbH_4 [InH, TlH ?] H_2Se, H_2Te $Cd^{(0)}/CdH_2$, $Hg^{(0)}$
BH_4^-	Unknown	Cu, Ag, Au, Ni, Pd, Rh, Pt, Ti, Ir, Mn, Co, Fe, Cr, Zn	Unidentifiable yields 1-50%
BH_3-CN^- BH_3NR_3	Formation of hydrides and atomic species	As, Sb, Bi Se, Te, Hg	Same products obtained with BH_4^-
$NaB(Et)_4$ $NaB(Ph)_4$	Formation of metal alkyls	Pb, Sn, Hg Se Bi, Cd Hg	$PbEt_4$, $SnEt_4$, $HgEt_2$ Et_2Se $BiEt_3$, ($CdEt_2$) $HgPh_2$

F^-	Volatile Complex	Si	SiF_4
Cl^-	Volatile complex	As, Bi, Sb, Cd, Zn, Ge, Tl, Pb	MCl_x
CO	Metal-carbonyl complexes	Ni	$Ni(CO)_4$
MnO_4^-	Formation of oxides	Os	OsO_4
MeOH/ H_2SO_4	Formation of ester	B	$B(OMe)_3$

Formation of metal alkyls: The most used agents in aqueous solution are tatralkylborates, that give volatile alkyl derivatives[5], more stable than the relative hydrides. Tetraethylborate is sensitive to atmospheric oxygen and decomposes exothermically, while tetraphenylborate is more stable.

The use of cyanoborohydride and aminoboranes: They can be considered as complexes of BH_3 with cyanide and with amines. The reactivity of these complexes varies depending on the donor group. Cyanoborohydride and aminoboranes have lower reducing power. They are more stable in protic conditions and the acidic hydrolysis speed is lower than the that of borohydride.

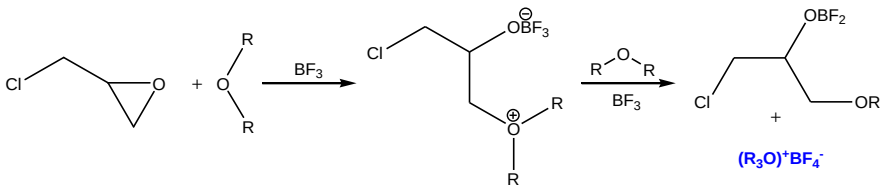
1.2 Trialkyloxonium salts[6]

At present, the chemical vapour generation is commonly applied to the analysis of the metals and semi-metals listed in *Table 1.1*. Recently CVG has been applied to the determination of anionic species, with promising results[7]. From these considerations, it was decided to investigate the reactivity of trialkyloxonium tetrafluoroborate salts, which

are powerful alkylating agents, as formal donors of carbocations, for the derivatization of anionic/nucleophilic species in aqueous phase. Oxonium salts are organic compounds containing an oxygen atom positively charged and can be classified in saturated and unsaturated, depending on whether the ligands at the oxygen atom are connected to the eteroatom by single or multiple bonds. In saturated compounds, the charge is localized on the oxygen while, in the unsaturated compounds, charge is localized on the adjacent atom. Saturated compounds, depending on the number of R groups linked to oxygen, can also be classified in primary ($[RH_2O]^+X^-$), secondary ($[R_2HO]^+X^-$) and tertiary ($[R_3O]^+X^-$). Trialkyloxonium salts were discovered by H. Meerwein in 1937[8] and were immediately classified as a new class of high potential alkylating compounds[9]. These salts are soluble in water and can be handled in air. They are able to alkylate anionic/nucleophilic substances, producing derivatives that are either volatile or soluble in organic solvents. The applications of these reagents have been explored in organic chemistry over the 20th century[6, 8, 10] but their application in analytical chemistry for the determination of inorganic anions has only been recently introduced[7].

1.2.1 Preparation of $R_3O^+BF_4^-$

The method commonly used to prepare trialkyloxonium tetrafluoroborate salts is based on the reaction between 2-chloromethyloxirane and ether with trifluoroborane[6, 8, 11].



The mechanism of this reaction is confirmed by the possibility of isolate the first reaction intermediate that, reacting with the ether in presence of trifluoroborane, gives trialkyloxonium tetrafluoroborate salt. Boric ester obtained can also be converted, by NaOH, in 2-alkoxy-methyl-oxirane, that is the precursor of our product. Elevated yields

are obtained with methyl and ethyl derivates; reagents with a greater number of carbon atoms give derivatives with a high stability in water and their synthesis is not more complicated[12]. Other synthesis methods have been proposed by G.A. Olah et al[6].

1.2.2 Decomposition of $R_3O^+BF_4^-$ [8]

Trialkyloxonium salts are soluble in polar solvents and they can also be soluble in less polar solvents, depending on the length of the alkyl radical. A characteristic property of tertiary oxonium salts is their dissociation at high temperatures with the separation of ether complexes of boron trifluoride and alkyl fluorides. Trialkyloxonium salts decompose not only at high temperature but also when their solutions are allowed to stand. In particular, triethyloxonium fluoroborate is unstable in solution, decomposing into diethyl ether, boron trifluoride, and ethyl fluoride, in relatively little time. Decomposition is accelerated by the addition of ethers to the solutions.

1.2.3 Chemistry of $R_3O^+BF_4^-$

The use of trialkyloxonium salts in organic chemistry is due to their great alkylating strength and to the possibility of alkylate substrates with heteroatoms such as O, N and S, or carbon atoms, depending on the reaction conditions.

1.2.3.1 O-alkylation

For labile compounds, the use of these salts for oxygen alkylation is very useful, thanks to the particularly mild reaction conditions.

- ***Hydrolysis of trialkyloxonium salts***

The most simple reaction of this category is the hydrolysis of oxonium salts[8].

$$R_3O^+BF_4^- + H_2O \rightarrow R_2O + ROH + H^+BF_4^-$$

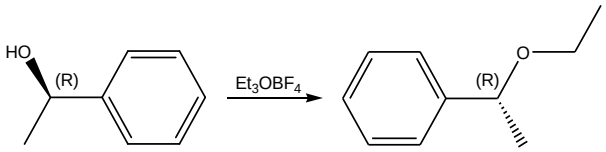
This reaction involves the formation of an alcohol and an ether and shows different reaction times for the complete hydrolysis of the salt, depending on the length of the alkyl substituent (*Table 1.2*).

Table 1.2 Hydrolysis times of different oxonium salts

Oxonium salt	***Time [min]***
$(Me_3O)^+BF_4^-$	8
$(Et_3O)^+BF_4^-$	80
$(n\text{-}Pr)_3O^+BF_4^-$	120

- ***Esterification of alcohols, carboxylic acids and ethers***

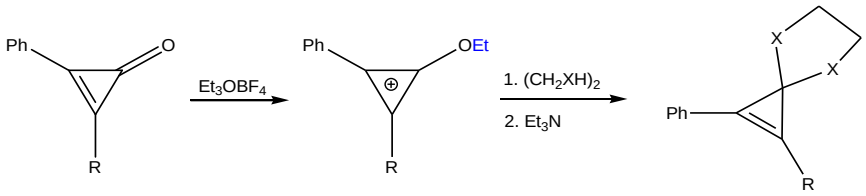
For the alkylation of organic substrates such as alcohols, phenols and carboxylic acids, the presence of strong alkylating agents such as trialkyloxonium salts is not necessary. However, when the systems require reaction conditions not too energetic or higher yields, these salts are widely used for alkylation reactions. These salts are used for the alkylation of carboxylic acids, thanks to the quantitative yields with simple carboxylic acids. D.J. Raber et al.[13] described the advantages of the application of the trimethyloxonium and triethyloxonium salts for the alkylation of carboxylic acids. Esterification is carried out by adding the trialkyloxonium salt and the N,N-diisopropylethylamine to the CH_2Cl_2 solution of the carboxylic acid. Below the alkylation reaction of an alcohol to obtain an ether[6, 14] is shown. This reaction is suitable if we want to maintain the stereochemistry of a particular product.



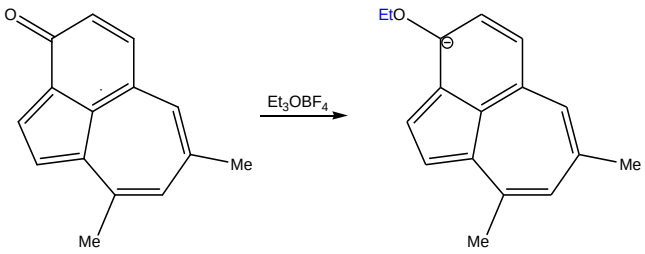
N,N-diisopropylethylamine is necessary in order to buffer the acidity and avoid isomerization problems. The reactivity of the trialkyloxonium salts with the ethers leads to the formation of a new trialkyloxonium salt and can be exploited for the synthesis of these reagents. This reaction has been employed by Meerwein in order to prepare the trimethyloxonium tetrafluoroborate salt[15].

- ***Reaction with the carbonyl group***

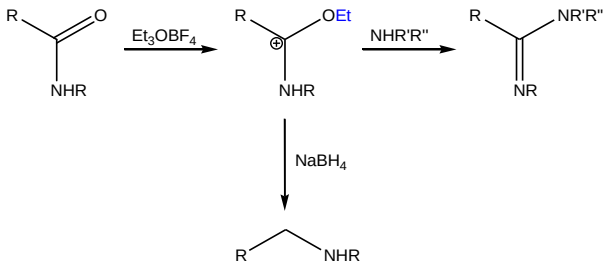
Trialkyloxonium salts can also be employed for the alkylation of ketones, followed by the formation of a stable positive ion. These alkoxyalkyl salts can be used as synthetic intermediates[16].



The intermediate salt is stable when the positive charge is delocalized. An example is the reaction reported by R. Neidlein et al.[17].

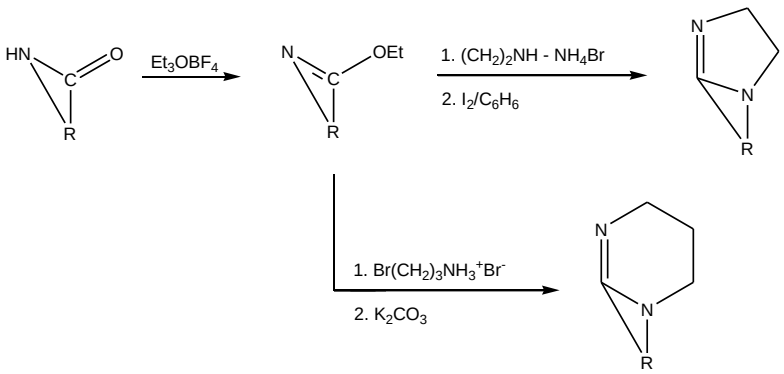


The use of trialkyloxonium salts also allows the *O*-alkylation of amides. The intermediates are used in organic chemistry for synthetic purpose. The attack of the ethylating agent is on the oxygen and leads to the formation of a very reactive immidio salt that, in a second step, may be reduced to an amine group by using $NaBH_4$[18], or converted to amidine, by treatment with an amine[19].



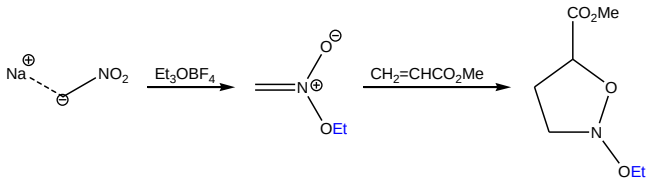
This reaction can convert an amide group in an amine, using softer conditions than organic chemistry reduction by $LiAlH_4$, and it was employed by S. Hanessian[20] to remove a *N*-acetyl group introduced before to protect the amine.

The reaction between triethyloxonium tetrafluoroborate salt and cyclic amides is interesting for synthetic purposes as it leads to the formation of excellent synthetic intermediates. For example H. Yamamoto et al.[21] proposed a method for the preparation of cyclic poliammidine, starting from lactams ethylation.



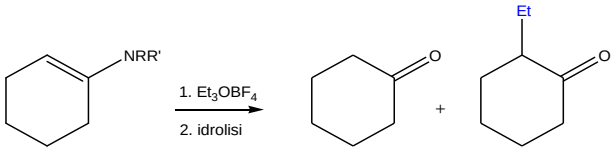
- ***Reaction with the carbonyl group***

Sulfoxides and phosphinoxides undergo *O*-alkylation by trialkyloxonium salts to give the corresponding salts of sulfonium and phosphonium. The alkylation does not occur on sulfur or phosphorus atoms. Also the nitroso-compounds prefer the attack to oxygen rather than nitrogen, as well as the nitro-compounds which are transformed into the analogous nitronic esters as shown by H. Sato et al.[22].



1.2.3.2 C-alkylation

This reaction leads to the formation of C-C bonds. In presence of nucleophilic heteroatoms on the hydrocarbon chain, the alkylation reaction on the chain could be competitive with the same reaction on the heteroatoms.



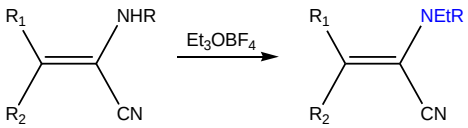
In this particular example, the data reported[23] show that the relative proportions between the two products change depending on the groups R of the amine and the kind of alkylating agent used, even if the favourite product resulted from the attack of the carbocation on the carbon atom in beta.

1.2.3.3 N-alkylation

Trialkyloxonium salts are not widely employed for the alkylation of amines because of their excessive electrophilicity. Therefore, their application is still limited at the alkylation of compounds bearing strong electron attractor substituents, capable of limiting the nucleophilicity of nitrogen[8]**.** In this paragraph only the most important examples will be mentioned, ignoring the applications only used in organic synthesis.

- ***Alkylation of amines***

The reaction between amines and trialkyloxonium salts is a salification reaction of the amino nitrogen atom. Primary and secondary amines undergo the alkylation of the nitrogen atom with formation of the corresponding ammonium salt; by the use of a base, the acid ammonium salt is deprotonated and we obtain the secondary or tertiary amine. This reaction has been exploited by N. De Kimpe et al.[24] to obtain α-tertiary cyanoenamine.

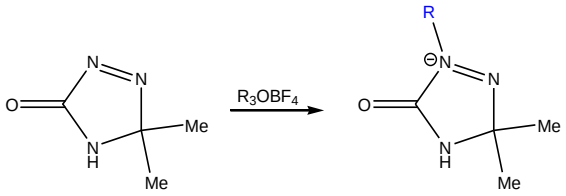


When a particular compound has two different amino groups competitive, there may be differences between the behaviour of the trialkyloxonium salt and other alkylating classics, as described by B. Rouot et al.[25]. About the reactivity with tertiary amines, the trialkyloxonium salt, in mild conditions, leads to the formation of a stable quaternary ammonium salt.

- ***Alkylation of imines and azo-compounds***

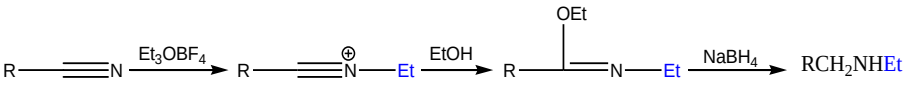
The *N*-alkylation by trialkyloxonium salts is also possible on compounds bearing a nitrogen atom bonded to a carbon by a double bond. The resulting iminium salt could be isolable sometimes. Subsequently these intermediates may be reduced by $NaBH_4$[26] or subject to basic treatment[27]. The reactivity of azo-compound with the trialkyloxonium salt

leads to the salification of the nitrogen atom more suitable for an electrophilic attack. The attack is favored on the nitrogen atom that is less hindered by the groups and better suited to the delocalization of the positive charge[28].



- ***Alkylation of nitriles and azides***

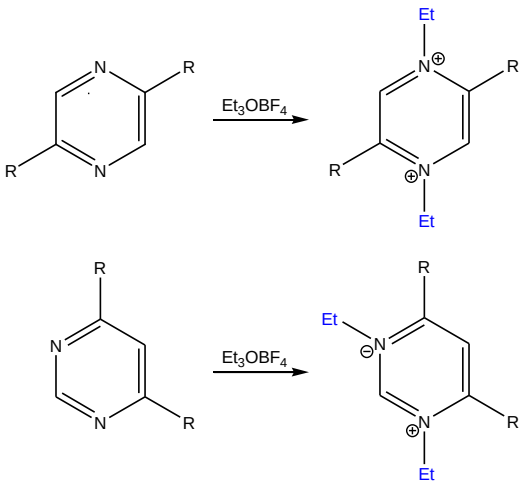
The reaction between trialkyloxonium salts and nitriles leads to the formation of nitrilio salts, by the alkylation on the nitrogen atom. Such intermediates are liable to undergo nucleophilic attack on the carbon due to a strong deficiency electronics. They can also be reduced to amines using an alcoholic solution of $NaBH_4$[29].



The alkyl azides undergo the alkylating attack by trialkyloxonium salts[30] on the nitrogen bound to the hydrocarbon chain.

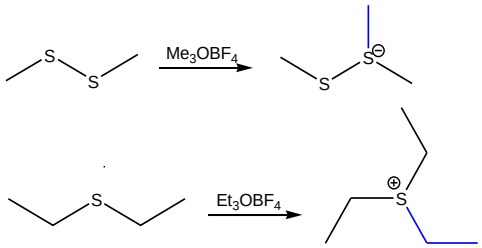
- ***Alkylation of cyclic compounds heteroaromatic with six-membered***

Even this type of organic compounds is capable of undergoing the alkylation by trialkyloxonium salts. Pyridines undergo the nitrogen alkylation giving the corresponding pyridinium salts, that are important synthetic intermediates[31]. T.J. Curphey[32] also investigated the behavior of pyrazine and pyrimidine with the triethyloxonium tetrafluoroborate salt. For these compounds we observed the alkylation of both nitrogen atoms and the alkylation yield depends on the α-substituents of these hetero aromatic rings: when the hindrance is great, the yield is low.

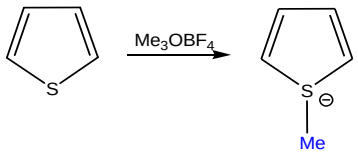


1.2.3.4 S-alkylation

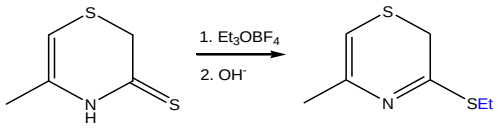
Trialkyloxonium salts are also sulfur alkylating. Dimethylsulfide and diethylsulfide can be alkylated, leading to the formation of salts of sulfonium tetrafluoroborate[8].



The reaction between thiophene and the triethyloxonium salt, studied by G.C. Brumlik et al.[33], is another example of *S*-alkylation.



The alkylation of thiolactams is also described. The reaction products are the corresponding esters[8]. An example is provided by the experiments of Č.R. Johnson et al.[34].



1.3 Analytical application: alkylation of anions in aqueous media

Trialkyloxonium salts are able to alkylate some anions in aqueous solution and the yields obtained are different depending on the type of anion/nucleophile that is considered[8]. O.A. Reutov et al.[35] studied the alkylation yields in aqueous media of some anions, summarized in the table below[8] (*Table 1.3*).

Table 1.3 Alkylation yields

X^-	F^-	Cl^-	Br^-	I^-	SCN^-	CN^-
Ethylation yield	trace	12%	23%	53%	64%	55%

Aqueous phase reaction of trialkyloxonium tetrafluoroborates, $R_3O^+BF_4^-$ (R = Me, Et) has been recently tested[7] in the alkylation of simple inorganic anionic substrates such as halogen ions, cyanide, thiocyanate, sulfide, nitrite and nitrate. Volatile products were separated and identified by gas chromatography mass spectrometry. Alkylated derivatives RI, RBr, RCl were obtained for iodide, bromide, and chloride, respectively, while for fluoride its conversion efficiency to RF was very poor. RCN and R_2S were obtained for cyanide and sulfide, while thiocyanate gave RSCN as a main reaction product with a minor amount of alkylisothiocyanate, RNCS (<7%). Among the tested oxoanions only nitrites and nitrates gave volatile derivatives in the form of R-O-NO and R-O-NO_2, respectively. Reaction performed by adding $R_3O^+BF_4^-$ in CH_2Cl_2 to aqueous samples followed by extraction in organic phase of the reaction products, failed to identify other less volatile reaction products[7].

1.3.1 Quantification of nitrite and nitrate

Nitrite and nitrate are non volatile anions and their derivatization with trialkyloxonium salts allows to generate the organic volatile *O*-derivatives Et-ONO and Et-ONO_2[36]. The simultaneous determination of these anions at trace and ultra-trace levels in complex matrices is important in the field of environmental contamination and biological process. Nitrite and nitrate are the final states of degradation a variety of *N*-compounds in some different metabolic pathways. NO is important as regulator in the vascular system, both in the brain and in the immune system. Nitrite and nitrate in environmental samples can occur from industrial and domestic combustion and from agricultural sources[37]. Their determination in samples of different origin has been discussed by Jobgen at al.[38] and Moocroft et al.[39]. Their determination by GC-MS can be achieved by derivatization to generate volatile species. This approach, however, does not work for nitrite and requires the use of strong acid conditions which may be critical if any nitrite is present because of its possible conversion to nitrate. In this conditions, nitrite is protonated to nitrous acid, which is then oxidized to nitric acid, therefore subtracting NO_2^- from the reaction environment[36]. The use of isotopic dilution method for the simultaneous determination of nitrite and nitrate by negative chemical ionization (CI-) GC-MS together with pretreating the sample with ammonium hydroxide to maintain an alkaline pH during alkylation, avoids the problem of the conversion of nitrite to nitrate[36].

1.3.2 Determination of fluoride

Sodium fluoride and sodium mono fluoro phosphate are chemical ingredients used for the manufacturing of toothpastes, gels and also in the foams used for the radiation therapy. Fluoride is one of the inevitable constituent of prescription and non prescription mouth washes available and people are frequently exposed to fluoride. Several studies have confirmed that fluoride toothpastes are risk factors for dental fluorosis[40]. Elevated levels of fluoride occur in natural waters over an extensive geographical belt, so the monitoring of this anion in water and food products is important from a toxicologically point of view[41]. Determination of fluoride is affected by the presence of common fluoride complexing agents, such as $Al^{(III)}$ and $Fe^{(III)}$. Its derivatization has traditionally been

performed using silicon-based chemistry in order to convert F^- to Me_3SiF[40, 42] or Ph_3SiF[43]. The resulting volatile fluorosilanes can be determinated by gas chromatography following either liquid extraction with organic solvents or SPME from the headspace. However, the Si-F bond is not stable in aqueous solution, especially at high pH. Consequently, Kage et al.[44] proposed carbon-based alkylation of fluoride with pentafluorobenzylbromide (F_5BzBr), yielding volatile F_5BzF to be analysed by GC-MS. Oxonium salts have only recently been introduced for fluoride derivatization[41]. The use of $Et_3O^+BF_4^-$ is not suitable for the determination of fluoride, because tetrafluoroborate slowly hydrolyzes, releasing fluoride ions. New methods[41] employ triethyloxonium tetrachloroferrate$^{(III)}$ to convert fluoride to stable fluoroethane which can be than analysed by GC-MS.

Chapter 2

Materials and methods

2.1 Reactants and products

For the preparation and the dilution of the working solutions we used ultra pure water, obtained by a Milli-Q system (Purelab Pro + Purelab Classic, USA). *Table 2.1* shows all the reagents used for the analyses with their relative degree of purity:

Table 2.1 Reagents employed for the analysis, the supplier and the purity

Reagent	***Supplier***	***Purity***
KSCN	Sigma-Aldrich	1000 μg/mL in H_2O
KCN	Sigma-Aldrich	1000 μg/mL in 1% KOH
Na_2S	Sigma-Aldrich	1000 μg/mL in 1% NaOH
NaBr	Sigma-Aldrich	1000 μg/mL Br^- in H_2O
KI	Sigma-Aldrich	1000 μg/mL I^- in H_2O
$Na_2SO_4 \cdot 10H_2O$	Carlo Erba	99%
Isopropanolo- D8	Armar Chemicals	99.8%
$KS^{13}CN$	Sigma-Aldrich	99 atom % ^{13}C
$K^{15}NO_3$	Cambridge Isotope Laboratories	99 atom % ^{15}N
NaOH	Sigma-Aldrich	30% in H_2O
NH_3	Sigma-Aldrich	30% in H_2O
CH_3CH_2SCN	Sigma-Aldrich	98%

Reagent	***Supplier***	***Purity***
CH_3CH_2CN	Sigma-Aldrich	99%
$CH_3CH_2SCH_2CH_3$	Sigma-Aldrich	98%
$C_4H_{11}N$	Sigma-Aldrich	≥99.5%
$C_6H_{15}N$	Sigma-Aldrich	≥99.5%
$Et_3O^+BF_4^-$	Sigma-Aldrich	≥97%
EtI	Sigma-Aldrich	99%
EtBr	Sigma-Aldrich	≥99%
$CF_3(CF_2)_5CF_2COOH$	Sigma-Aldrich	96%
Acetate standard for IC	Sigma-Aldrich	1000 µg/mL acetate in H_2O
Butyrate standard for IC	Sigma-Aldrich	1000 µg/mL butyrate in H_2O
Propionate standard for IC	Sigma-Aldrich	1000 µg/mL propionate in H_2O
Lactate standard for IC	Sigma-Aldrich	1000 µg/mL lactate in H_2O
C_6H_5OH	Sigma-Aldrich	99%
$Cl_3C_6H_2OH$	Sigma-Aldrich	98%
Cl_5C_6OH	Sigma-Aldrich	99%

2.1.1 Manipulation of trialkyloxonium salt

The salt used in this work is triethyloxonium tetrafluoroborate ($Et_3O^+BF_4^-$). This salt is very hygroscopic, so its manipulation is not easy. It is able to absorb the moisture present in the atmosphere, liquefying and hydrolyzing in little time. Therefore its transfer into the vial must be conducted quickly, if possible within a glove box in a nitrogen atmosphere in the presence of dried silica gel; in many cases a dryer could been employed. Once extracted from the bottle, the salt was transferred into the vials and stored in a freezer at -20°C. After adding cold water (+4°C), the salt is stable for a limited period of time, therefore it is necessary to proceed immediately to its addition within the reaction vial.

2.1.2 Choice of the internal standard

The choice of the internal standard plays a very important role in the analytical field. The internal standard for the measurement of the headspace must be a substance with a not very high boiling temperature. It should have also similar physical-chemical properties as the

analyte in order to provide a similar instrumental response but well chromatographically or spectroscopically separated from the analyte. In this work we employed three different internal standards, in order to normalize the areas of the signals of the chromatograms: Isopropyl alchol-D8, $K^{15}NO_3$ and $KS^{13}CN$. The choice of the internal standard was based on the kind of the analysis performed and on the characteristics of the desired compounds to be detected. The following table (*Table 2.2*) shows the relative concentration of each internal standard prepared.

Table 2.2 Internal standards preparation

Compound	***Final concentration [µg/mL]***
Isopropyl alchol-D8 (liquid)	880
$K^{15}NO_3$ (solid)	1006
$KS^{13}CN$ (solid)	1037

Standard solution of $KS^{13}CN$ was subsequently diluted 10 times and the final concentration was 103 µg/mL.

2.2 Materials and instrumentation

2.2.1 Laboratory equipment

For the headspace analysis 10 mL vials were used (Agilent Technologies, 8010-0038, CrossLab Vial, Screw Top Headspace) fitted with a screw cap and equipped with septum PTFE/silicone (Agilent Technologies, 8010-0139, CrossLab headspace cap, magnetic, 18 mm, PTFE/silicone septa). The choice of these septa was dictated by the requirements to have a membrane able to ensure the sealing of small dimensions of gases that develop inside, even after perforation of the septum. To monitor the pH in each vial (necessary for

the experimental design) a pH meter was used (Oakaton Instruments, USA). For the transfer of small volumes in each vials and for the preparation of standard solutions, micro pipette Eppendorf Research were used:

- 10 - 100 µL
- 100 - 1000 µL
- 1000 - 5000 µL

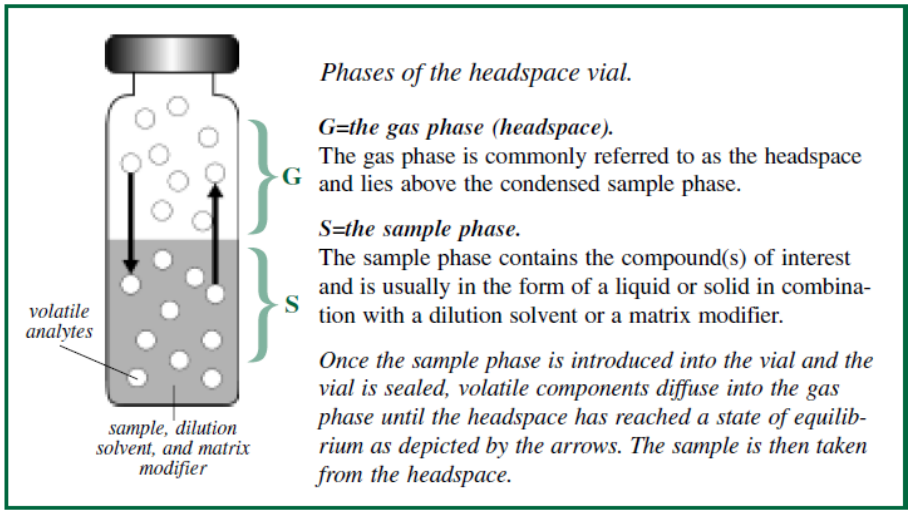
To accelerate the separation between substances with different density, a centrifuge (Eppendorf Centrifuge 5804R) has been used, and for the homogenization of the solutions, a sonicator (Ultrasonik 28x NDI, USA) and a vortex (Velp Scientifica) have been used. In addition to the materials mentioned above, common laboratory objects have been used (such as Pasteur pipettes, beakers and flasks) whose accurate description will not be given.

2.2.2 Headspace-gas chromatography[45]

Most of the products and biological samples are composed of a wide variety of compounds that differ in molecular weight, polarity and volatility. Headspace sampling is the fastest and cleanest method to analyze volatile organic compounds. Headspace-gas chromatography is not a new technique: it has been practiced since the early days of gas chromatography. However, there is still an increasing interest, probably given to the several advantages of this technique, among which the reduced costs. In order to achieve the best performance when using the HS/GC, careful attention should be used in sample preparation and instrument setup. A headspace sample is normally prepared in a vial containing the sample, the dilution solvent and the headspace (*Figure 2.1*). Volatile components from complex sample mixture can be extracted from non-volatile sample components and isolated in the headspace of a sample vial.

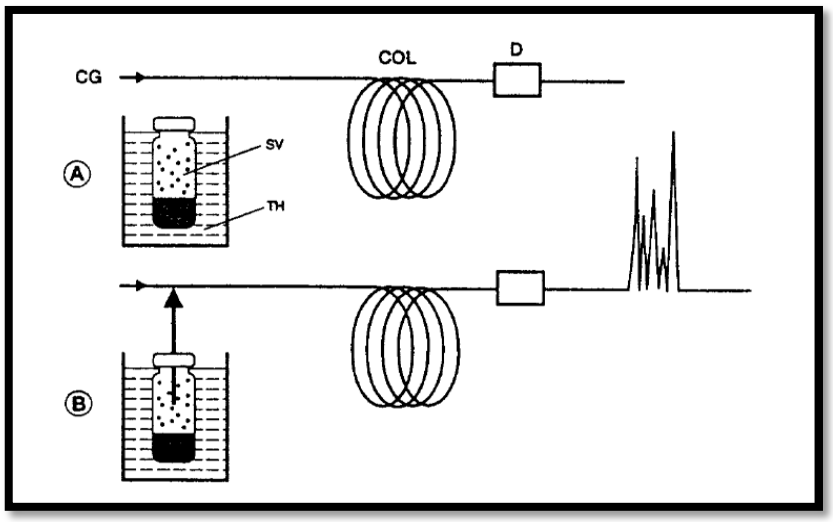
This technique consists of two steps. First, the sample – a liquid or a solid – is placed in a vessel having a gas volume above it, and the vessel, usually a vial, is closed.

Figure 2.1 Phases of the headspace vial



This vial is then thermostatted at a constant temperature, in some cases under agitation, until equilibrium is reached between the two phases. Then an aliquot of the vial gas phase (the *headspace*) is introduced into the carrier gas stream, which carries it into the column, where it is analyzed. *Figure 2.2* visualizes the two steps of HS-GC. Sample transfer can be carried out manually, by using a gas-tight syringe, or automatically, by the use of an autosampler.

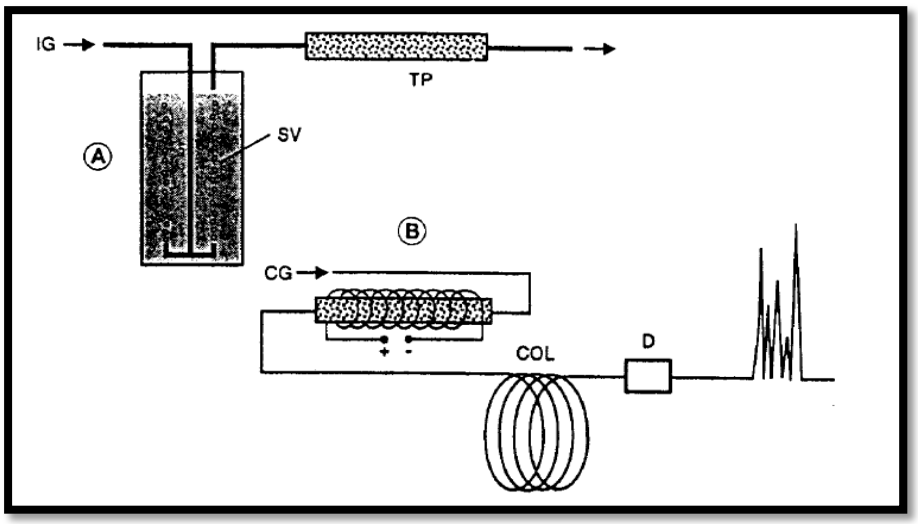
Figure 2.2 Principles of static (equilibrium) headspace-gas chromatography. (A) equilibration and (B) sample transfer. ***GC***= carrier gas, ***SV***= sample vial, ***TH***= thermostat, ***COL***= gas chromatographic column, ***D***= detector



In this procedure, the two phases in the same vial are under *static conditions* and sample transfer is carried out after they reach equilibrium. This type of HS analysis is called *static* or *equilibrium HS analysis*. Today static headspace-gas chromatography is widely used in laboratories for various official measurements and for the determination of toxic impurities in the environment. Static HS-GC is used for example in the determination of the vinyl chloride content of wastewater and of PVC resin, slurry, wet cake and latex samples[46-48]. The federal Food and Drug Administration has accepted official methods for the analysis of vinyl chloride in corn oil and food-simulating solvents[49], in oils and vinegar[50] and in PVC food packaging[51], which use static HS-GC. Machata's work[52] described a semiautomatic system for the determination of ethanol in blood. This work opened the way for the development of the first automated instrument to carry out the gas chromatographic analysis of headspace samples. In many countries, this technique is the accepted official method for the determination of ethanol in the blood of drivers

There is, however, another way to carry out gas extraction. In this version we do not wait for equilibrium and we do not analyze an aliquot of the gas phase: gas extracted-ion is carried out continuously. By continuously removing the gas phase, we rely on the fact that volatile analytes will re-establish the equilibrium state, which, however, is never reached, by moving into the vapour phase. At the end, the total amount of volatile analytes is thus removed from the sample. This technique is called *continuous gas extraction*. In the most frequently used form of continuous gas extracted-ion, an inert gas (the *purge gas*) flowes through the (liquid) sample and the sample solution is extracted by the gas bubbles. The gas effluent is usually conducted through a *trap* containing an adsorbent, which will retain the volatile analytes carried there by the purge gas. When extraction is completed, the collected analytes may be analyzed by rapidly releasing them from the trap (usually by heating and backflushing) and conducting the "plug" into the gas chromatograph. *Figure 2.3* visualizes this procedure, which may be called *dynamic headspace analysis* or, more specifically, the *purge-and-trap technique*.

Figure 2.3 Principles of dynamic headspace-gas chromatography ("purge-and-trap"). (A) sample purging and collection of the removed volatiles in a trap and (B) desorption from the trap and transfer into the gas chromatograph. ***IG***= inert purge gas, ***GC***= carrier gas, ***SV***= sample vial, ***TP***= trap, ***COL***= gas chromatographic column, ***D***= detector



In order to determine the working conditions, we need to carefully monitor the termostatting temperature, in order to ensure reproducibility in transferring the analytes from the aqueous/solid phase to the gaseous phase. Present-day thermostatting systems specify temperature control as precise as ±0.01°C.

2.2.3 Instrument description and detection conditions

All of the experiments were performed by a GC-MS system, consisting of an Agilent 6850 gas chromatograph, equipped with a split/splitless injector, connected to a 5975c mass spectrometer, equipped with an autosampler and an incubator Combi PAL CTC. For all of the analyses a special Agilent capillary column DB-624, 6% cyanopropyl-phenil 94% dimethylpolisiloxane (60m x 0.250mm x 1.40µm) was used. We prepared three different methods for the analysis, changing the oven temperature. The first method (*METHOD 1*) had a slow temperature rave increment, in order to obtain the maximum chromatographic separation of all of the compounds present in the sample. Chromatographic conditions were: 30°C, held for 12 min, to 250°C at 10°C/min for 15 min. The second method

(*METHOD 2*), faster than the first, was employed to determine the analytical figures of merit of each anion and to the analysis of human saliva samples to determine thiocyanate concentration. Chromatographic conditions of the method were: 60°C, held for 2 min, to 200°C at 15°C/min, to 250°C at 30°C/min for 2 min. The third method (*METHOD 3*) was employed to built the PFOA calibration plot. Chromatographic conditions were: 70°C, held for 5 min, to 250°C at 25°C/min for 10 min.

Tables 2.3 and *2.4* summarize the ions monitored and the main characteristics of the three methods respectively.

Table 2.3 Ions monitored: quantifier and qualifier

Alkyl derivative	***m/z quantifier***	***m/z qualifier***	***ratio [quantifier/qualifier]***
EtSCN	87	59	2.58
EtCN	54	52	4.63
EtSEt	90	75	1.91
EtI	127	156	2.20
EtBr	110	108	2.40
$EtS^{13}CN$	88	60	2.58
i-propOH D8	49	46	6.40
$K^{15}NO_3$	47	77	1.07
PFOA	169	69	-

Table 2.4 Method 1, 2 and 3 characteristics

Method	***runtime [min]***	***chromatographic conditions***
METHOD 1	49.00	isotherm 30°C for 12’ 10°C/min for 15’ up to 250°C
METHOD 2	15.00	isotherm 60°C for 2’ 15°C/min up to 200°C 30°C/min for 2’ up to 250°C
METHOD 3	22.00	isotherm 70°C for 5’ 25°C/min for 10’ up to 250°C

For all of the three methods, injector was set at 200°C and flow at 1 mL/min; carrier gas was helium (purity 99.9995%). Incubation temperature was set at 75°C for 10 min and syringe temperature was 80°C in order to avoid the condensation of some compounds (syringe size: 2.5 mL). The injection was split mode, with 8:1 ratio. The transfer line temperature was 260°C. The temperature of the mass spectrometer source was set at 250°C and quadrupole temperature was 150°C. The source has been working in electron ionization (EI) positive mode (70 eV). All of the chromatograms were acquired in *Total Ion Current* (TIC) and *Single Ion Monitoring* (SIM) mode.

2.2.4 Data processing

All of the chromatograms obtained were analyzed using the program Excalibur™ 1.4 SR1 of Thermo Electron Corporation. For the identification of derivatives through their mass spectrum, the libraries NIST 2005 were used (NIST Mass Spectral Search Program version 2.0). Detection programs employed include the investigation of a wide range of masses, for the qualitative analysis, restricted for the quantitative analysis, in order to improve the signal/noise ratio. The chromatographic signal for the quantitative analysis, was integrated time to time on a specific ion chosen, according to its abundance in the mass spectrum. Also for internal standard a m/z ratio was chosen. The analytical signals obtained were always normalized for the peak area of the internal standard. For the statistical treatment of the data, Excel 2007 by Microsoft Corporation and the OriginLab Origin 8.6® Corporation were used.

2.3 Standard solutions

For the qualitative and the quantitative analysis standard solutions of known concentration were prepared. These solutions were prepared in volumetric flasks by dilution of stock solutions with higher concentration. Once prepared, the solutions were transferred to 50 mL conical tubes BD Falcon, 30x115mm, in clarified polypropylene and stored in the refrigerator, at a temperature of +4°C.

- ***Working solution 1***: solution with SCN^-, CN^-, S^{2-}, Br^- e I^-, 10 µg/mL, prepared by dilution of the stock solutions.

- ***Working solution 2***: solution with SCN^-, CN^-, S^{2-}, Br^- e I^-, 100 µg/mL, prepared by dilution of the stock solutions.

- ***Working solution 3***: solution with EtSCN, EtCN and EtSEt, 2 µg/mL, prepared by dilution of the stock solutions.

- ***Working solution 4:*** solution with CN^-, SCN^- and S^{2-}, 2 µg/mL, prepared by dilution of stock the solutions.

- ***Working solution 5***: solution with SCN^-, CN^-, S^{2-}, Br^- e I^-, 3 µg/mL, prepared by dilution of the stock solutions.

2.4 Vials preparation

For all of the analyses, 1g of the triethyloxonium tetrafluoroborate salt was dissolved in 1 mL of water; the final concentration obtained was about 2.5M. After the addition of the reagent, the alkylation reaction starts and the volatile organic compounds begin to form into each vial. The addition of $Et_3O^+BF_4^-$ as well as the closure of the vial must thus be fast, in order to avoid analyte losses. In particular reagents were added in the following order: the inorganic salt, the working solution, the base, the internal standard and the reagent at last. All the substances added were weighed.

2.5 Saliva samples

Saliva samples were collected by STARSTED Salivette® for Cortisol Testing. These allow sampling the saliva thanks to the presence of a polyester swab. All of the salivette were

stored in a refrigerator at -20°C. Samples came from ten different volunteers, men and women of different age (from 20 to 57 years old). Among these, there were five non-smokers (samples C1-C5) and five smokers (samples C6-C10). Below (*Table 2.5*) the list of the saliva samples collected is reported with their characteristics. Samples were collected early morning, before breakfast and before washing teeth. The swab was kept in the mouth for ten minutes, without chewing it, passing it from side to side of the mouth (*Figure 2.4*). Before the analysis, the salivette were centrifuged at 3000 rpm for ten minutes, in order to collect the saliva from the swab. Subsequently, the swab was removed and the samples, diluted 1:10 with water and stored at +4°C.

Figure 2.4 Saliva sampling by STARSTED Salivette® for Cortisol Testing

The results obtained from the analysis of each sample were compared with three different pool of saliva samples, that were prepared according to the procedure reported above

- MIX-1: 100 µL of each non-smokers sample were taken and mixed all together
- MIX-2: 100 µL of each smokers sample were taken and mixed all together
- MIX-3: 100 µL of all of the samples were taken and mixed all together

Table 2.5 List of the saliva samples collected with their characteristics

Sample	***Age***	***Sex***	***Non-Smoker***	***Smoker***	***Cigarette/die***
C1	27	male	✓		-
C2	53	female	✓		-
C3	58	male	✓		-
C4	24	female	✓		-
C5	41	female	✓		-
C6	25	female		✓	4-5
C7	33	male		✓	12-15
C8	28	female		✓	8-10
C9	25	male		✓	10-12
C10	50	male		✓	12-15

Chapter 3

Experimental results

3.1 Reactions

Trialkyloxonium salts are able to alkylate anions in aqueous solution and the reaction yields obtained are different depending on the type of anion/nucleophic considered. The reactivity of trialkyloxonium salts in aqueous media can be described as follow:

$$X^- + R_3O^+ \rightarrow RX + R_2O \qquad \text{(derivatization reaction)}$$

$$R_3O^+ + H_2O \rightarrow R_2O + ROH + H^+ \qquad \text{(hydrolysis)}$$

Where X^- is an anion (such as I^-, Br^-, CN^-, SCN^-, S^{2-}) and R is an alkyl group (such as Me, Et or Ph). These two reactions are competitive and depending on the alkyl group chosen, the time of hydrolysis changes. For example trimethyloxonium hydrolyzes much faster than the triethyloxonium, while triphenyloxonium is rather stable in aqueous media and long times and severe conditions are required for its reaction with nucleophiles (as reported in *Table 1.2*)[6]. For the analytical applications explored so far, triethyloxonium salts give the best compromise between the rate of hydrolysis and the analyte alkylation. The hydrolysis of triethyloxonium salt generates an equivalent amount of protons, which means the sample solution becomes increasingly acidic. Under acidic conditions some of the

anions generated by weak acids (cyanide, sulfide, etc) can undergo protonation (HCN, H_2S) and escape from the solution. Furthermore, their anionic forms are more activated toward Et_3O^+ derivatization with respect to the protonated forms. Adjustment of pH could be problematic as the buffer can react with Et_3O^+. In general, all the reactions that can take place can be classified as *analytical reactions*, *competitive reactions* and *interfering reactions*:

Analytical reactions

$R_3O^+_{(aq)} + X^-_{(aq)} \rightarrow RX_{(g)} + R_2O$ (1) primary derivatization reaction

$RX_{(aq)} \rightleftharpoons RX_{(g)}$ (2) phase transfer of analytical derivate

Competitive reactions

$R_3O^+ + H_2O \rightarrow ROH + R_2O + H^+$ (3) reagent hydrolysis

$X^-_{(aq)} + H^+_{(aq)} \rightleftharpoons HX_{(aq)}$ (4) protonation of analytical substrate

$HX_{(aq)} \rightleftharpoons HX_{(g)}$ (5) phase transfer of protonated analyte

$RX + R_3O^+ \rightarrow R_2X^+ + R_2O$ (6) alkylation of analytical derivate

Interfering reactions

$X^- + M^{n+} \rightleftharpoons MX^{(n-1)+}$ (7) metal complex formation

$R_3O^+ + Y^- \rightarrow RY + R_2O$ (8) alkylation of anionic species other than X^-

$R_3O^+ + L \rightarrow RL^+ + R_2O$ (9) alkylation of ligand/donor species

In order to optimize the experimental conditions, it was necessary to consider all of the experimental parameters that play role in controlling all the possible reactions mentioned

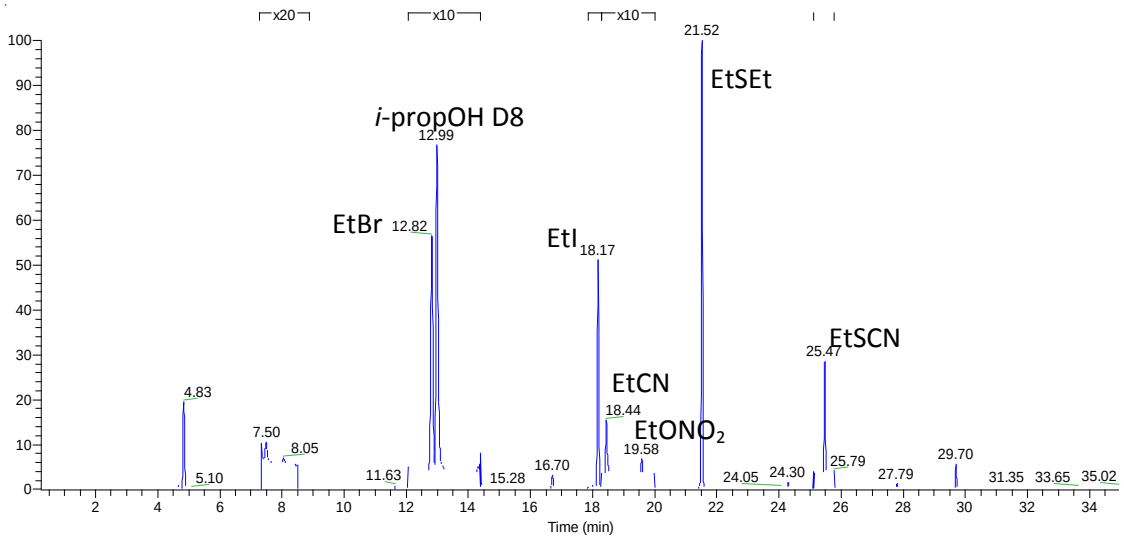
above. The most important parameters studied are: the reaction time (the time between the addition of the alkylating agent and the GC-MS analysis), the incubation temperature, the amount of a non-reactive salt to induce salting out effect and the pH solution. The choice of chemical additives, such as buffers and masking agents, represents a critical aspect of this derivatization procedure due to the reactivity of the trialkyloxonium salts with other anionic species or ligand/donor species.

A fundamental aspect to be considered is the different behavior of the different anions studied varying these parameters. The amount of alkylating agents and the amount of base added in each vial, both affecting the final pH, are maybe the most important parameter to be evaluated and that most influence our yields.

3.2 Qualitative results

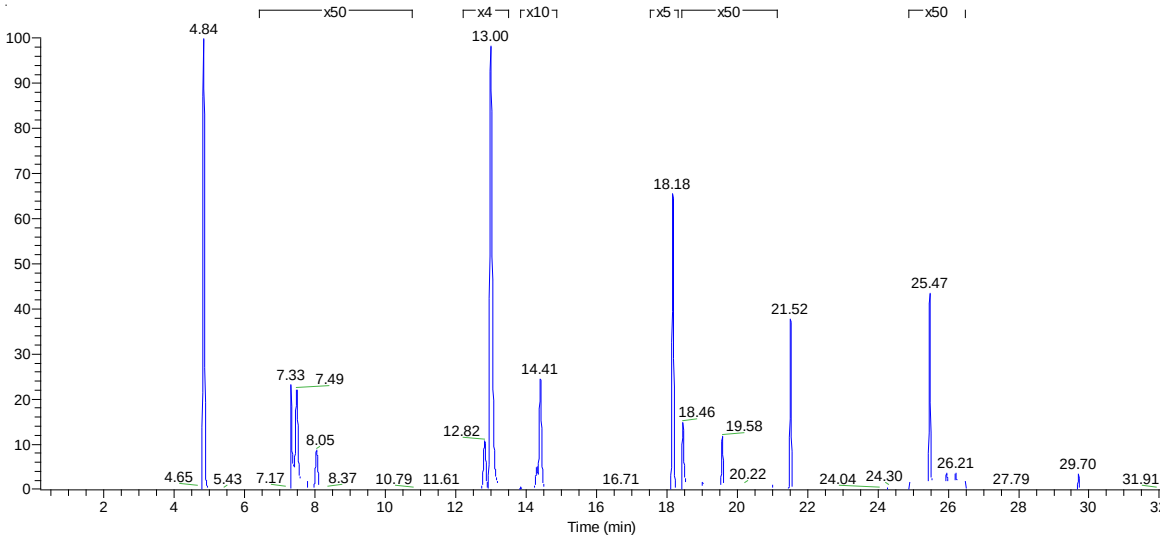
The study of the reactivity of the trialkyloxonium salts with anionic species is very interesting, in order to improve the reaction yields. In this work we tried to set up an analytical method for the determination of anions in aqueous solution by the use of the triethyloxonium tetrafluoroborate salt, exploiting the CVG for analysis of the headspace (HS) by GC-MS. Once the method was optimized, it was applied to the characterization of samples of saliva, investigating the problems arising when dealing with such complex matrices. This application is particularly interesting given the possibility to correlate the presence within these matrices of certain anions (CN^-, SCN^-, I^-) to specific diseases. This session will describe the reaction products detected in the headspace to be related to the concentration in the solutions. More attention has been dedicated to the anion SCN^- because of the subsequent studies on biological matrices, in order to identify and quantify the compound EtSCN. For this purpose, our chromatographic method, *METHOD 1*, (*Section 2.2.3*), was optimized. Our aim was to be able to discriminate all the alkyl derivatives and to obtain chromatograms in SIM (*Single Ion Monitoring*) and TIC (*Total Ion current Chromatogram*) mode, with good separation of the chromatographic peaks. In this respect, *working solution 1*, containing the anions SCN^-, CN^-, S^{2-}, I^- and Br^-, was analyzed by *METHOD 1*. *Figure 3.1* shows the TIC chromatogram obtained after the reaction with triethyloxonium salt, by GC-MS analysis.

Figure 3.1 TIC Chromatogram of the analysis of the 10 µg/mL working solution containing all of the anions



As shown (*Figure 3.1*), the method allows to separate each chromatographic peak. This method has also been applied to the analysis of working solutions at lower concentration, respectively 1 and 0.1 µg/mL, in order to test its sensitivity. *Figure 3.2* shows the TIC chromatogram obtained from the analysis of the 0.1 µg/mL working solution. Even in this case, the method allows the detection of all of the chromatographic peaks, so it is functional for the analysis of our analytes in trace.

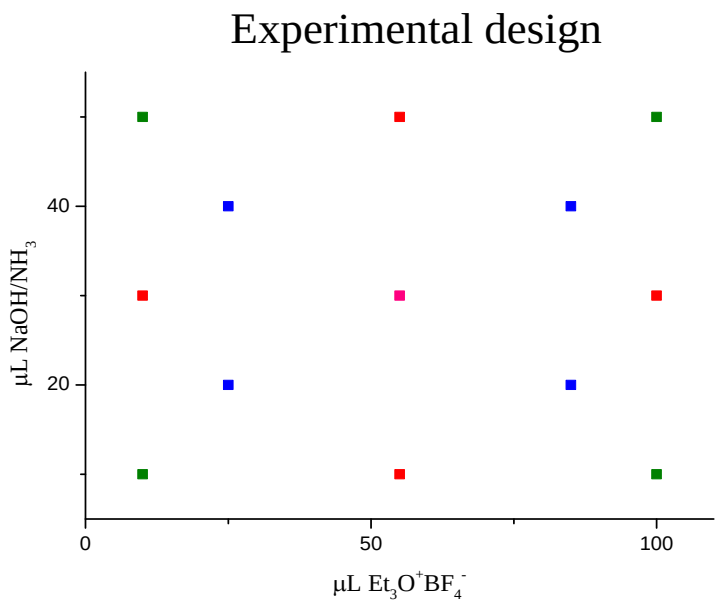
Figure 3.2 TIC Chromatogram of the analysis of the 0.1 µg/mL working solution containing all of the anions



3.3 Choice of the base: the role of pH

The addition of a base in the alkylation reaction with trialkyloxonium salts is maybe the most important experimental parameter to be considered in order to maximize the reaction yields. In fact, is necessary to counteract the acidic character of the triethyloxonium salt. The pH solution plays an important role because anions protonation or side reactions are affected by acidity. Owing to their different alkaline properties, NH_3 and NaOH were taken into consideration. To adequately choose which base could be added in the reaction environment and its concentration, we conducted two different experimental designs. Therefore we decided to perform an experimental design with two factors (concentration of the base and of the ethylating agent $Et_3O^+BF_4^-$) and five levels: a *Central Composite Design* (*Figure 3.3*). We tested NH_3 0.02M - 0.1M, NaOH 0.02M – 0.1M and $Et_3O^+BF_4^-$ 0.01M – 0.1M. Therefore, in *Figure 3.3* the volume range that was chosen is reported with the minimum and maximum volumes of $Et_3O^+BF_4^-$ chosen. Each experimental design consist of thirteen experimental points. For both the experimental designs we employed the *working solution 2*, containing all of the anions at 100 µg/mL concentration; all of the analysis were performed by the *METHOD 1*. The internal standard used for the areas normalization of the anions was *i*-propOH D8, because it doesn't participate to the reaction and it allows to correct the variations of the headspace and of the instrumental response.

Figure 3.3 Thirteen points of the experimental design with two factors and five levels for the optimization of the pH solution



From the analysis of the chromatograms we derived the areas of each reaction derivative, obtained from the vials where ammonia or sodium hydroxide was added as the base. The data were processed in order to correlate the experimental areas of each compound to the respective concentration of NH_3/NaOH and of the reagent added. After processing our data, we obtained the "*response surfaces*", that show the efficiency of the process as a function of the base and the reactive concentration. These surfaces can simplify the optimization work, providing instantly and intuitively the best reaction conditions. It is evident that the concentrations of NH_3/NaOH and of the alkylating agent to be added into the vials are different, depending on the anion that we want to study. Response surface of the species studied are reported below. *Figures 3.4(a)(b)* show the experimental results obtained for the thiocyanate anion. We observed that this anion requires a reaction environment preferably acid in order to give the alkylation reaction. As we can see from the *Figure 3.4(a)*, when we used NaOH as base, the maximum response was obtained for values of pH equal to 2. When the base used was NH_3 (*Figure 3.4(b)*), the maximum response was always in acidic conditions. We observe that the range of concentration of base and alkylating agent to add in each vial is very narrow in the case of NaOH while, for NH_3, is broader. This is because NH_3 is a soft base and the OH^- concentration in the reaction environment is low and related to the amount of alkylating agent added for the reaction. Conversely NaOH is a strong base and in aqueous phase is completely dissociated. The reaction with this base and the triethyloxonium is immediate.

Figure 3.4(a) Response surface of the thiocyanate ion employing NaOH as base

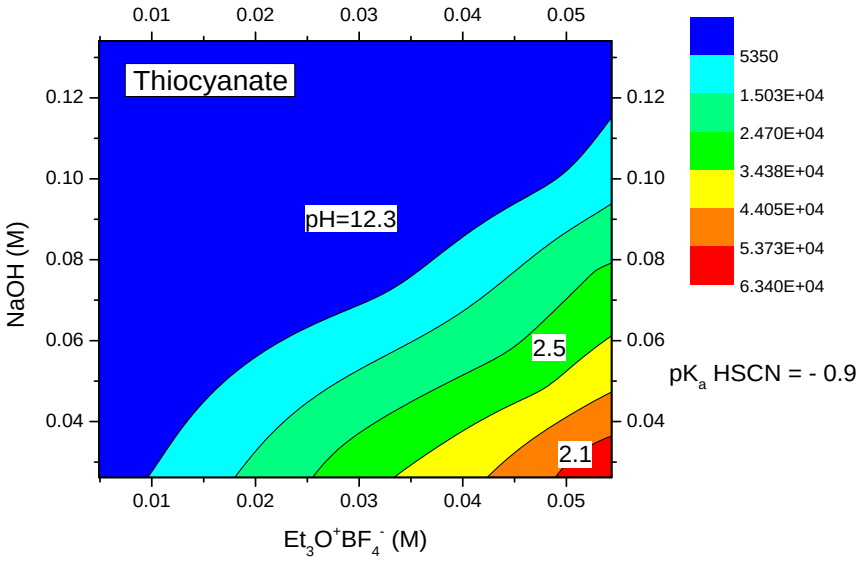
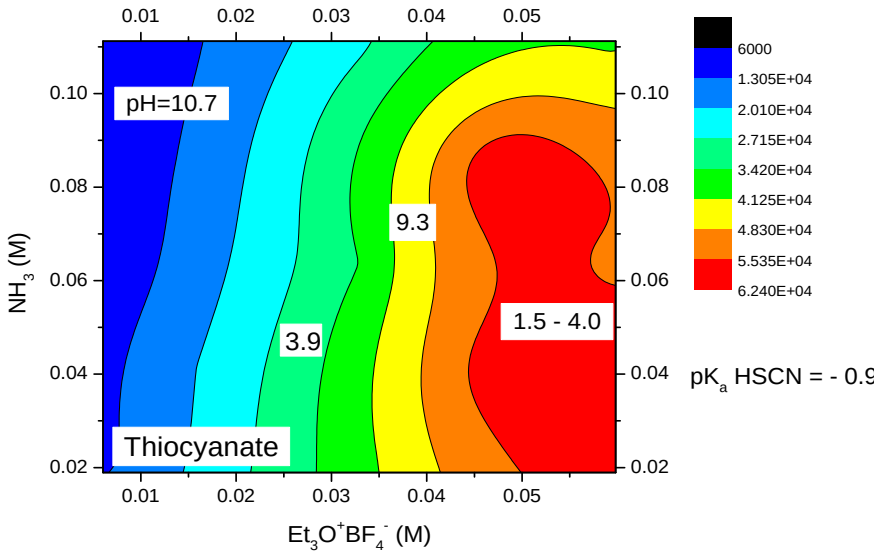


Figure 3.4(b) Response surface of the thiocyanate ion employing NH_3 as base



As far as the sulfide anion is concerned, we observed from *Figures 3.5(a)(b)* that this anion requires an alkaline reaction environment, with pH values of 10-12.

Figure 3.5(a) Response surface of the sulfide ion employing NaOH as base

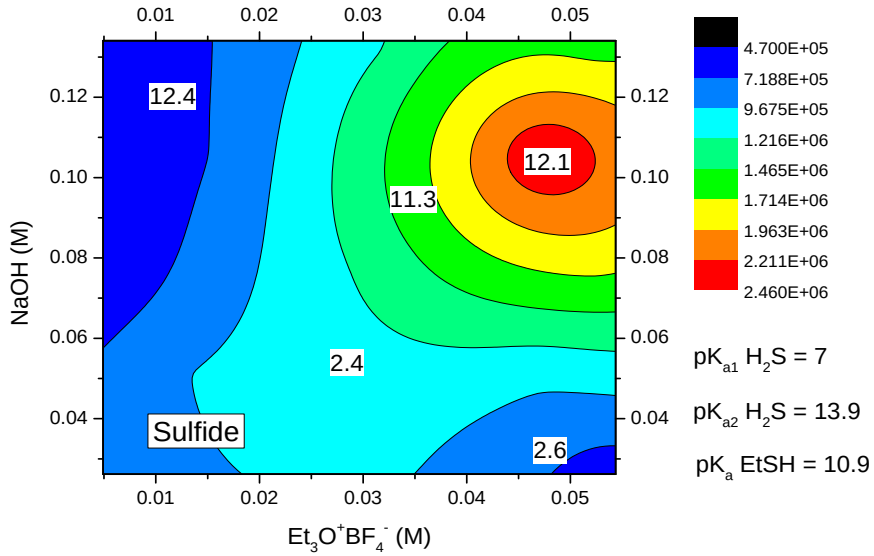
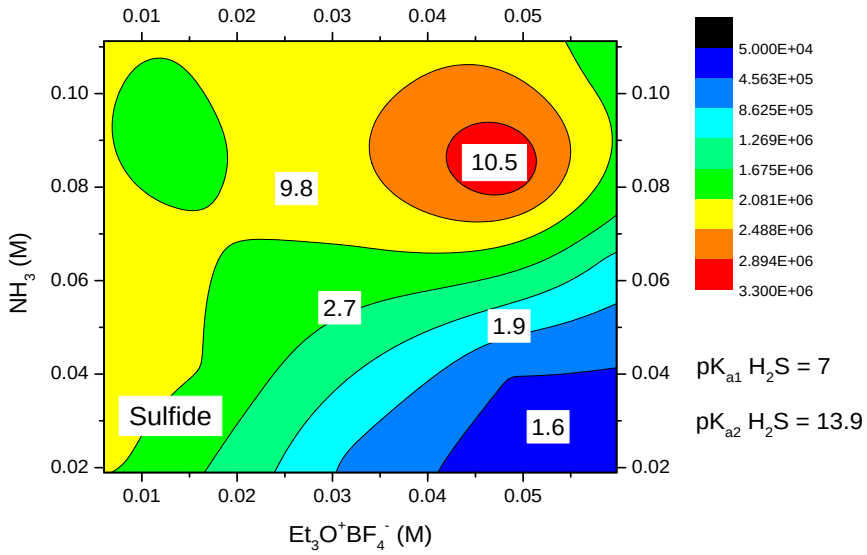


Figure 3.5(b) Response surface of the sulfide ion employing NH_3 as base



As far as the halides Br^- and I^- are concerned, the response surfaces (*Figures 3.6(a)(b)* and *Figures 3.7(a)(b)*) obtained with the use of NaOH as base are similar and show that they required a reaction environment preferably acidic. As the thiocyanate anion, we observe that the range of concentration of base and alkylating agent to add in each vial is very narrow about NaOH while, for NH_3, is broader.

Figure 3.6(a) Response surface of the bromide ion employing NaOH as base

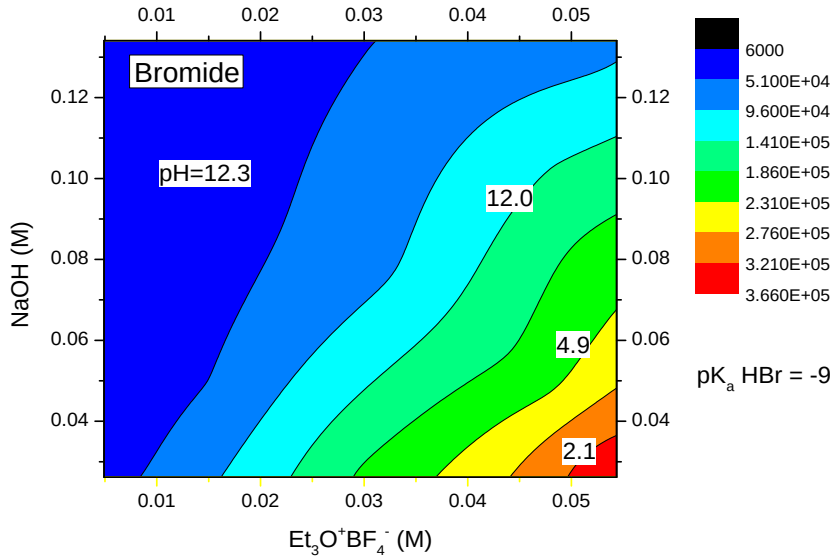


Figure 3.6(b) Response surface of the bromide ion employing NH_3 as base

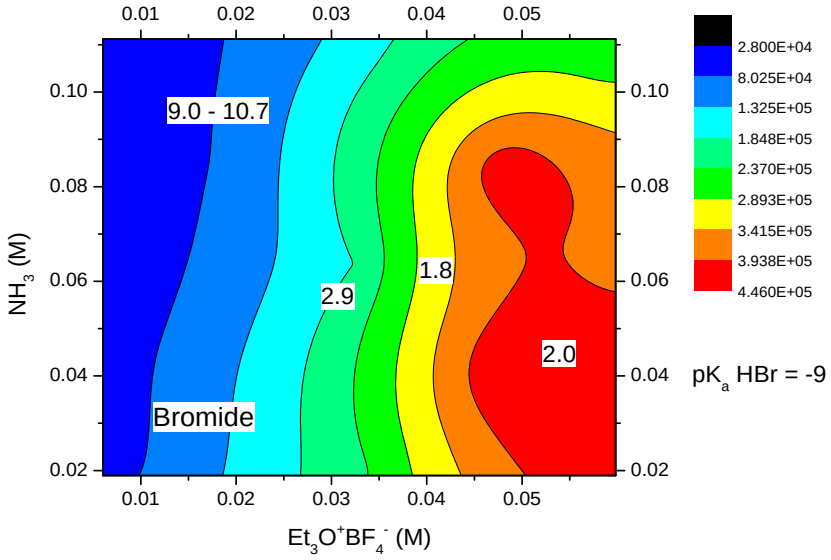


Figure 3.7(a) Response surface of the iodide ion employing NaOH as base

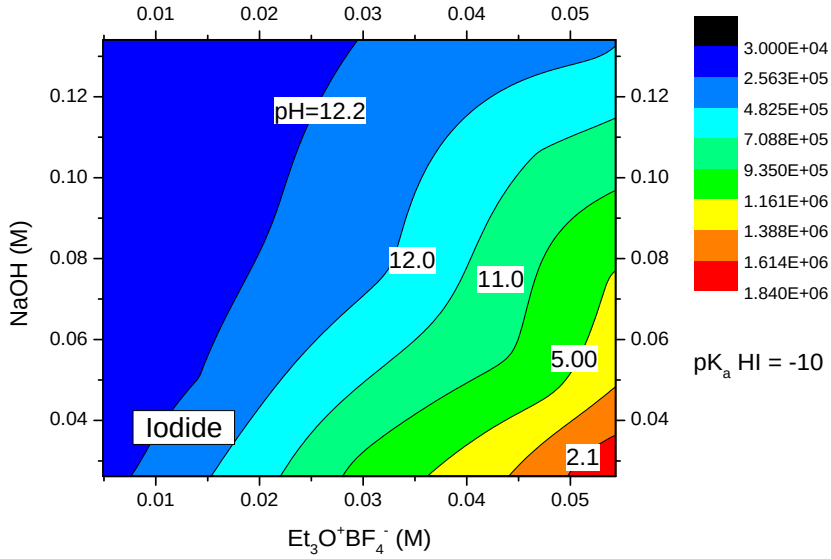
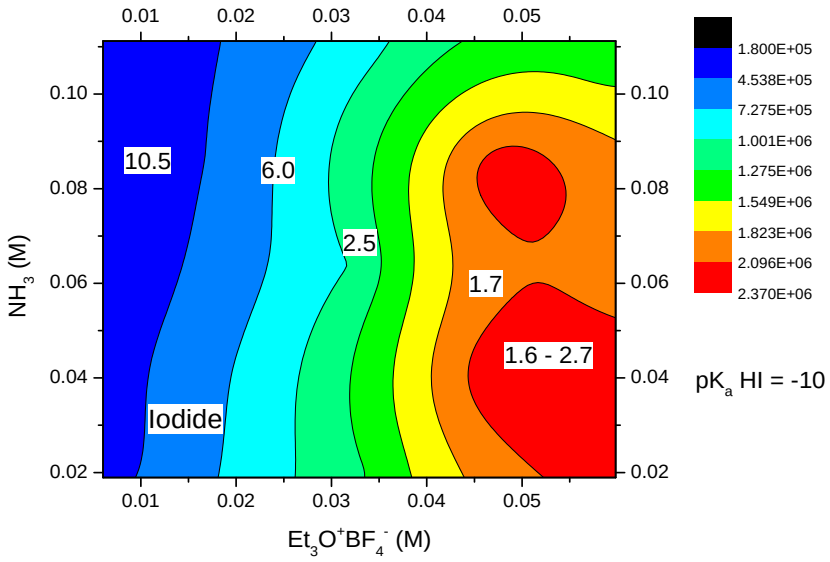


Figure 3.7(b) Response surface of the iodide ion employing NH_3 as base



Cyanide anion shows preferred an alkaline reaction environment (*Figures 3.8(a)(b)*). From the response surface is evident that the use of NaOH as base allows to obtain higher yields compared to NH_3.

Figure 3.8(a) Response surface of the cyanide ion employing NaOH as base

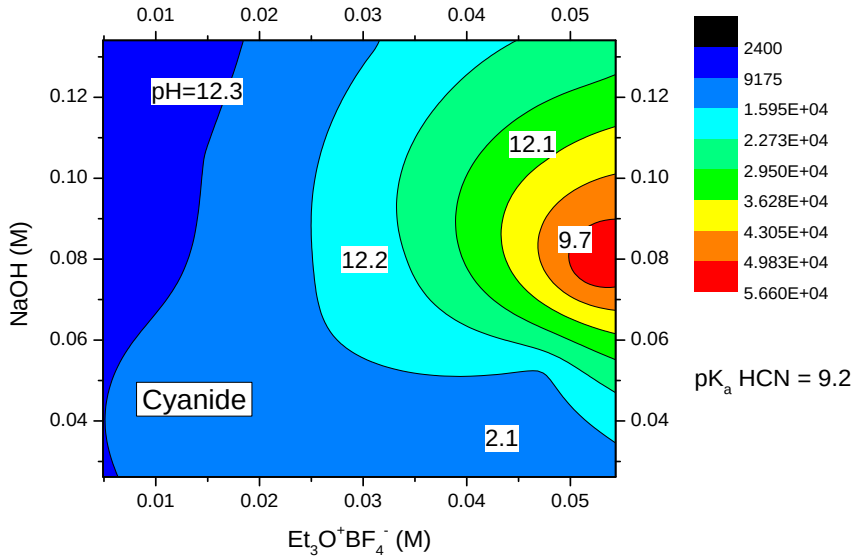
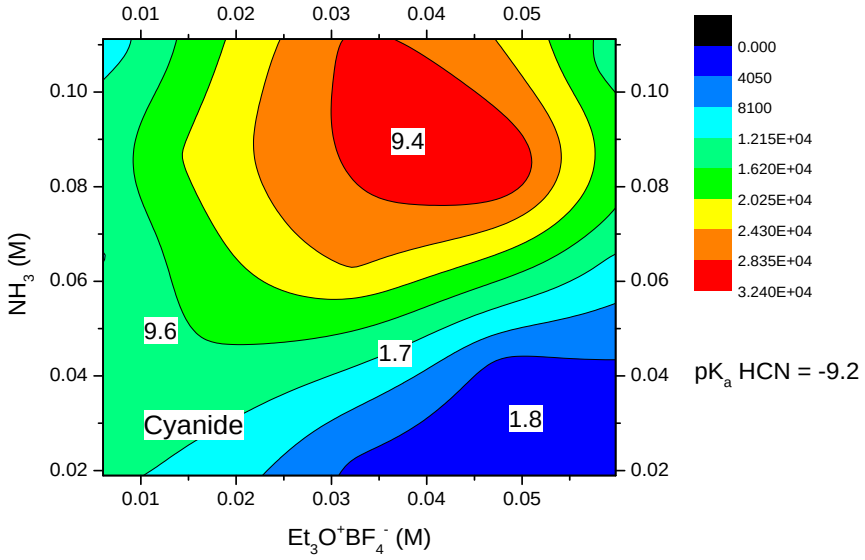
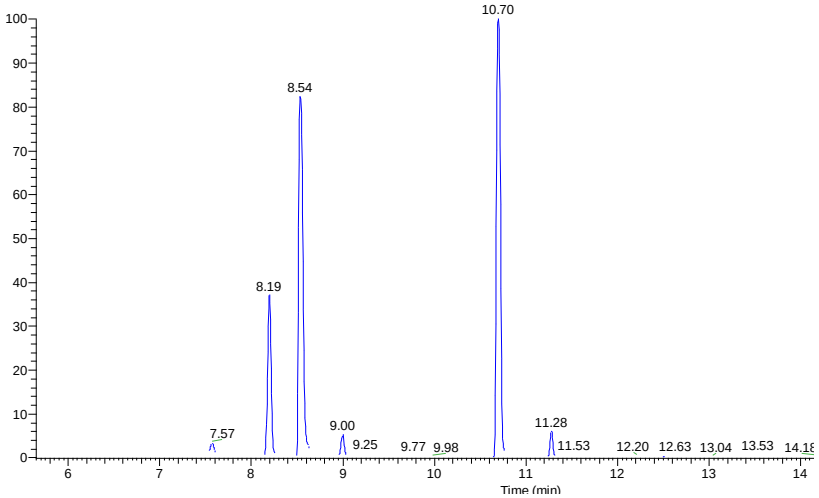


Figure 3.8(b) Response surface of the cyanide ion employing NH_3 as base



Comparing the areas obtained employing ammonia or NaOH as base, we observe that, for some anions, cyanide and thiocyanate, with NaOH we obtain higher yields of derivatization than using NH_3. Furthermore, the chromatograms obtained from solutions prepared by adding ammonia, show the presence of unwanted organic compounds such as diethylamine (m/z = 73) and triethylamine (m/z = 101), due to ammonia alkylation, absent treating with NaOH (*Figure 3.9*). Maybe the formation of these compounds subtracts the triethyloxonium salt from the reaction environment, leading to an increase of the pH solution. Therefore NaOH was the base chosen.

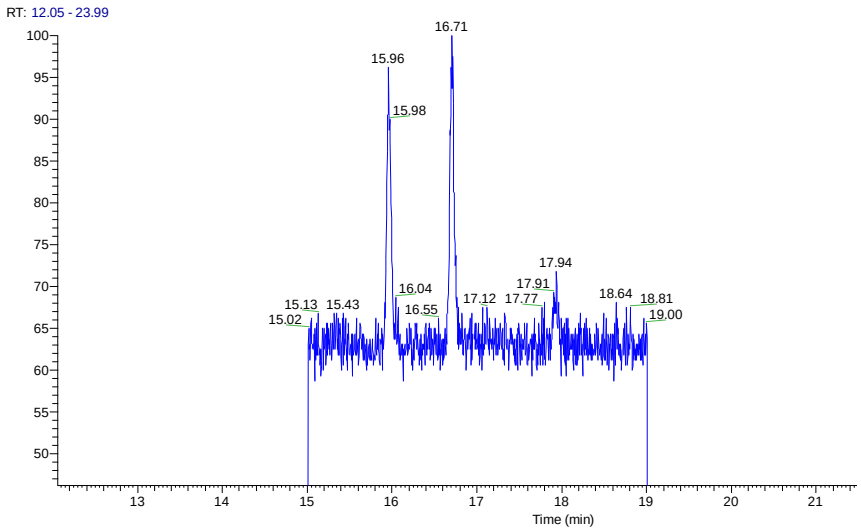
Figure 3.9 Chromatographic peaks of diethylamine (t_R= 8.54 min) and triethylamine (t_R= 10.7 min)



3.3.1 Stability of the thiocyanate ion in alkaline environment

In literature it is reported that, in alkaline reaction environments, thiocyanate ion can be oxidized to cyanide ion[53]. This conversion could influence our quantitative analysis for the determination of the two anions. Therefore we decided to test the stability of the thiocyanate ion in an alkaline solution preparing a 10 µg/mL of SCN^- in 0.1M NaOH. This solution, immediately after its preparation, one hour later, two hours later and the next day (more than 24 hours), was treated with the triethyloxonium salt and analyzed by HS/GC-MS. Three blanks were also prepared and analyzed, to exclude the presence of EtSCN and EtCN in water. The method employed for the analysis was the *METHOD 1* (*Section 2.2.3*).

Figure 3.10 Extracted ion of EtCN (m/z = 54; t_R = 18.44')



The propionitrile has a retention time (t_R) of 18.44'. (*Figure 3.10*) The extracted-ion chromatogram of EtCN (m/z =54) doesn't show the presence of this compound. The absence of the propionitrile in all the chromatograms obtained allows to exclude the conversion of the thiocyanate anion to the cyanide ion in alkaline environment. Furthermore, the EtSCN areas didn't change despite the different times of analysis, thus we concluded that thiocyanate decomposition doesn't take place in an alkaline environment.

3.4 The "salting out" and the temperature

Samples must be prepared to maximize the distribution of the volatile components in the headspace, and minimize unwanted contamination from other compounds in the sample matrix. To help determine the concentration of an analyte in the headspace, is necessary to consider the partition coefficient (*K*), which is defined as the equilibrium distribution of an analyte between the sample phase and the gas phase. This coefficient is defined as:

$$K = \frac{C_l}{C_g}$$

Where C_l is the concentration of the analyte in the sample phase and C_g is the concentration of the analyte in the gas phase. Compounds that have low *K* values will tend to partition more readily into the gas phase and have relatively high response and low limits of detection. On the other hand, compounds with high *K* values will tend to partition less readily into the gas phase and have low relatively low response and high limits of detection. *Table 3.1* shows the partition coefficient values for common compounds.

Table 3.1 *K* values of common solvents in air-water systems at 40°C

Solvent	***K value***
cyclohexane	0.077
n-hexane	0.14
tetrachloroethylene	1.48
o-xilene	2.44
toluene	2.82
benzene	2.90
dichloromethane	5.65
ethyl acetate	62.4
n-butanol	647
isopropanol	825
ethanol	1355
dioxane	1618

K value can be lowered by changing the temperature at which the vial is equilibrated or by changing the composition of the sample matrix. The addition of an high salt concentrations in aqueous samples decrease the solubility of polar organic volatiles in the sample matrix and promote their transfer into the headspace, resulting in lower *K* values. This technique

is commonly called as *salting out* (or drowning out) and is widely employed in chemistry. However, the magnitude of the salting out effect on *K* is not the same for all compounds. Compounds with *K* values that are already relatively low will experience very little change in partition coefficient after adding a salt to an aqueous sample matrix. Generally, volatile polar compounds in polar matrices will experience the largest shifts in *K* and have higher responses after the addition of salt to the sample matrix. *Table 3.2* lists most of the common salts used for salting out procedures.

Table 3.2 Common salts used to decrease matrix effects

Salt
ammonium chloride
ammonium sulfate
sodium chloride
sodium citrate
sodium sulfate
potassium carbonate

The phase ratio β is the relative volume of the headspace compared to volume of the liquid phase in the sample vial and it is defined as:

$$\beta = \frac{V_g}{V_l}$$

Lower values for β will yield higher responses for volatile compounds. However, decreasing the β value will not always yield the increase in response needed to improve sensitivity.

Partition coefficients and phase ratios work together to determine the final concentration of volatile compounds in the headspace of sample vials. The concentration of volatile compounds in the gas can be expressed as

$$C_g = \frac{C_0}{(K + \beta)}$$

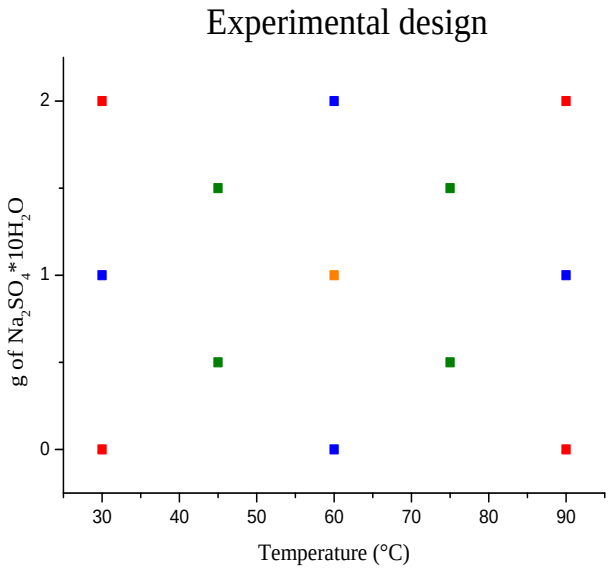
where C_g is the concentration of volatile analytes in the gas phase and C_0 is the original concentration of volatile analytes in the sample. Striving for the lowest values for both *K* and β will results in higher concentration of volatile analytes in the gas phase and,

therefore, better sensitivity. In addition to working with K and β, sensitivity also can be improved by simply increasing the size of the headspace sample that is withdrawn from the sample vial and transferred to the GC.

3.4.1 Temperature and salt addition: the effects

We studied the effect of the incubation temperature and of the addition of a non-reactive salt in the reaction environment in order to estimate their role in the transfer of the products from the aqueous phase to the headspace. For this purpose, we studied the behaviour of EtCN, EtSCN and EtSEt, that are the alkylation products of the reaction between CN^-, SCN^- and S^{2-} with the triethyloxonium salt. We built an experimental design with two factors (incubation temperature and amount of the salt) and five levels: a *Central Composite Design* (*Figure 3.11*). Thirteen experiments were performed using the *working solution 3* (*Section 2.3*), containing the derivatized anions at 2 µg/mL concentration. The temperature range investigated has been chosen in accordance with the instrumental limits (min 30°C, max 90°C). For salting out effect, we added an increasing amount of a salt (0, 0.5, 1, 1.5 and 2g of salt) in each vial. The salt $Na_2SO_4 \cdot 10H_2O$ was used for the analysis; results are reported below. The method employed for the analysis was *METHOD 1*.

Figure 3.11 Thirteen points of the experimental design of the salting out effect and the temperature, with two factors and five levels



We observed that the addition of the salt in each vial causes an increase of the EtCN, EtSCN and EtSEt areas in the same range of temperature. Thus it was confirmed that the salting out effect allows to increase the transfer of the products from the aqueous phase to the headspace. As expected we observed that in all cases, the higher the amount of the salt added in each vial, the higher the salting out effect. Experimental results are reported in *Figures 3.12(a)(b)(c).*

Figure 3.12(a) Increase of the alkylation yield of the thiocyanate ion in function of the temperature and the amount of salt added in each vial

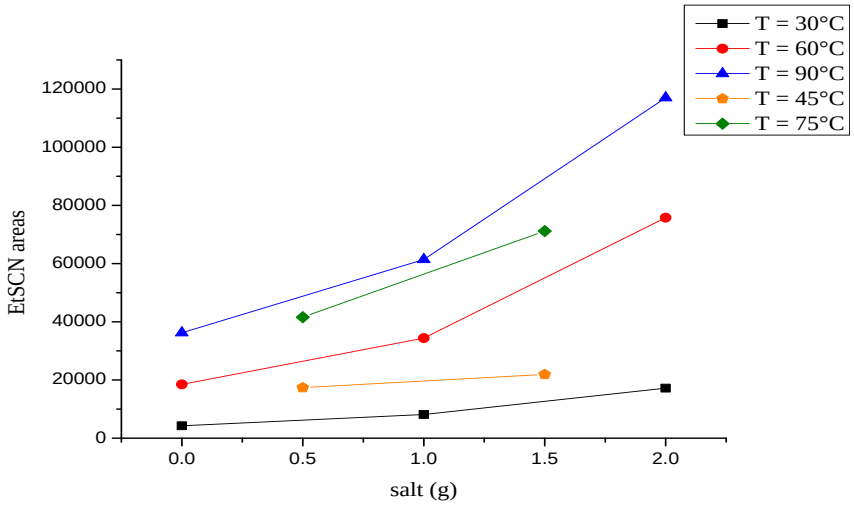


Figure 3.12(b) Increase of the alkylation yield of the cyanide ion in function of the temperature and the amount of salt added in each vial

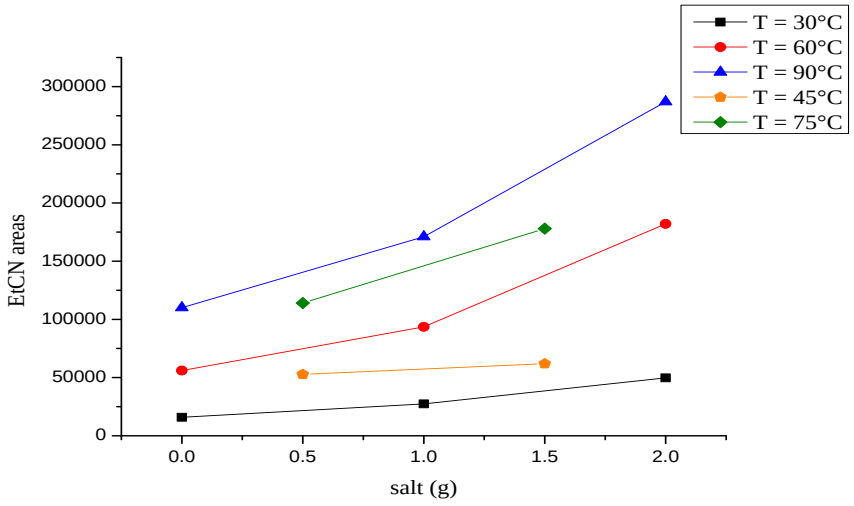
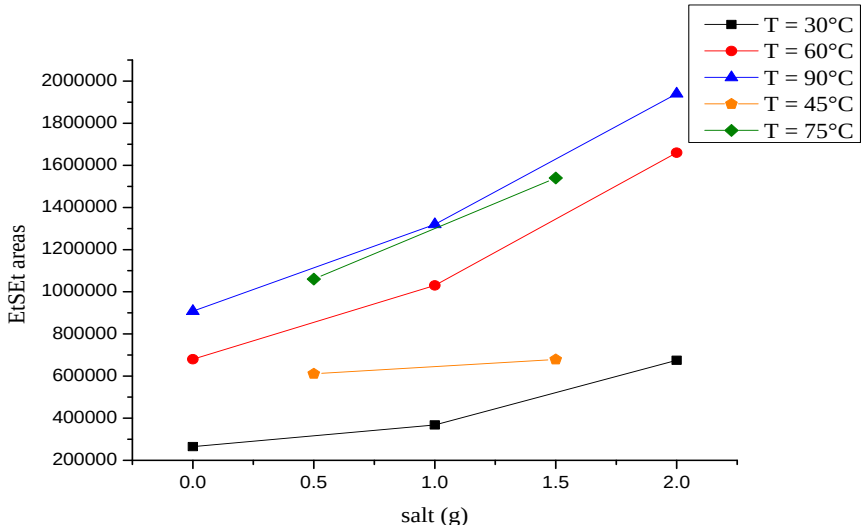


Figure 3.12(c) Increase of the alkylation yield of the sulfide ion in function of the temperature and the amount of salt added in each vial



We observed that for all analytes, the best reaction conditions provide an incubation temperature of 90°C and an amount of the non-reactive salt of 2g. We also studied the effect of the incubation temperature without adding the salt in the reaction environment. The temperature investigated were again 30, 45, 60, 75 and 90 °C. As shown below (*Figures 3.13(a)(b)(c)*), increasing the temperature, we obtain an increase of the reaction products transfer in the headspace, even without the addition of the salt. This confirms that the temperature plays a fundamental role in the transfer of analytes from the aqueous phase to the headspace and can be modulated to obtain best results in the analytical response.

Figure 3.13(a) Increase of the generation yield of the thiocyanate ion by the temperature

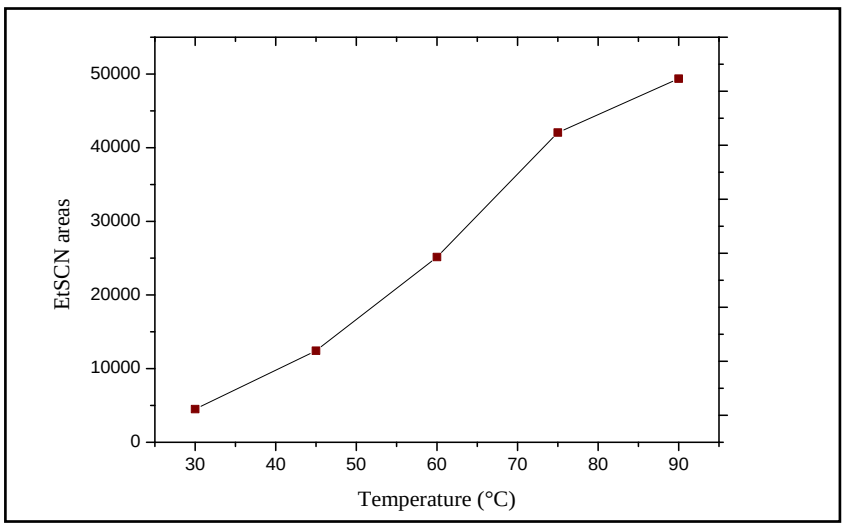


Figure 3.13(b) Increase of the generation yield of the cyanide ion by the temperature

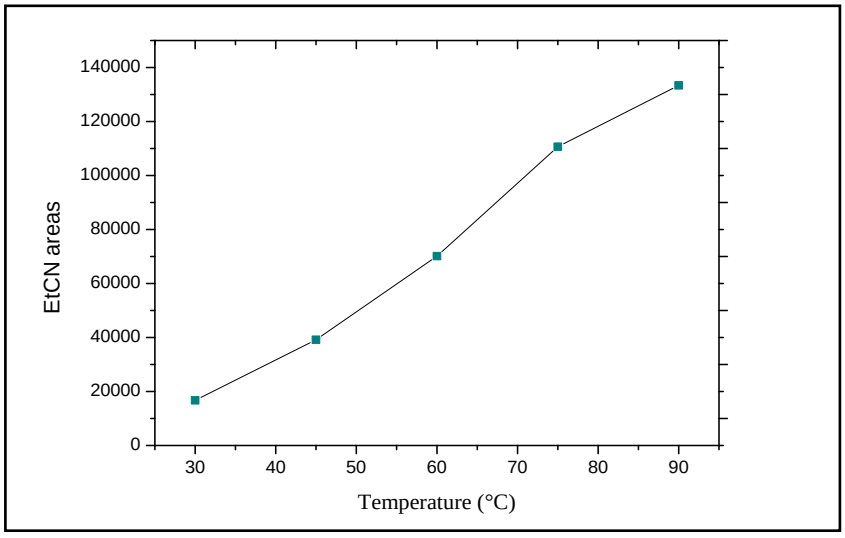
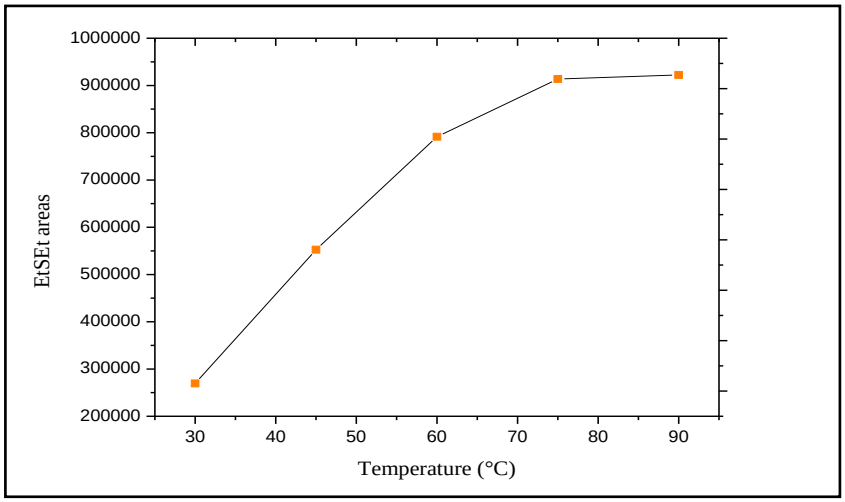


Figure 3.13(c) Increase of the generation yield of the sulfide ion by the temperature



Therefore, the incubation temperature and the amount of the salt play an important role in the transfer of the analytes from the aqueous phase to the headspace. Thus working on both parameters we chose the best reaction conditions in order to obtain an efficient transfer in the headspace. From the experimental results, we decided to work at an incubation

temperature of 75°C, that in most cases had an effect close to 90°C, in order to hindered the boiling of water and limited the amount of water vapour in the headspace. We also decided to add 1g of the salt in each vial because the salting out effect was already present with 1g of the salt and in order to limited the contaminations due to the presence of some anions, as impurities, in the salt.

3.5 Effect of reaction time

After the optimization of the pH solution, the salting out and the temperature effect, it is interesting to know the time required from the addition of the ethylating agent, the triethyloxonium salt, and the headspace analysis by GC-MS. To this aim, we analyzed a working solution containing the anions, the *working solution 4*, containing the anions CN^-, SCN^- and S^{2-} (*Section 2.3*), at different times from the addition of the reagent in each vial. An analysis sequence was set where different vials, prepared with the reactant and the salt, were analyzed at different times: 30', 60', 90', 180', 360', 14h30' and more than 24 h after the addition of the triethyloxonium salt. *Figures 3.14(a)(b)* summarize the experimental results obtained. The method employed for the analyses was *METHOD 2*.

Figure 3.14(a) Time course of the alkylation reaction of thiocyanate and cyanide anions

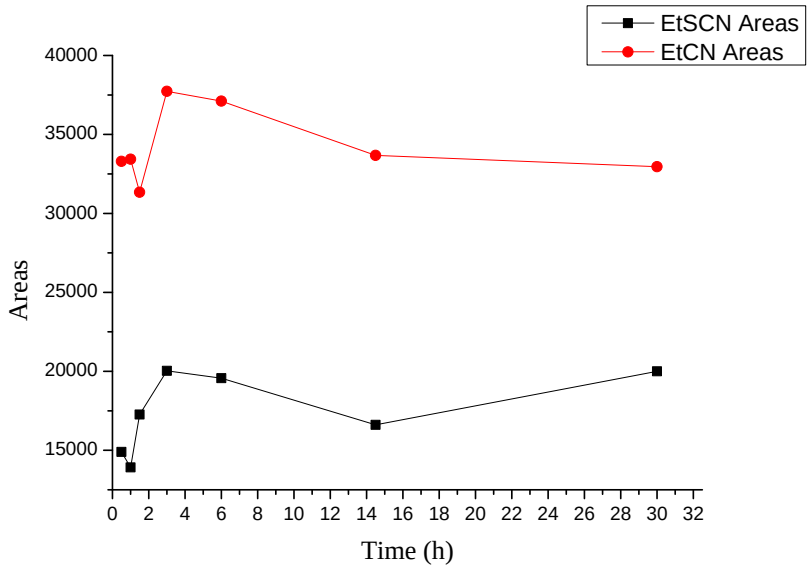
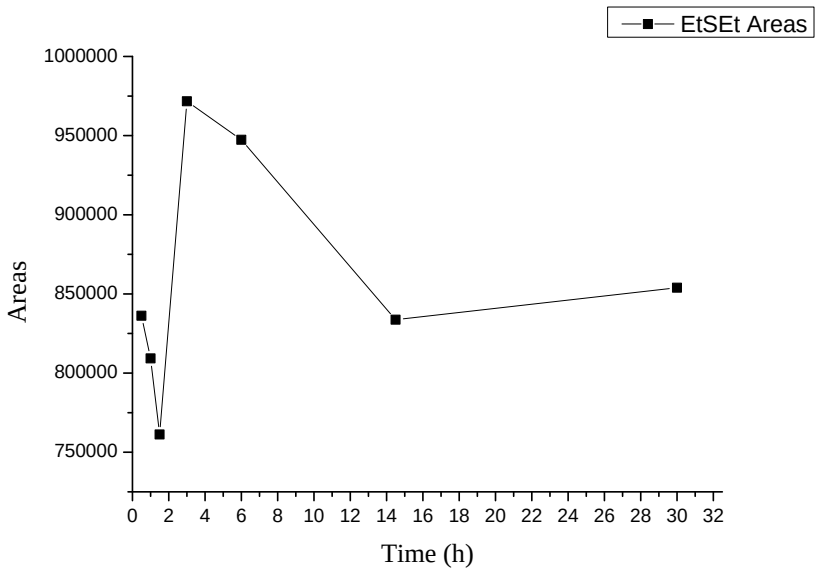


Figure 3.14(b) Time course of the alkylation reaction of the sulfide anion



The study of the chromatograms obtained shows a maximum of the alkylation yields at three hours after the addition of the trialkyloxonium salt for all analytes, with the greatest increase for S^{2-} anion (*Figure 3.14(b)*).

We can assume that after three hours we can proceed with the GC-MS analysis. For EtSCN, an increase of the areas has been found from the analysis of the last vial. However, this increase is not enough to justify a waiting time of 24 hours between the addition of $Et_3O^+BF_4^-$ and the analysis.

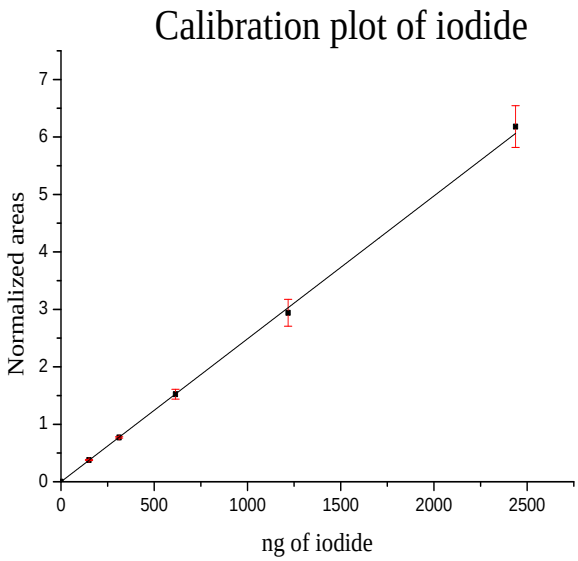
We concluded that these three reactions have a similar time course and a derivatization of three hours was chosen for the future analysis. This also allows to have all of the results in the same conditions.

3.6 Calibration of the anions and analytical figures of merit

Once optimized the reaction conditions of our method, we decided to build the calibration plot of each analyte. Aliquots (50, 100, 200, 400 and 800 µL) of the *working solution 5* (*Section 2.3*), containing the anions SCN^-, CN^-, S^{2-}, I^- and Br^-, were added to the vials and analyzed. We prepared also five blanks and for each aliquot three replicates were prepared, analyzed the same day. The method employed for the analysis was the *METHOD 2* (*Section 2.2.3*).

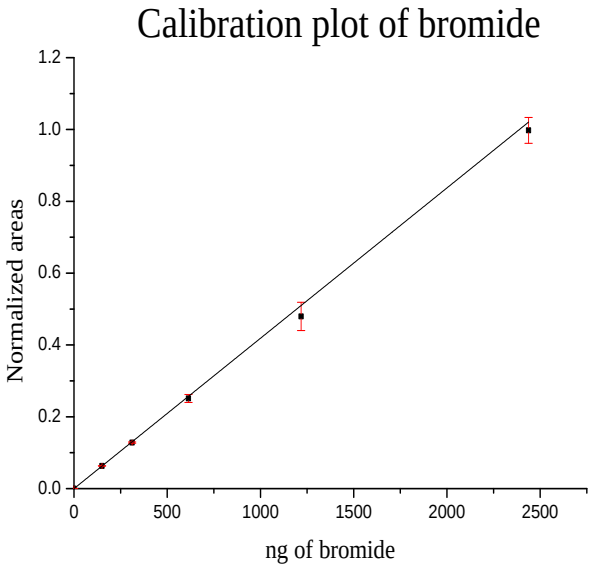
Below the calibration plot of each analyte is reported (*Figures 3.15(a)(b)(c)(d)(e)*). Thiocyanate areas were normalized to the labelled internal standard $S^{13}CN^-$ areas while cyanide, sulfide, iodide and bromide areas were normalized to the area of the labelled internal standard $^{15}NO_3^-$.

Figure 3.15(a) Calibration plot of iodide



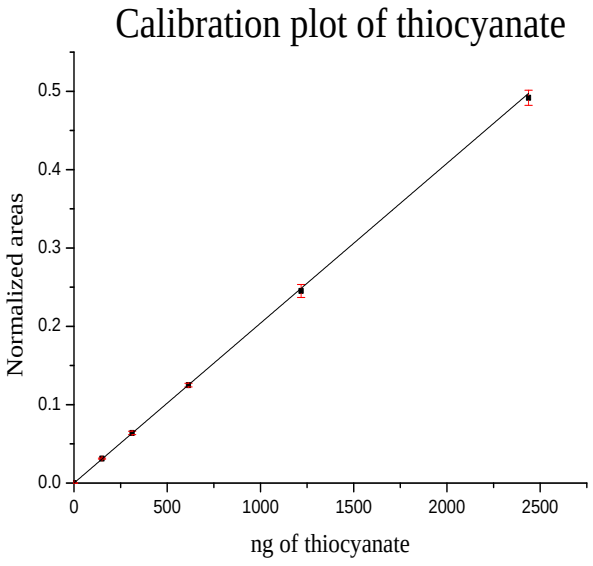
Slope	$2.52 \cdot 10^{-3}$
R^2	0.9999
Linearity	150-2440 ng

Figure 3.15(b) Calibration plot of bromide



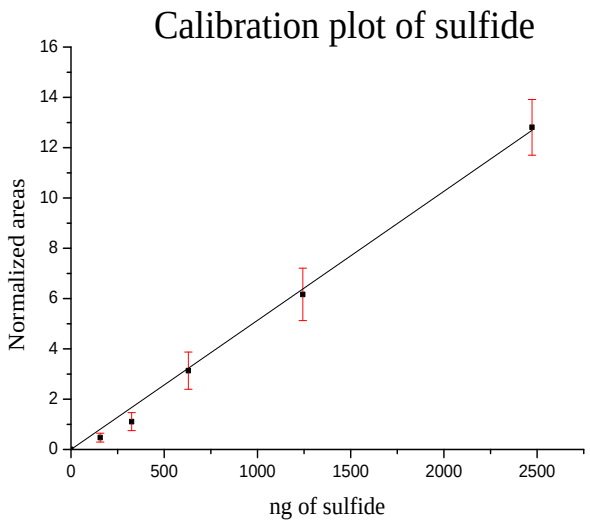
Slope	$4.18 \cdot 10^{-4}$
R^2	0.9999
Linearity	150-2440 ng

Figure 3.15(c) Calibration plot of thiocyanate



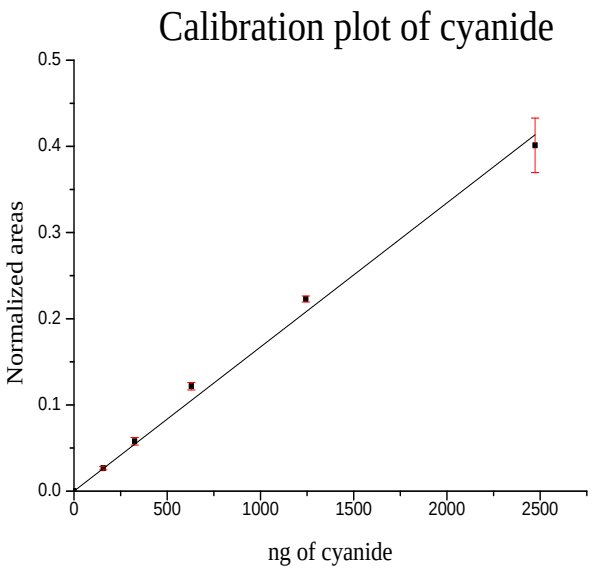
Slope	$2.04 \cdot 10^{-4}$
R^2	0.9999
Linearity	150-2440 ng

Figure 3.15(d) Calibration plot of sulfide



Slope	$5.13 \cdot 10^{-3}$
R^2	0.9937
Linearity	150-2440 ng

Figure 3.15(e) Calibration plot of cyanide



Slope	$1.67 \cdot 10^{-4}$
R^2	0.9986
Linearity	150-2440 ng

Our results show good linearity and good R^2 values. The calibration curves of the anions CN^- and S^{2-} show R^2 values lower than the other anions. Despite the calibration of these anions has been repeated several times, we were unable to obtain better results. Therefore, we can hypothesise that better results could be obtained by using a labelled standard for the normalization of the areas. Moreover we observed that the error that affecting each point in the calibration plot of sulfide anion is very high. This is actually due to the simultaneous formation of EtS-SEt besides EtSEt, as observed in the *TIC* chromatogram (Appendix A, Figure D).

Once the reaction conditions of our method were optimized, we estimated the analytical figures of merit for each analyte studied in this work. The figures of merit serve as indicator for the characteristics of an analytical technique with regards to a specific analyte.

In this work we estimated the linearity, the sensitivity, the reproducibility intra and inter-day, the LOD (*Limit of Detection*) and the LOQ (*Limit of Quantification*) for each analyte.

- ***Linearity***

  Linearity was estimated as: $\log_{10}$ (ng of analyte/LOQ) where the LOQ was calculated as $10\sigma/m$.

- ***Sensitivity***

  Sensitivity was assumed as the average slope (*m*) measured for three different calibration plots of each anion with the relative confidence interval at 95%. The calibration plots were reproducible over 4 months.

- ***Intra and inter-day repeatability***

  The relative standard deviation (RDS) of normalized areas has been calculated as the standard deviation of a set of measurement divided by the mean of all of the measurements. The repeatability is reported as the percent relative standard deviation (%RDS).

- ***LOD and LOQ of our method***

  The LOD and LOQ values were calculated as 3 times the standard deviation of the blank divided by the calibration curve slope (3σ/m) and 10 times the standard deviation of the blank divided by the calibration curve slope (10σ/m) respectively.

- ***Instrumental LOD and LOQ***

  LOD and LOQ values were estimated as 3S/N and 10S/N respectively. These values were estimated by the software SigNoise of Thermo Electron Corporation.

All of the parameters were estimated under optimized conditions. Analytical figures of merit are reported below in *Table 3.3*.

Table 3.3 Analytical figures of merit: linearity, sensibility, intra and inter-day repeatability, LOD and LOQ

Analyte	*Linearity (decades)* [(a)]	*Sensitivity*	*Repeatability [%]* [(b)]		*LOD [ng/mL]*		*LOQ [ng/mL]*	
			Intra-day	*Inter-day*	*3S/N*	*3σ/m*	*10S/N*	*10σ/m*
I^-	1.4 – 2.6	$2.52\cdot10^{-3}\pm6.57\cdot10^{-5}$	0.61	0.82	0.05	1.99	0.16	6.65
Br^-	1.8 - 3.0	$4.09\cdot10^{-4}\pm3.52\cdot10^{-5}$	0.52	0.57	0.51	0.68	1.71	2.26
SCN^-	1.9 – 3.1	$2.02\cdot10^{-4}\pm1.24\cdot10^{-6}$	1.13	0.99	2.24	0.59	7.47	1.98
S^{2-}	1.6 – 2.8	$4.97\cdot10^{-3}\pm4.2\cdot10^{-3}$	6.24	5.17	0.12	1.24	0.38	4.14
CN^-	1.0 – 2.2	$1.69\cdot10^{-4}\pm1.41\cdot10^{-5}$	2.44	2.82	48.97	4.80	163.22	16.00

[(a)] Maximum tested concentration was 2440 ng/6 mL = 407 ng/mL

[(b)] Expressed as RSD%, n = 3 (intra-day), n = 9 (inter-day: 3 analysis in 3 different days)

Chapter 4

Analytical applications and analysis of saliva samples

4.1 Biological matrices

The analysis of complex matrices, such as biological ones, can be very laborious and complex, from the analytical point of view, because of the presence of endogenous compounds produced by the human metabolism. These matrices are difficult to be analyzed and rich of interfering compounds that may hinder the identification and the quantification of the analytes of interest. Sugars, proteins, enzymes, hormones and minerals are the main components, and to remove or reduce their interference it is often necessary dilute or to pretreat the samples, causing extended analysis time, as well as an increased risk of losses and contaminations. *Chemical Vapour Generation*, by formation of volatile alkyl derivatives of anions could be applied to this kind of matrices because interferences are controlled, thanks to the analyte-matrix separation obtained by derivatization. In this work, the analytical method developed for the determination of anions with $Et_3O^+BF_4^-$ by HS/GC-MS was used to determine thiocyanate in human saliva.

4.1.1 Saliva[54]

Saliva is a hyposmotic liquid secreted from the salivary glands, located in the oral cavity. Like all secretions, saliva is mainly composed of water (99%), while only 1% is represented by organic and inorganic substances. The inorganic substances are mineral salts (*Table 4.1*), such as chlorides and sodium, potassium and calcium bicarbonates, while the organic fraction is constituted by enzymes, such as amylase, mucin, lysozyme, and immunoglobulins. Saliva plays many important functions. The digestion begins in the mouth due to chewing and to the presence of some enzymes, such as ptyalin, capable of digesting polysaccharides. For the oral cavity, saliva plays some different function: hygienic, especially for the presence of water and mineral salts, antibacterial and lubricating, thanks to the presence of lysozyme, lactoferrin, thiocyanate ion and antibodies, which allow to oppose to the life and to the growth of the bacteria. Saliva can therefore act as a natural disinfectant. Generally it has a neutral pH, between 6.5 and 7.5 approximately. Thanks to the presence of bicarbonates, saliva is a buffer system that corrects the acidity of the mouth after eating or drinking. Average 1500 mL of saliva are secreted daily: the normal production is about 20 mL/h, while under stimulation can reach 250 mL/h.

Table 4.1 Saliva composition, main inorganic components (mmol/L)

Components	***mmol/L***
Na^+	5.2-24
K^+	14-41
Ca^{2+}	1.2-2.8
SCN^-	0.4-6.6
Cl^-	6.5-43
Br^-	2.5-13
I^-	0.3-1.9
HCO_3^-	2.1-13
phosphates	1.9-5.5
F^-	< 2.6

Saliva is a body fluid with great diagnostic potential for non-invasive analysis. Several diseases such as alcoholism cirrhosis, cardiovascular diseases, cystic fibrosis and diabetes mellitus are known to directly or indirectly affect the functions of salivary glands[55-56].

Moreover, changes in salivary composition correlate with disease susceptibility and progression. Human saliva is, therefore, a potential source of novel diagnostic markers and therapeutic targets[57]. In the last years there has been a growing interest in identifying "salivary biomarkers" as a means of monitoring general health and for the early diagnosis of diseases including bacterial infection, human immunodeficiency virus (HIV) and oral cancer[58]. In comparison with other body fluids such as serum or urine, several further advantages exist in analyzing saliva including straightforward sample collection, sufficient quantities for analysis, and lower costs of storage and shipping.

4.1.1.1 Presence of cyanide and thiocyanate in human saliva

Cyanide is a powerful chemical asphyxiant found in some forensic cases following voluntary (suicide, by ingestion of KCN or NaCN) or involuntary ingestion (fire, accidental exposure)[59]. It acts through fixation to cytochrome C oxidase, the mitochondrial enzyme responsible for the last step in cell respiration, and the blood oxygen transporter hemoglobin[60-61]. Cyanide was reported to be responsible for a ''knockout effect'' in some fires, rendering the person unconscious well before carbon monoxide reaches lethal levels. Finally, low levels of cyanide are generated in the body as a metabolite of nitroprusside, acetonitrile, acrylonitrile and cyanogenic glycosides[62]. Thiocyanate ion is usually present in low concentrations in biological fluids, such as saliva[53, 63]. Its toxicity is far lower than cyanide, but high concentration of thiocyanate can inhibit the absorption of iodine by the thyroid gland, thereby, reducing the formation of thyroxine, one of the major thyroid hormones[64]. The presence of thiocyanate in body fluids (saliva, serum, urine) is due to the digestion of some vegetables such as cassava (mainly present in tropical countries such as Africa, Asia and Latin America)[65], cabbage, turnips, kale[66], but also milk and cheese[67]. Some clinical disorders like ataxic neuropathy[68], tropical diabetes[69], endemic goiter and cretinism[53] may be due to high levels of SCN in body fluids from ingestion of cyanogenic glycosides in cassava. It's also present in drugs that are used in the treatment of thyroid problems and arterial hypertension[70]. High concentrations may also be due to the detoxification of HCN from the tobacco smoke. The thiocyanate is the major metabolite of cyanide, one of the products of the combustion process of tobacco[67]. Actually, the determination of the cyanide and the thiocyanate concentrations in saliva plays a very important role because it allows to

monitor the clinical condition of patients as well as distinguishing smokers from non-smokers[53]. As thiocyanate is significantly correlated with the daily cigarette consumption[71]. However, variations in the volume of saliva can influence SCN levels, making it more difficult to ascertain the relation between tobacco consumption and SCN concentration[72].

4.1.2 Analytical techniques for cyanide and thiocyanate determination in biological matrices

From the qualitative point of view, the complexation of the thiocyanate with the Fe^{3+} leads to the formation of a complex with a characteristic red colour[63]. Over the years, many analytical techniques have been developed and tested for the determination of cyanide and thiocyanate in different biological matrices such as saliva, urine, plasma and blood[64]. The most used techniques are capillary electrophoresis[73-74], spectrophotometry[75-76], ion chromatography (IC)[77-80], liquid chromatography (HPLC)[81], atomic absorption spectrometry[82], cathodic stripping voltammetry[83], gas chromatography (GC) with capture electron[84] or mass spectrometric detection[85], and micellar electrokinetic capillary chromatography[86]. In literature some works are reported that employ the headspace GC-MS analysis for the quantification of cyanide and thiocyanate in human fluids[59, 62]. Over the past two decades, the most widely used analytical technique for the determination of these anions is ion chromatography (IC)[64] with conductively detector. However, this type of detector is not very selective for the determination of thiocyanate, especially in biological samples where there are simultaneously present other anions (such as Cl^-, SO_4^- etc.).

4.2 Detection of thiocyanate in saliva samples

As discussed in Chapter 1, *Chemical Vapour Generation* can be easily applied to the analysis of biological samples because it is a separation technique that, by derivatization, allows to remove the analytes from the matrix, removing interferences due to the sample

matrix. In this section we show the analytical results obtained by applying our method to the analysis of saliva samples to determine the thiocyanate concentration. The derivatization reaction, by triethyloxonium salt, was exploited to conduct qualitative and quantitative analysis and the data obtained were compared with the literature data[54]. All of the analysis were performed under optimized conditions with regards to pH solution, incubation temperature, amount of inorganic salt added and waiting time from the addition of the triethyloxonium salt to the GC-MS analysis, in order to obtain high yields.

4.2.1 Calibration plot of SCN^-

Before the analysis of real samples, the calibration plot of the thiocyanate ion, was repeated, employing the *METHOD 2*, shorter than the *METHOD 1* (*Section 2.2.3*). Aliquots (50, 100, 200, 400 and 800 µL) of a 3 µg/mL working solution (corresponding to 150, 300, 600, 1200 and 2400 ng) containing SCN^- were added into the vials and for each aliquot five replicates were prepared and analyzed the same day, as well as five blank samples. Thiocyanate areas were normalized to the labelled internal standard areas. The calibration curve was obtained by determining the normalized areas as a function of the ng of thiocyanate added in each vial. (*Figure 4.1*). The experimental parameters obtained for the calibration curve are reported in *Table 4.2*.

Figure 4.1 Calibration plot of the thiocyanate anion

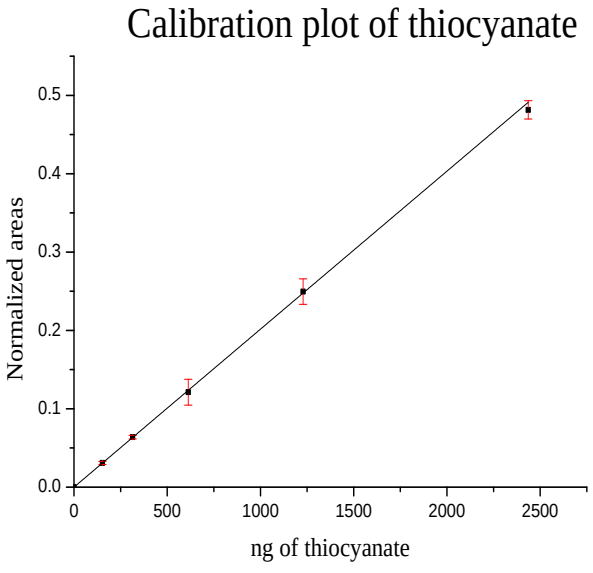


Table 4.2 Experimental parameters of the calibration plot of the thiocyanate anion

Slope	$2.02 \cdot 10^{-4}$
R^2	0.9994
Linearity	150-2440 ng

4.2.2 Analytical addition method

Before the analysis of saliva samples, we made the analytical addition method to a pool of saliva samples, in order to verify the presence of matrix effects. The sample was diluted 1:10 and 50 µL were added in each vial. The analytical procedure for the preparation of the vial is the same as adopted for the construction of calibration plots. Thiocyanate areas obtained were normalized to the labelled internal standard areas. *Table 4.3* summarizes the results obtained.

Table 4.3 Comparison between the experimental parameters of the calibration plot and the experimental parameters of the analytical addition method

	calibration plot	***analytical addition method***
Slope	$2.02 \cdot 10^{-4} \pm 0.02$	$2.04 \cdot 10^{-4} \pm 0.01$
R^2	0.9994	0.9999

As shown, comparing the slope values obtained, there's no matrix effect, probably owing either to the separation carried out by HS analysis method and to the use of the labelled internal standard. We thus proceeded with the analysis of the saliva samples by using the calibration plot reported above.

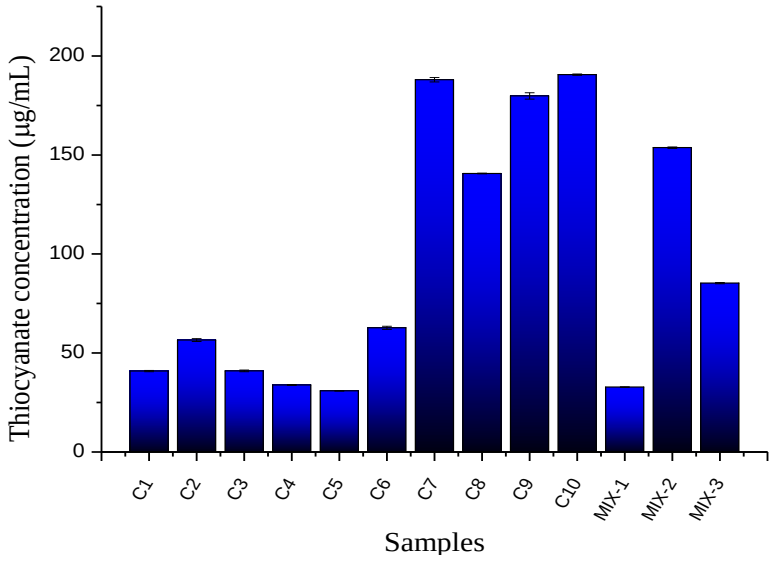
4.2.3 HS/GC-MS analysis

Following results obtained about thiocyanate ion from the experimental design, 10 µL of NaOH and 100 µL of the ethylating agent were added. For each sample four replicates were prepared and analyzed the same day; we also prepared four blanks in order to exclude the presence of EtSCN in water. The method used for the analysis was the *METHOD 2* (*Section 2.2.3*).

4.2.4 Analytical results

From the chromatograms obtained, we derived the amount (ng) of SCN^- present in each sample, from the calibration plot. Considering the dilution of the samples and the volume of saliva added in each vial, we obtained the thiocyanate concentration in µg/mL. The concentration of SCN^- obtained from each sample are shown in *Figure 4.2*. Different concentrations were obtained from each sample, not only the different smoking habit of the people investigated, but also because SCN^- content also depends on the body mass, age, sex and diet[54] of each subject.

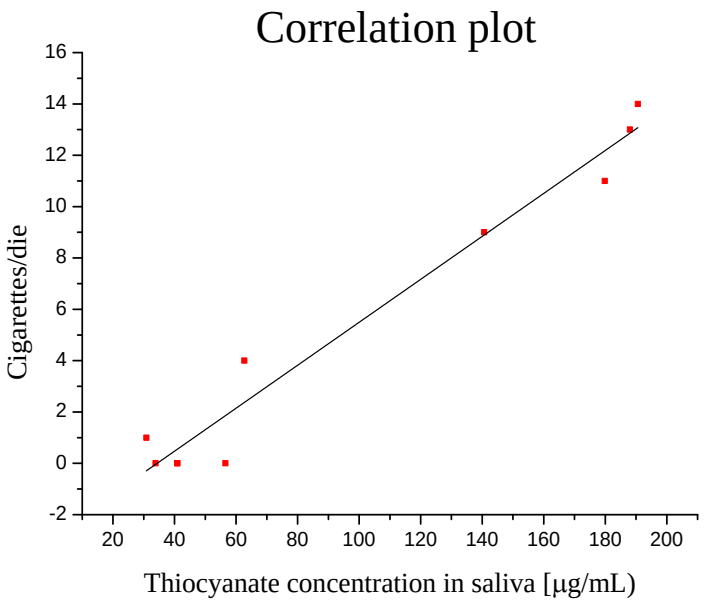
Figure 4.2 Histogram of the thiocyanate concentration obtained from the amount of the anion from the calibration plot



As shown in *Figure 4.2* the amount of thiocyanate detected in each sample is closely related to the amount detected in the three MIX prepared before the analysis. As expected, comparing the results obtained from samples C1-C5 with the MIX-1 and from samples C6-C10 with the MIX-2 we observe that thiocyanate concentration levels detected are comparable.

Below is reported the correlation plot between the number of cigarette/die and the thiocyanate concentration detected for each sample by the calibration plot, with the experimental parameters.

Figure 4.3 Correlation plot of the number of cigarette/die and thiocyanate concentration

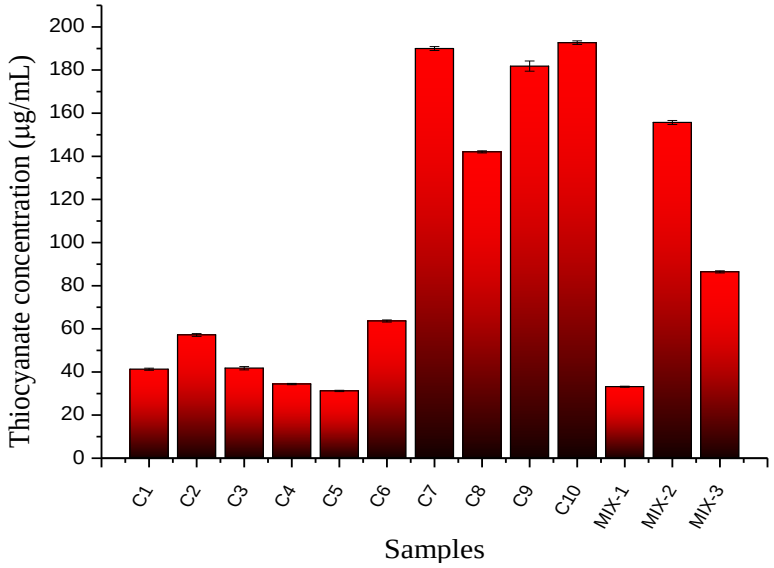


Slope	$8.4 \cdot 10^{-4}$
Intercept	-2.87
R^2	0.9829

The number of samples analyzed is low and didn't allow to make a reliable statistical analysis. Despite this aspect, we tried to make a correlation plot with the available values. *Figure 4.3* shows a good correlation between thiocyanate concentration and the number of cigarettes smoked. This suggests that probably the amount of thiocyanate was mainly derived from smoking through cyanide metabolism with only a minor part from the alimentation. Further studies are necessary in order to confirm this correlation.

Thiocyanate concentration by isotopic dilution (*Figure 4.4*) was also estimated. For this purpose it is necessary to know the exact amount of labelled internal standard added in each vial.

Figure 4.4 Histogram of the thiocyanate concentration obtained by isotopic dilution



The use of a labelled internal standard, that presents a behaviour similar to the analyte in solution, allows to correct the areas of the analyte of interest by reducing the presence of the matrix effect. From the ratio between thiocyanate areas and labelled internal standard areas, knowing the amount of internal standard added in each vial, we obtain the amount of thiocyanate present in each sample. From the ng obtained, we can derive the thiocyanate concentration.

$$\frac{A\ EtSCN}{A\ EtS^{13}CN} = \frac{ng\ EtSCN}{ng\ EtS^{13}CN}$$

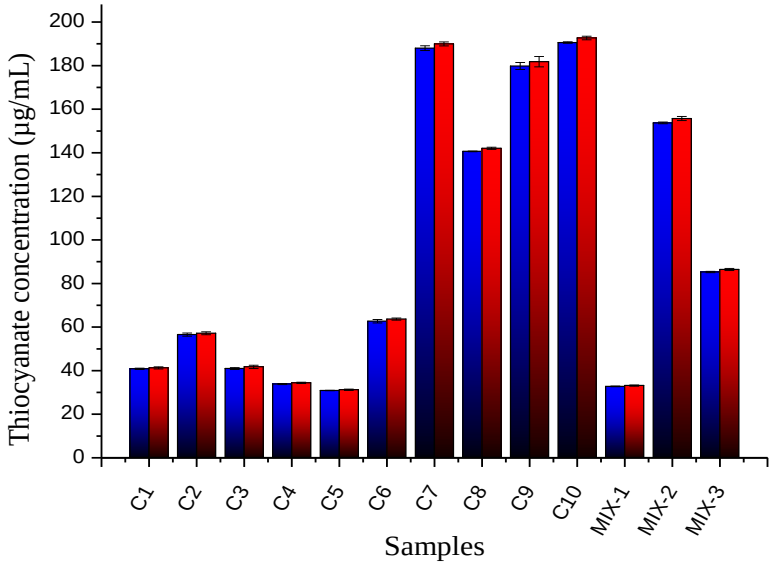
Table 4.4 and *Figure 4.5* show the concentrations of the anion in each samples estimated by calibration plot and isotopic dilution. In order to compare the results obtained, we proceeded with the *F*-test, to assess whether the variance of the values found differ significantly and to determine if the accuracy of the two methods is the same, within the limits of probability data (95% confidence interval, n=3). Subsequently, after that the *F*-

test was found to be positive, we proceeded with the *t*-test, in order to decide if the difference between the two means is statistically significant.

Table 4.4 Thiocyanate concentration from each samples obtained by calibration plot and isotopic dilution with the 95% confidence interval

Sample	***Thiocyanate concentration [µg/mL]***	
	Calibration plot	***Isotopic dilution***
C1	40.86 ± 0.32	41.27 ± 0.48
C2	56.53 ± 0.72	57.16 ± 0.67
C3	40.96 ± 0.41	41.74 ± 0.76
C4	33.84 ± 0.21	34.40 ± 0.29
C5	30.87 ± 0.09	31.21 ± 0.31
C6	62.71 ± 0.79	63.65 ± 0.50
C7	188.0 ± 1.1	190.0 ± 0.9
C8	140.7 ± 0.2	142.0 ± 0.5
C9	179.8 ± 1.6	181.8 ± 2.4
C10	190.6 ± 0.3	192.7 ± 0.8
MIX-1	32.77 ± 0.13	33.17 ± 0.27
MIX-2	153.7 ± 0.38	155.7 ± 0.9
MIX-3	85.27 ± 0.33	86.43 ± 0.49

Figure 4.5 Comparison between thiocyanate concentration obtained with the calibration plot and with the isotopic dilution for each sample



Thiocyanate values found in each saliva samples are in agreement with the data available in literature. The concentration of this anion in human saliva is between 23.8 and 380 µg/mL for non-smoker subjects while, for smokers, it is between 69.6 and 707 µg/mL[54].

From the *t*-test results obtained, we conclude that the concentration values found by the two methods are different, thus the difference between them is statistically significant. Maybe, this is because the labeled internal standard $KS^{13}CN$ use for the analysis is not a certified material and because the 1% of the standard is compose of unlabeled thiocyanate.

4.2.5 Analytical figures of merit

Once we found the thiocyanate concentration in our samples, we estimated the analytical figures of merit: intra-day recovery and intra-day repeatability.

- ***Intra-day recovery***

  It was calculated as the ratio between the ng of thiocyanate added in a saliva sample (spike) and the difference between the ng of thiocyanate found and the ng present in the sample between the spike.

- ***Intra-day repeatability***

  Intra-day repeatability was calculated as the RDS% of the concentration of thiocyanate found in a saliva sample as such and in the same saliva sample with different spike of a thiocyanate standard solution

Table 4.5 summarizes the intra-day recovery and the intra-day repeatability for a pooled saliva samples

Table 4.5 Intra-day recovery and intra-day repeatability for a polled saliva samples

	Intra-day recovery	***Intra-day repeatability*** [(a)]
sample	-	2.14%
sample + spike	97.8-98.9%	1.14%

[(a)] Expressed as RSD%, n = 3 samples

As show in *Table 4.5* the intra-day recovery was high and the RDS% of our method is low, confirming a good intra-day repeatability.

Chapter 5

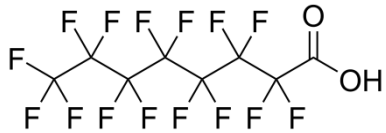
Other derivatization reactions: preliminary results

5.1 Detection of PFOA with $Et_3O^+BF_4^-$

5.1.1 Perfluorooctanoic acid

PFOA (Perfluorooctanoic acid, *Figure 5.1*) is one of the widely used perfluorinated compound. It has been detected in various environmental media and has polluted aquatic life and systems[87].

Figure 5.1 Perfluorooctanoic acid structure



Perfluoroalkyl compounds, such as perfluorooctanoic acid, because of their resistance to hydrolysis, photolysis, biodegradation, and metabolism, are pervasive environmental contaminants used in fire retardants and manufacture of consumer products as

stain/water/grease repellents in carpets and clothing or in non-stick surfaces for cooking utensils since the 1950s. PFOA is extremely stable in the environment, with long half lives in the human body of 2.3–3.5 years[88]. Concern has been raised over chronic exposure effects to human health, especially in relation to cholesterol metabolism. The properties of PFOA, such as its bioaccumulation, slow degradation, and relatively long half-life in organisms, make it worth to be checked, in particular in fatty matter, due to its lipophilicity. At certain doses, this substance causes a variety of adverse biological effects, including liver and body weight reduction, alveolar wall thickening, mitochondrial damage, gene induction, increases in larval mortality, and increased susceptibility to diseases[87]. There are a lot of recent works about the determination of the PFOA in some different animals such as mice[89-91], fish[92], birds[93] and in humans[94]. Liquid–liquid extraction (LLE) and SPE are two routine extraction methods[95]. In the last years the techniques employed for the analysis of the perfluoroalkyl compounds are liquid chromatography–tandem mass spectrometry[87] and liquid chromatography-ion trap mass spectrometry[96]. Our method, once optimized, was applied for the detection of the PFOA in order to estimate if triethyloxonium salt is able to derivatize this compound.

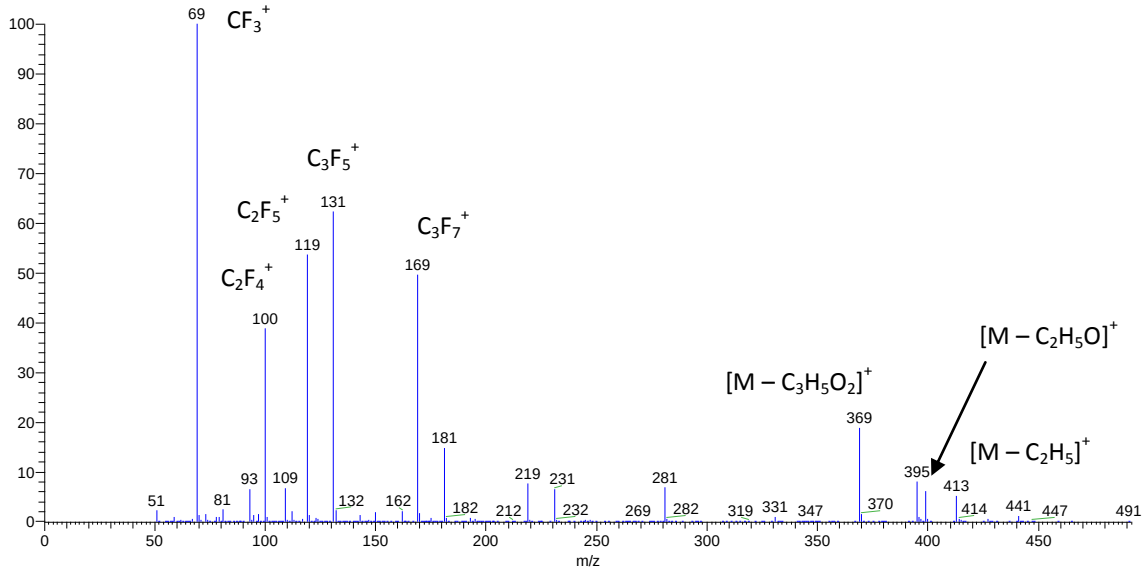
5.1.2 Derivatization of the PFOA by triethyloxonium salt

Detection of this compound is interesting because of its wide diffusion in various environmental media. We prepared a 100 µg/mL working solution of PFOA, 0.02M in NaOH. To facilitate the dissolution, the solution was sonicated for 15 minutes. We prepared a vial for the HS/GC-MS analysis, adding 2 mL of the PFOA solution, 10 µL of NaOH, 100 µL of the solution of triethyloxonium tetrafluoroborate salt and water, until a final volume of 5 mL. The method employed for the preliminary analysis of the sample was *METHOD 1* (*Section 2.2.3*) but, this time, the chromatogram was acquired only in *Total Ion Current* (TIC) mode, in order to identify our compound and to know its retention time. After the addition of the triethyloxonium tetrafluoroborate salt, we waited three hours before proceeding with the GC-MS analysis.

5.1.3 Analytical results

The mass spectrum of the chromatographic peak is in agreement with the proposed structure and evidences the presence of the ethylated species in the headspace (*Figure 5.2*).

Figure 5.2 Mass spectrum of the ethyl perfluorooctanoate



Thus we employed the *METHOD 3* (*Section 2.2.3*) for the analysis of this compound, in order to build its calibration plot. This time, the chromatograms were acquired both in *Total Ion Current* (TIC) and *Single Ion Monitoring* (SIM) mode.

5.1.4 Calibration plot

Six working solutions were employed for the calibration plot: 10 ng/mL, 50 ng/mL, 100 ng/mL, 500 ng/mL and 1000 ng/mL. In addition to the standard solutions, in each vial 10 µL of NaOH, 100 µL of the solution of $Et_3O^+BF_4^-$ and water were added (the same procedure adopted for the thiocyanate anion) until a final volume of 6 mL. We also prepared three blanks, in order to exclude the presence of the acid in the water. Since the addition to the salt and the HS/GC-MS analysis we waited for three hours. The calibration

plot obtained is reported below in *Figure 5.3* and the experimental parameter are reported in *Table 5.1*

Figure 5.3 Calibration plot of PFOA

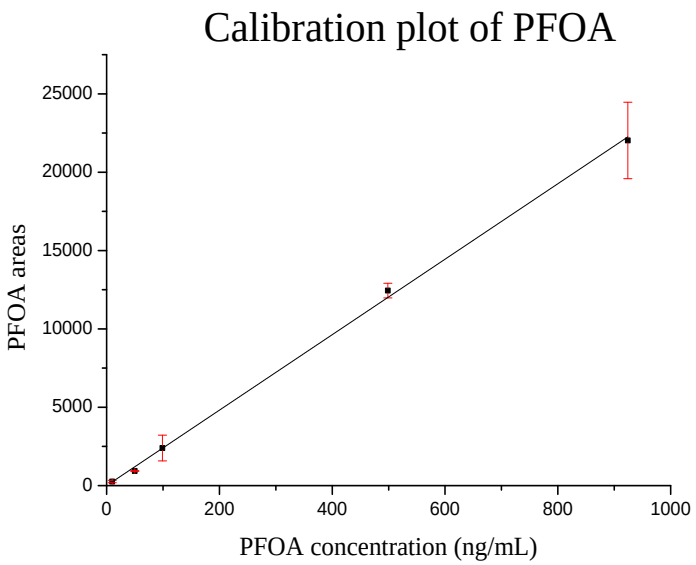


Table 5.1 Experimental parameters of the calibration plot of PFOA

Slope	24.1
R^2	0.9998
Linearity	10-1000 ng/mL

As shown in *Figure 5.3* and *Table 5.1* the calibration plot of this compound proves a good linearity and a good R^2 value. Further studies are necessary in order to optimize the reaction yields and to understand if parameters such as pH, reaction time, incubating temperature and salting out effect, could be modulated to obtain a better generation efficiency.

5.2 Detection of organic acids with $Et_3O^+BF_4^-$

5.2.1 Organic acids[54]

Our method was also applied to the detection of organic acids, such as lactic, butyric, propionic and acetic acid, in order to estimate if triethyloxonium salt is able to derivatize these compounds. The main classes of techniques employed to determine these organic compounds are: colorimetric, enzymatic, biosensor and chromatographic methods.

- ***Lactic acid***

Lactic acid (2-hydroxypropanoic acid) is one of the top 30 potential building-block chemicals obtained from biomass, and it has various applications in pharmaceutical technologies, cosmetic preparation, agriculture, detergents and in the food industry[97]. Food and food-related applications account for approximately 80% of total lactic acid production, whereas non-food-related industrial applications account for only 15%[98]. It is also product of anaerobic glicolyisis resulting from pyruvate by the enzyme lactate dehydrogenase (LDH). Lactic acid can be found in blood and biological fluids of human beings and animals. A healthy adult man normally produces about 120 g of lactic acid a day. Among these, 33% is produced by tissues characterized by an exclusively anaerobic metabolism (retina and blood red cells). The remaining 67% is produced by other tissues (most of all muscles) on the basis of the actual oxygen availability.

- ***Butyric acid***

Butyric acid (Butanoic acid) is a colorless organic acid with an unpleasant smell. Its and its derivatives are utilized in various industrial products, including plastics, fibers, food additives, and pharmaceuticals[99-100]. This acid is mainly produced by chemical and fermentation processes[101-102]. Butyric acid is a promising chemical that may hold the potential for future energy needs as it can be converted to butanol through biological transformation[103].

- ***Propionic acid***

Propionic acid (Propanoic acid) has many industrial applications as a specialty chemical and its calcium, potassium and sodium salts are widely used as food and feed preservatives[104]. Currently, propionic acid is produced almost exclusively via petrochemical processes[105]. To lower the product costs, recent efforts have focused on using industrial wastes or byproducts as low-cost renewable feedstocks for propionic acid fermentation[106]. Production of propionic acid from fermentation of glycerol is a promising development target since glycerol is obtained in large quantities as a by-product from biodiesel production[107].

- ***Acetic acid***

Acetic acid is found naturally throughout the nature or can be produced in large scale mainly by biotechnological methods. One of the most important applications of this organic acid is in the food industry[108]. It is generally added in different foods as acidity regulator, antimicrobial or flavouring agent to provide sour or fruity taste or flavor. Besides, it may have some effects on the functional properties of other food constituents.

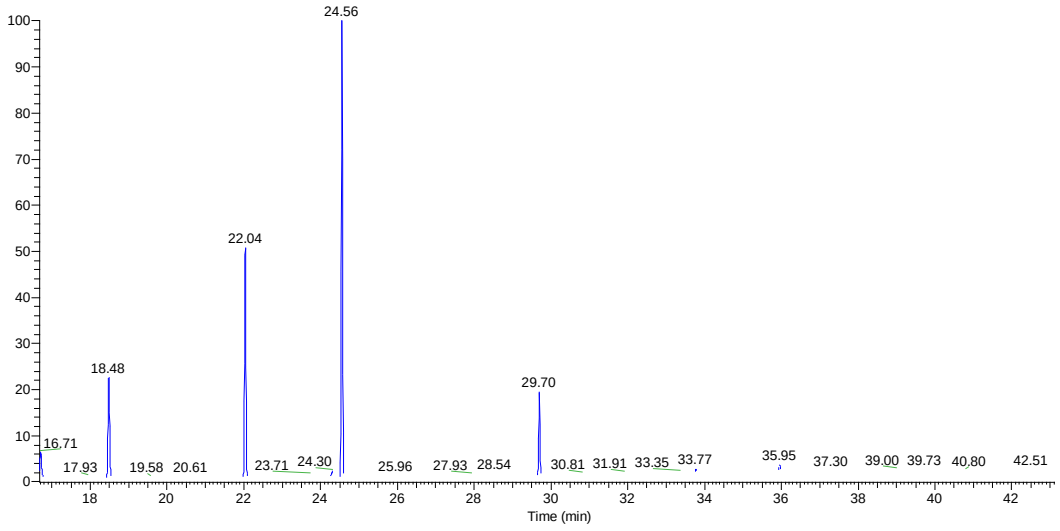
5.2.2 Derivatization of the acetic, propionic, butyric and lactic acids by triethyloxonium salt

For the analysis of these compounds we prepared a 100 µg/mL working solution of lactate, propionate, butyrate and acetate. We prepared a vial for the HS/GC-MS analysis, adding 2 mL of this working solution, 50 µL of NaOH (10M), 100 µL of the solution of $Et_3O^+BF_4^-$, 50 µL of the internal standard *i*-propOH D8 and water, until a final volume of 5 mL. The method employed for the preliminary analysis of the sample was *METHOD 1* (*Section 2.2.3*) but, this time, the chromatogram was acquired only in *Total Ion Current* (TIC) mode, in order to identify our compounds and to know their retention time. After the addition of the triethyloxonium tetrafluoroborate salt, we waited three hours before proceeding with the GC-MS analysis.

5.2.3 Analytical results

The mass spectra of the chromatogram obtained from the analyses show that the triethyloxonium tetrafluoroborate salt is able to derivatize the acetic, propionic and butyric acid, giving the ethyl acetate, ethyl propionate and ethyl butyrate. *Figure 5.4* shows the chromatogram obtained from the analysis of the working solution.

Figure 5.4 Chromatogram of acetate (t_R = 18.48'), propionate (t_R = 22.04') and butyrate (t_R = 24.56') mixture



We observed that acetate, propionate and butyrate peaks are well separated. *Figures 5.5(a)(b)(c)* show the comparison between the mass spectra obtained and the mass spectra present in the library.

Figure 5.5(a) Comparison between the mass spectra of the ethyl acetate

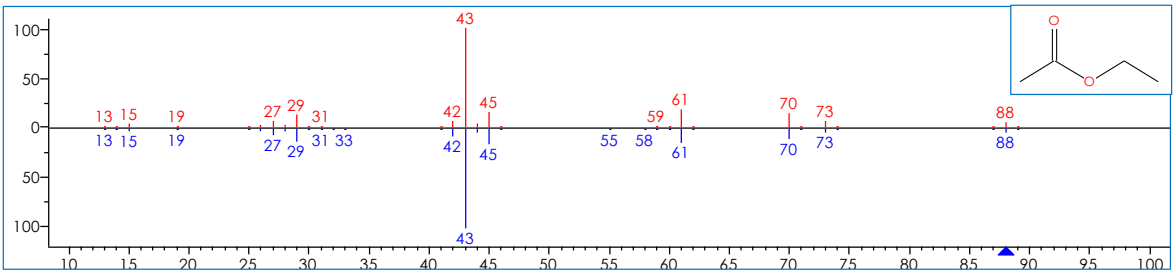


Figure 5.5(b) Comparison between the mass spectra of the ethyl propionate

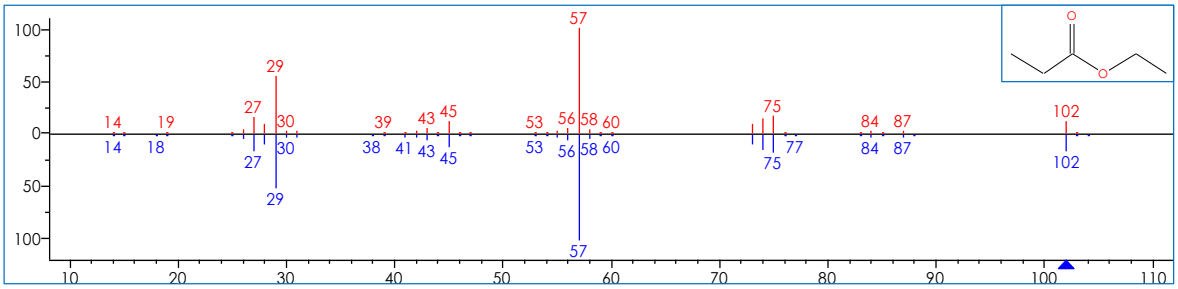
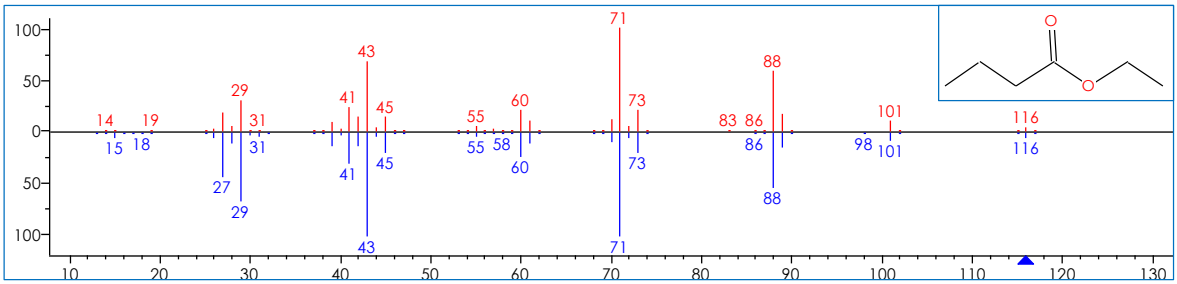


Figure 5.5(c) Comparison between the mass spectra of the ethyl butyrate



In the chromatogram obtained the ethyl lactate peak is not present. Even after other attempts, the lack of ethyl lactate in headspace could be explained suggesting that this product is formed by ethylation of lactate but it immediately undergoes hydrolysis, maybe promoted by the acidic environment. In fact the hydrolysis is a reversible reaction and the conversion is controlled by thermodynamic equilibrium[97]. Otherwise time needed for the alkylation could be different from the one employed in our procedure. Finally, the ethylation itself could be hindered by the actual form of lactate ion in solution. More studies are required to clarify this behaviour.

5.3 Detection of aromatic compounds with $Et_3O^+BF_4^-$

5.3.1 Aromatic compounds

Pentachlorophenol (PCP) is an organochlorine compound, widely present in the environment[109]. It has been used in the past as a biocide in wood preservation industries and other pesticide applications. Albeit its usage is banned or restricted in many countries, its historically worldwide usage and relative persistence make PCP a ubiquitous environmental pollutant. PCP had been detected in human body fluids (plasma or urine) of non-occupationally and occupationally exposed individuals[110]. Another organochlorine compound is the 2,4,6-trichlorophenol (2,4,6-TCP). This is a common chemical intermediate and a by-product of water chlorination and combustion processes, and it is a priority pollutant of the aquatic environment in many countries[111]. It has been used since the early 1930s, and it is the most frequently detected chlorophenol in surface waters. Due to its toxicity to aquatic life, resistance to degradation, and potential for bioaccumulation, 2,4,6-TCP is a priority pollutant of aquatic environments in the world. Phenol is invariably present in the effluents from industries engaged in the manufacture of a variety of chemicals such as plastics, dyes, and in plants. The presence of this compound in water also produces foul smelling, during chlorination treatment of water for domestic supply. Phenolic compounds are usually present in wastewater generated from the paint, solvent, petrochemical, coal conversion, pharmaceutical, plastic, iron-steel and paper and pulp industries. Several methods are currently used for the removal of phenol and its derivatives from wastewater: microbial degradation, chemical oxidation, incineration, solvent extraction and irradiation[112-114].

5.3.2 Derivatization of phenol and organochlorine compounds by triethyloxonium salt

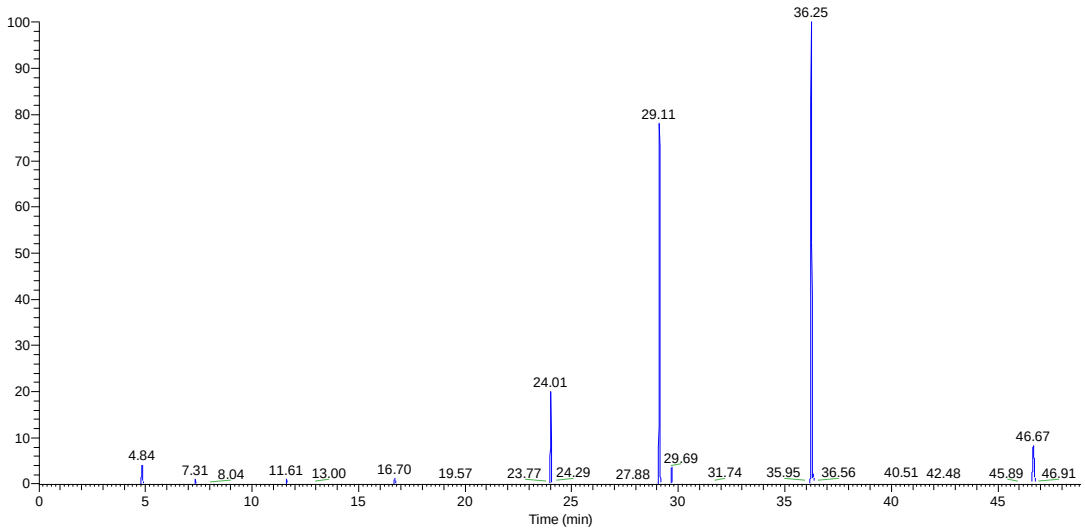
Detection of organic compounds as phenol, 2,4,6-trichlorophenol and pentachlorophenol has been mentioned in *Section 5.1.3*. Derivatization of these compounds with triethyloxonium salt was investigated. For the analysis of these compounds we prepared a 100 µg/mL working solution of phenol, 2,4,6-trichlorophenol and pentachlorophenol 0.02M in NaOH. To facilitate the dissolution, it was necessary to leave this solution in the sonicator for 15 minutes. We prepared a vial for the HS/GC-MS analysis, adding 2 mL of this solution, 10 µL of NaOH, 100 µL of the solution of $Et_3O^+BF_4^-$ (the same procedure adopted for the thiocyanate anion) and water, until a final volume of 6 mL. The method employed for the preliminary analysis of the sample was *METHOD 1* (*Section 2.2.3*). The chromatogram was acquired only in *Total Ion Current* (TIC) mode, in order to identify our compounds and to know their retention time. After the addition of the triethyloxonium tetrafluoroborate salt, we waited three hours before proceeding with the GC-MS analysis.

5.3.3 Analytical results

The chromatogram obtained from the analysis shows that the triethyloxonium tetrafluoroborate salt is able to derivatize phenol, 2,4,6-trichlorophenol and pentachlorophenol, giving ethoxybenzene, ethoxy-2,4,6-trichlorobenzene and ethoxypentachlorobenzene. *Figure 5.6* shows the chromatogram obtained from the analysis of the working solution mentioned above.

The chromatogram of the ethoxybenzene, (t_R = 29.11'), ethoxy-2,4,6-trichlorobenzene (t_R = 36.25') and ethoxypentachlorobenzene mixture (t_R = 46.67') show a good separation of the peaks.

Figure 5.6 Chromatogram of ethoxybenzene (t_R = 29.11'), ethoxy-2,4,6-trichlorobenzene (t_R = 36.25') and ethoxypentachlorobenzene (t_R = 46.67') mixture



Below, *Figures 5.7 (a)(b)(c)* show the mass spectrum of these ethylated compounds compared with the one present in the library.

Figure 5.7(a) Comparison between the mass spectra of the ethoxybenzene

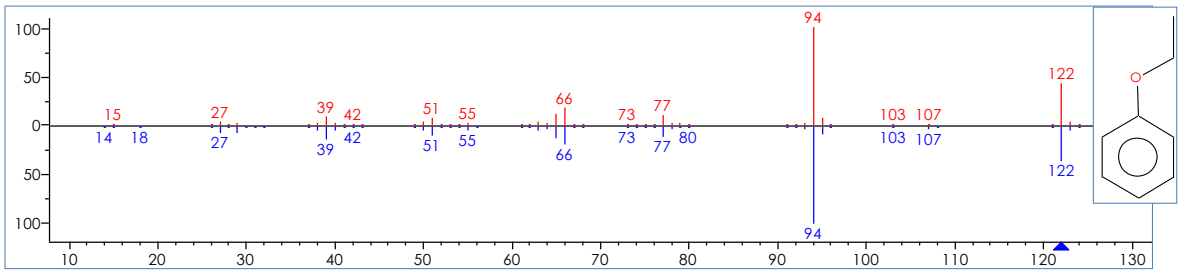


Figure 5.7(b) Comparison between the mass spectra of the ethoxy-2,4,6-trichlorobenzene

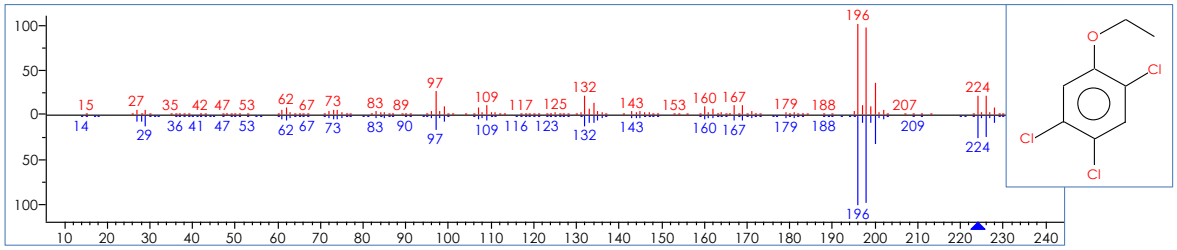
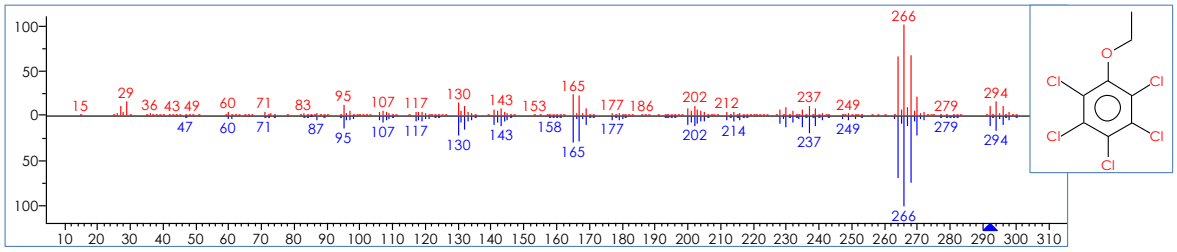


Figure 5.7(c) Comparison between the mass spectra of the ethoxypentachlorobenzene



From the chromatograms obtained, we can assume that triethyloxonium tetrafluoroborate salt is able to derivatize these organic compounds. Further studies will be necessary in order to optimize the reaction conditions and maximize the alkylation reaction yields.

Conclusions and further studies

In this work an analytical method for derivatization of the anions SCN^-, CN^-, S^{2-}, I^- and Br^- has been optimized by generation of alkylated volatile derivatives with triethyloxonium tetrafluoroborate salt in aqueous phase. The volatile derivatives, EtSCN, EtCN, Et_2S, EtI and EtBr, have been successfully analyzed and determined by Headspace/Gas Chromatography-Mass Spectrometry (HS/GC-MS). The optimization of some experimental parameters plays an important role in determining the yield of the derivatization reactions. The pH of sample solution controls both the rate of disappearance of the reagent and the structure of the substrate. Weak acids as CN^- and S^{2-} are much less reactive towards alkylation in the protonated form, as HCN and H_2S are volatile and they tend to leave the reaction solution. Considering that the hydrolysis of triethyloxonium decrease the pH, it is therefore mandatory to buffer the pH with non reactive buffers. For this purpose two different bases, NaOH and NH_3, were tested. In agreement with the expected behaviour, the anions CN^- and S^{2-} give better alkylation yields in a basic environment while the anions I^-, Br^- and SCN^- prefer an acidic environment. NaOH was preferred because of NH_3 alkylation, which is not interfering on the yield of alkylation process, but it generates volatile ethylamines which are detected during GC-MS analysis

The reaction time is also an important parameter involved in alkylation yields of the anions. We decided to wait three hours between the addition of the salt and the HS/GC-MS

analysis. Waiting for longer periods is useless and can lead to analyte losses through different chemical and physical processes.

In spite of the organic nature of the alkylated derivatives some of them show high affinity for the aqueous phase and, in addition, some of them are not so volatile. The dedicated studies regarding the transfer of the alkylated analytes from the aqueous phase to the headspace have shown that this is a very critical step. In order to improve the phase transfer efficiency the addition of an increasing amount of an inorganic salt and the increase of the incubation temperature (from 30 up to 90°C) proved that combination of these two factors can improve the overall yield of alkyl derivatives by one up to several orders of magnitude.

This method, once optimized, has been successfully applied to the analysis of a biological matrix, saliva, for the determination of SCN^-. The concentration of this anion is closely related to the presence of specific pathologies in human body, such as ataxic neuropathy, tropical diabetes, endemic goiter and cretinism. In particular, the amount of thiocyanate detected in saliva from different volunteers could be related with their smoking habit, in agreement with data reported in literature.

Further perspectives for the analytical applications of this alkylation method have been evaluated on a series of organic compounds as phenol, 2,4,6-trichlorophenol, pentachlorophenol, perfluorooctanoic acid and acetic, propionic and butyric acids. Qualitative analyses have shown that triethyloxonium tetrafluoroborate salt is able to derivatize these organic compounds even if further studies are necessary in order to evaluate their suitability for quantitative analysis.

Appendix A

Chromatograms and mass spectra

Figure A Extracted-ion of EtO-NO (t_R = 7.49') and EtBr (t_R = 12.83'): chromatogram peak and mass spectrum

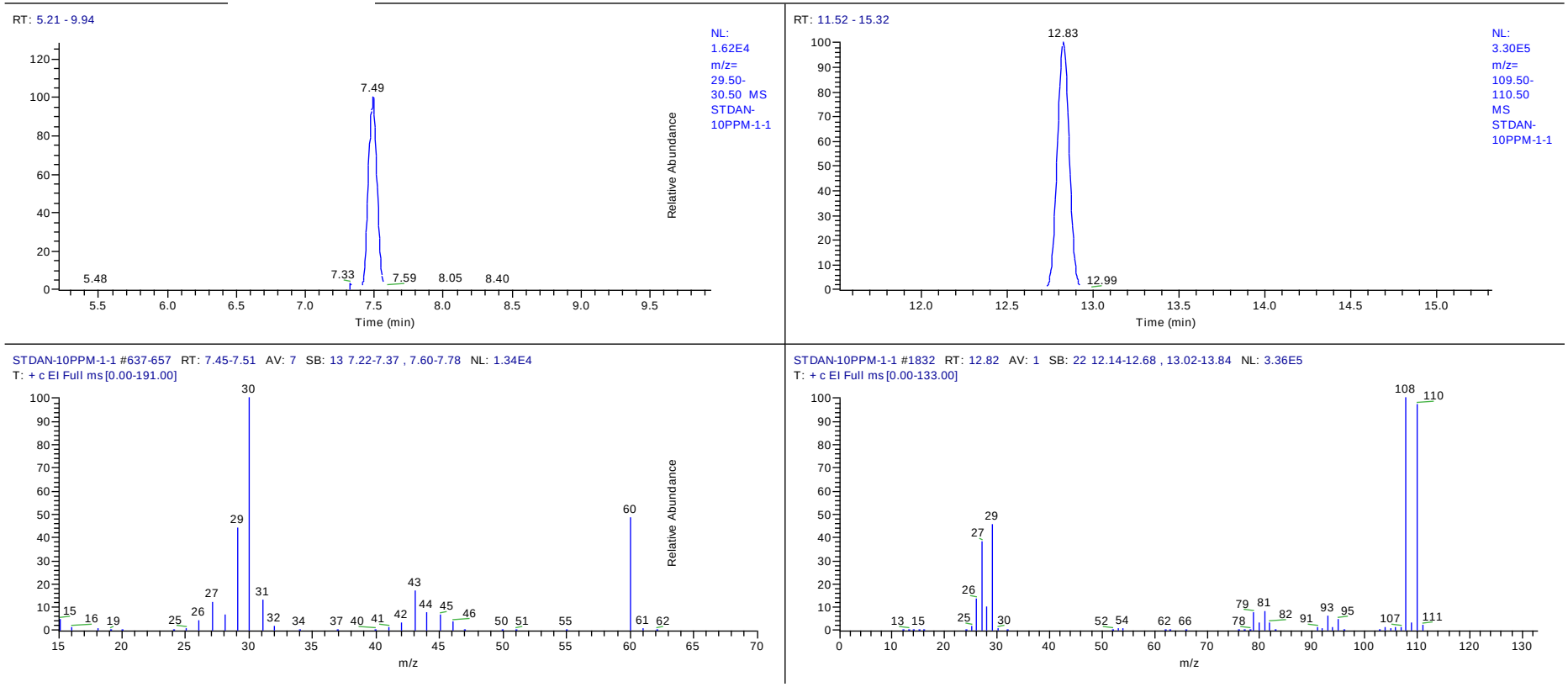


Figure B Extracted-ion of EtI (t_R = 18.17') and EtCN (t_R = 18.44'): chromatogram peak and mass spectrum

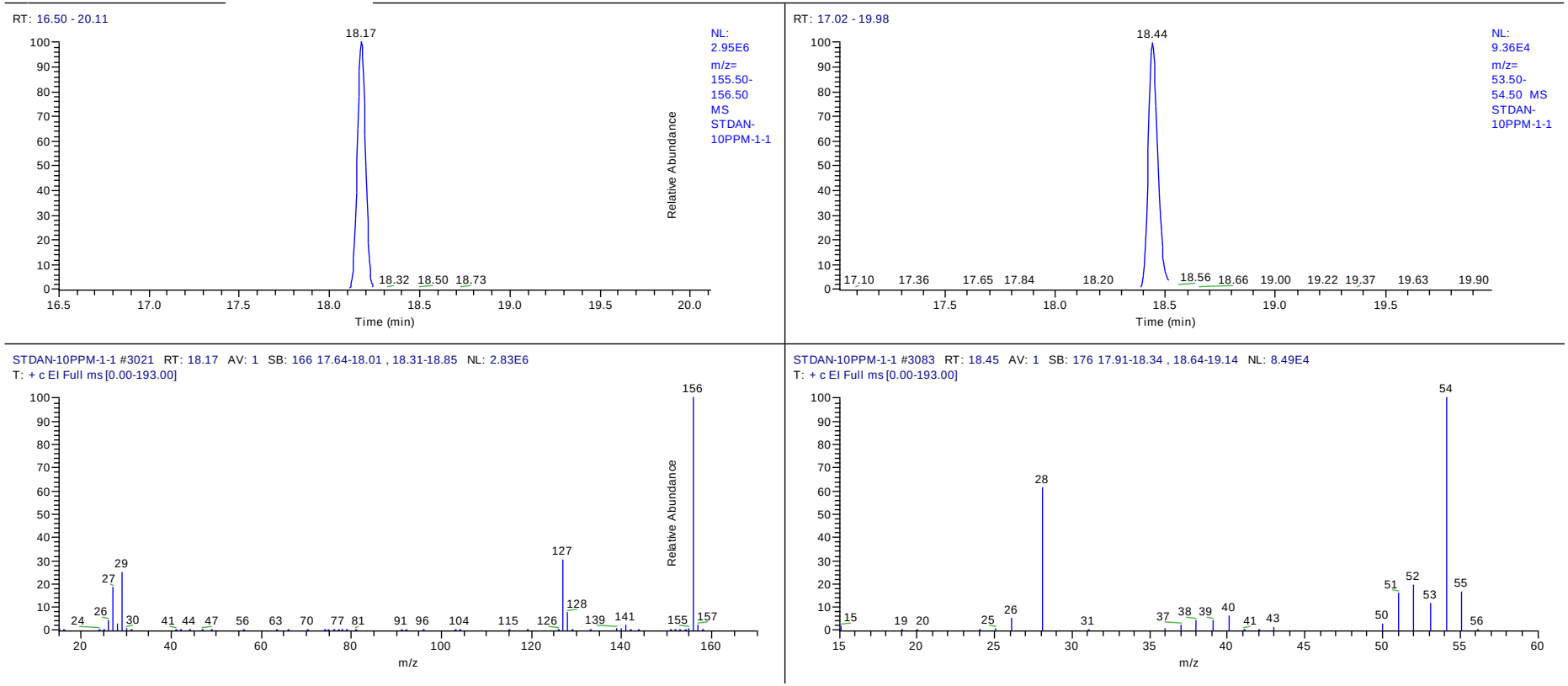


Figure C Extracted-ion of EtO-NO_2 (t_R = 19.58') and EtSEt (t_R = 21.52'): chromatogram peak and mass spectrum

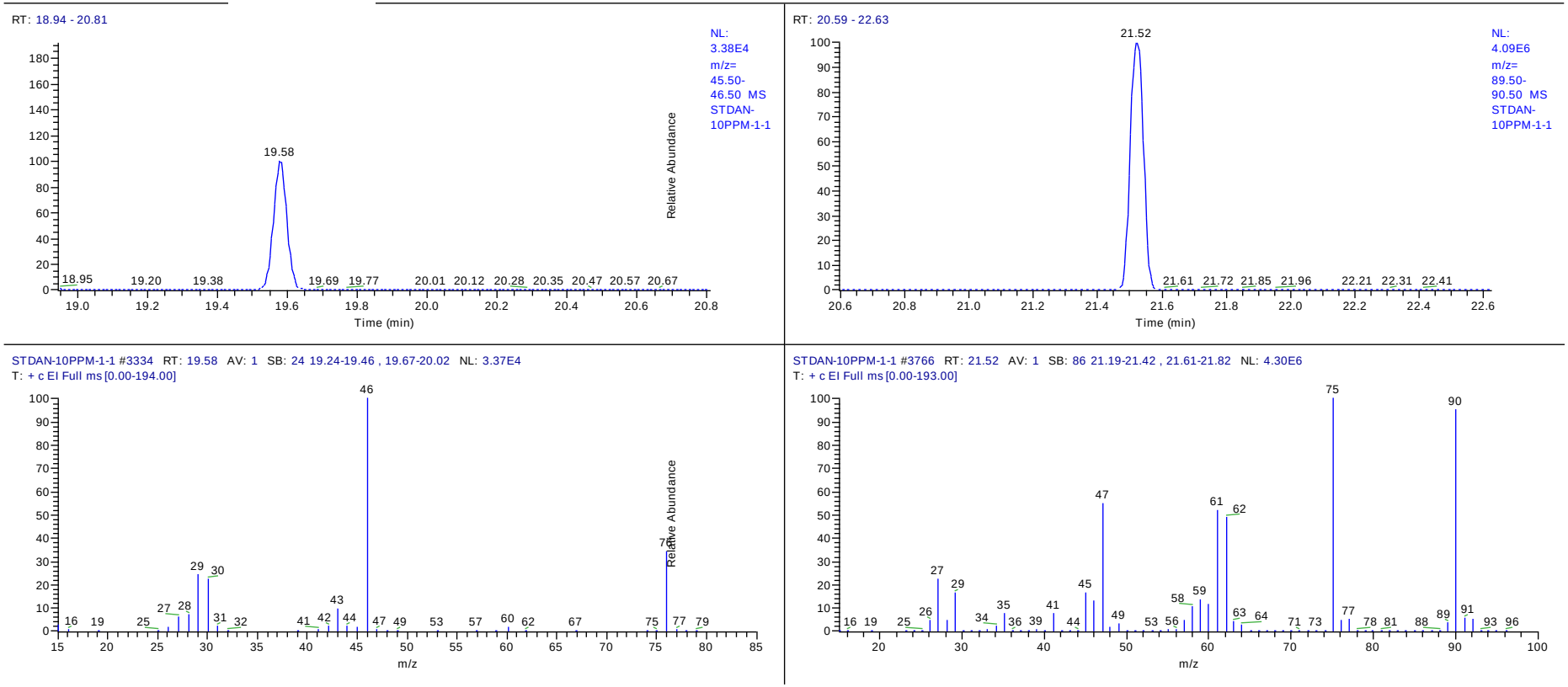


Figure D Extracted-ion of EtS-SEt (t_R = 14.57'): chromatogram peak and mass spectrum

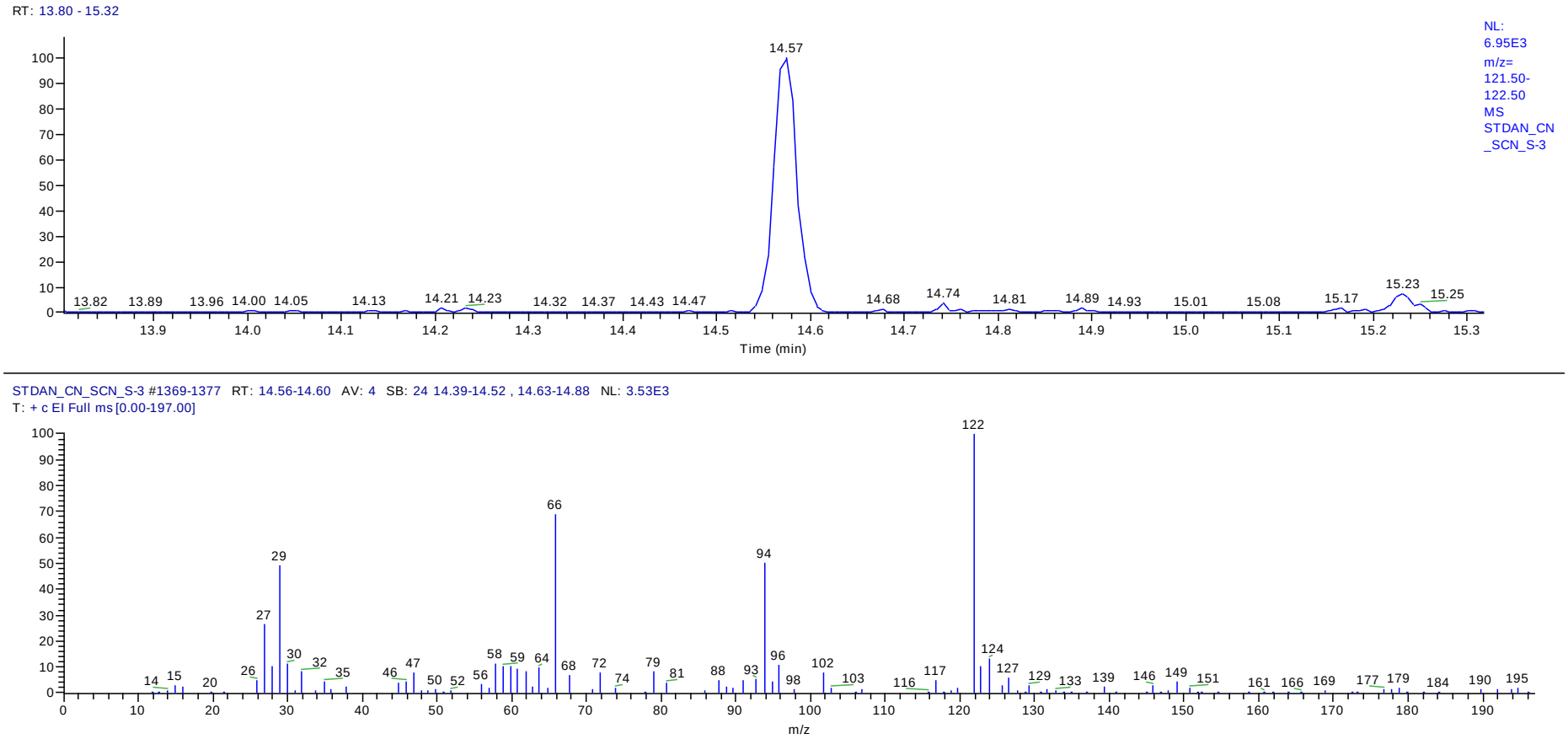


Figure E Extracted-ion of EtSCN (t_R = 25.48'): chromatogram peak and mass spectrum

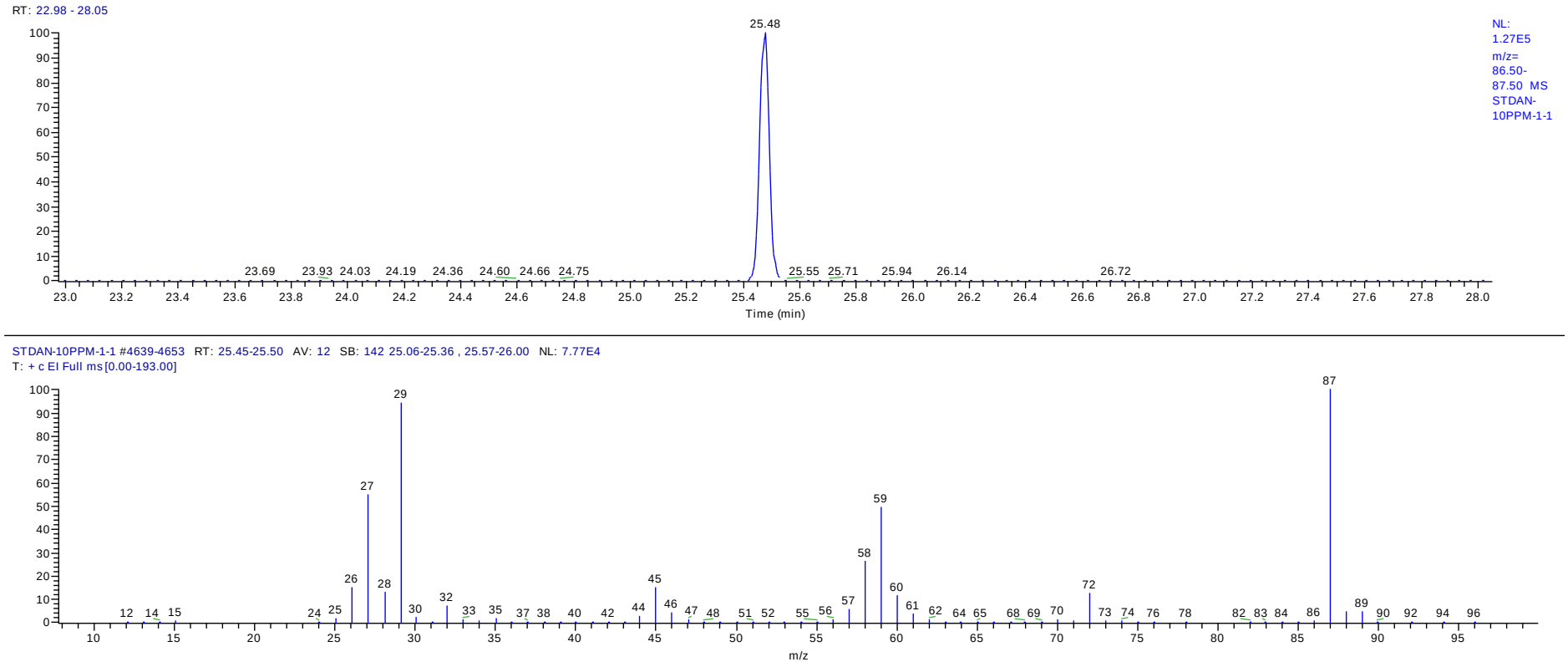


Figure F Extracted-ion of Perfluorooctanoic acid (t_R = 10.13') and ethoxybenzene (t_R = 12.82'): chromatogram peak and mass spectrum

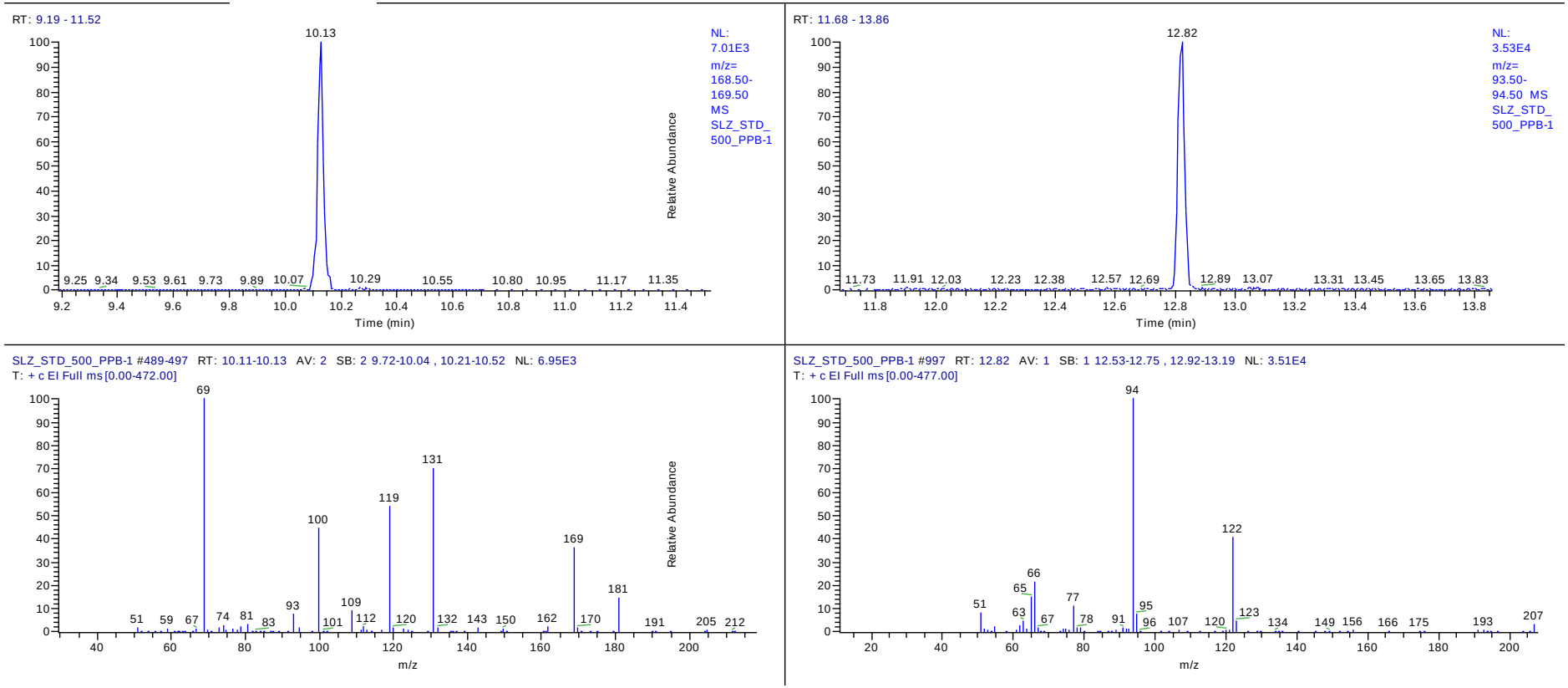


Figure G Extracted-ion 3of ethyl acetate (t_R = 18.18') and ethyl propionate (t_R = 22.04'): chromatogram peak and mass spectrum

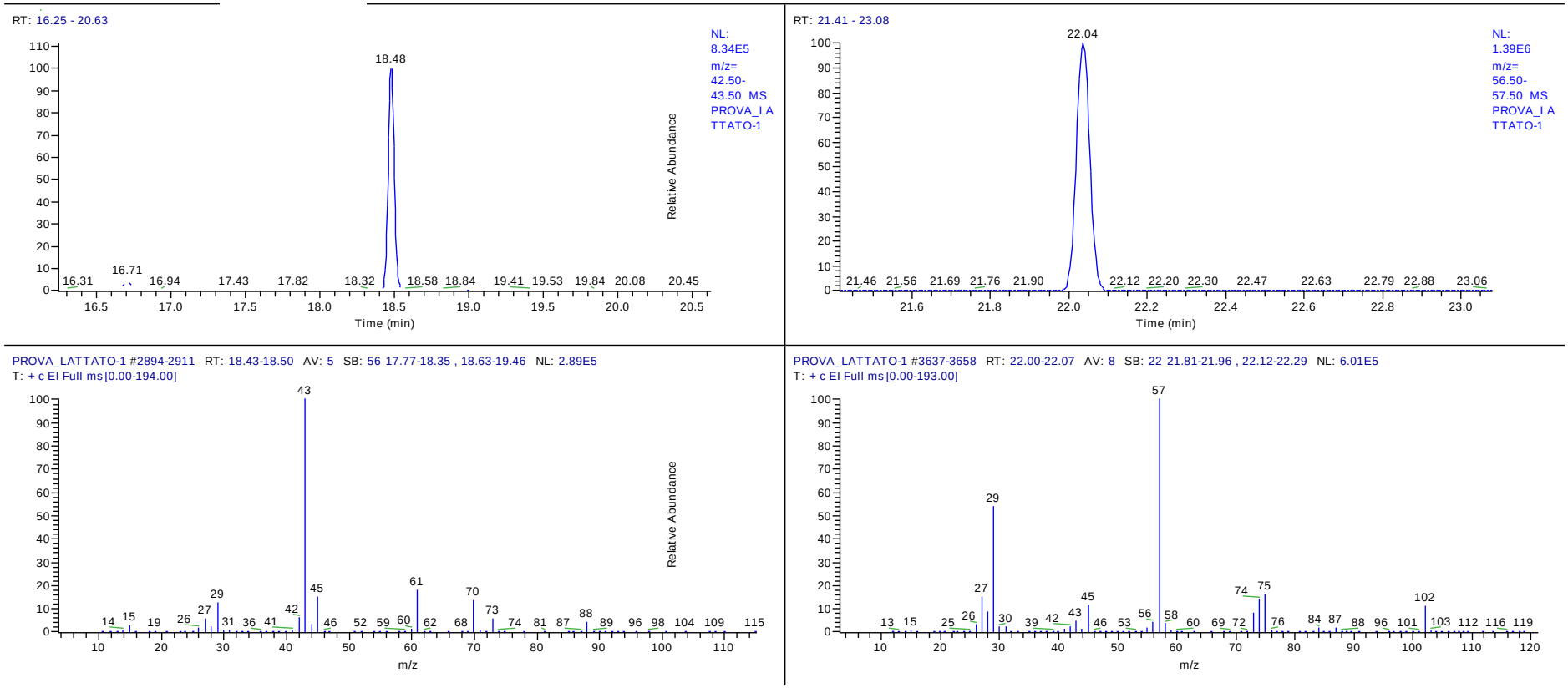


Figure H Extracted-ion of ethyl butyrate (t_R = 24.56'): chromatogram peak and mass spectrum

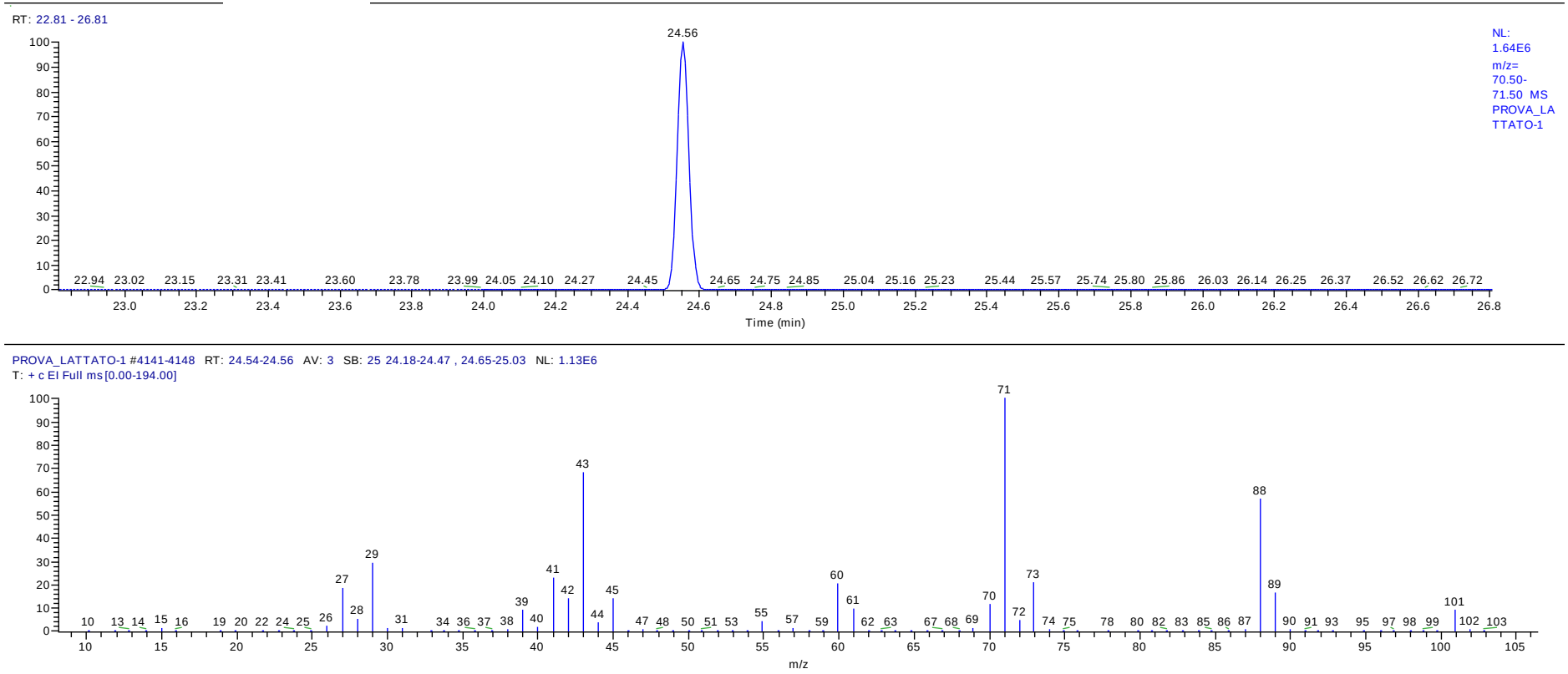


Figure I Extracted-ion of ethoxybenzene (t_R = 29.11') and ethoxy-2,4,6-trichlorobenzene: chromatogram peak and mass spectrum

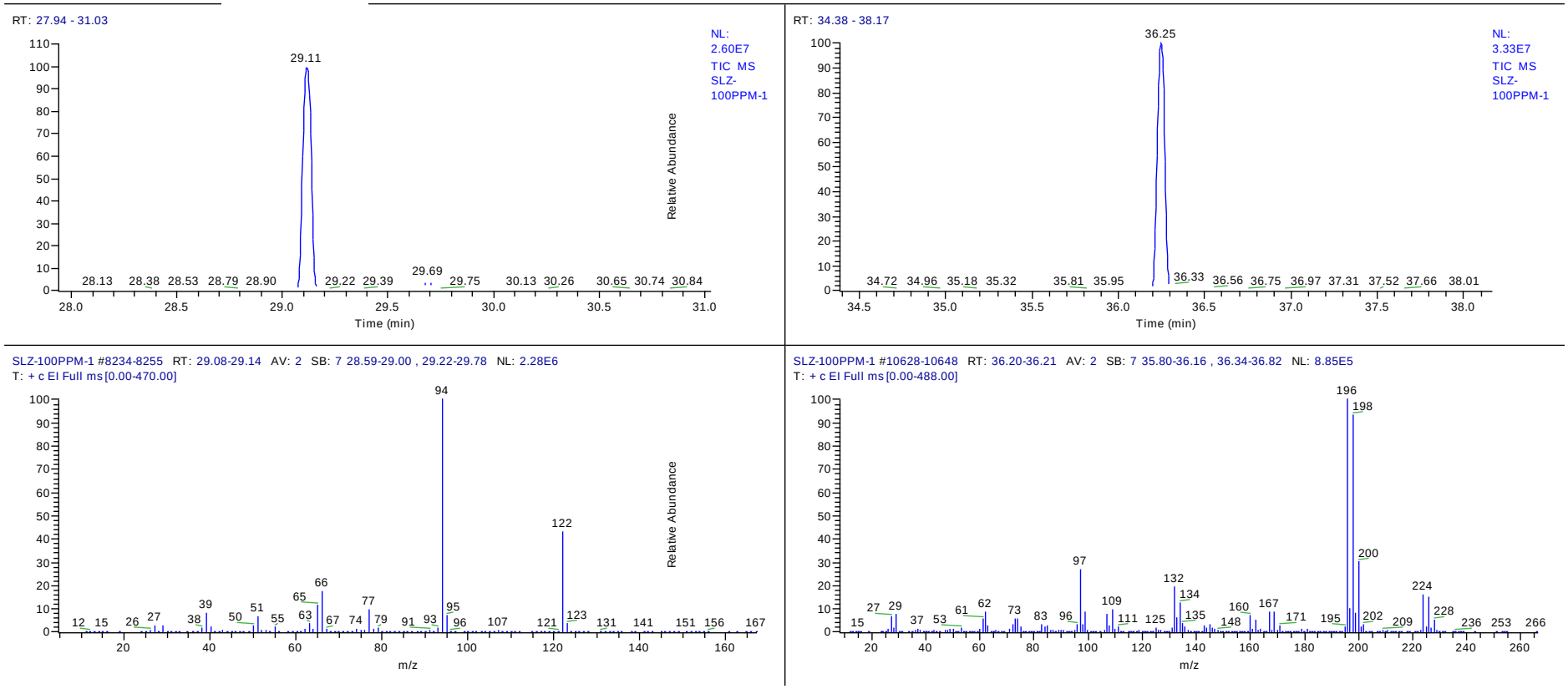
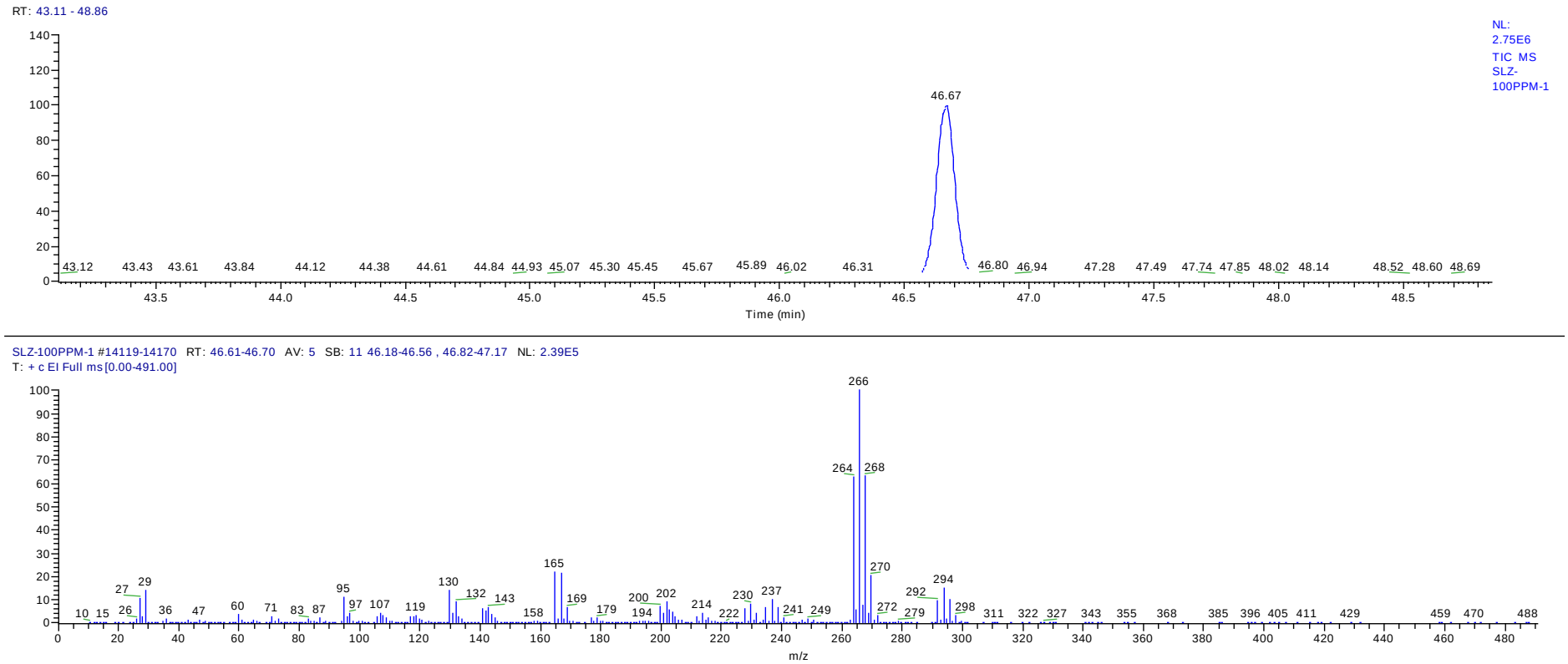


Figure L Extracted-ion of ethoxypentachlorobenzene (t_R = 46.67'): chromatogram peak and mass spectrum



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