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Abstract

3-Nitrotyrosine and the 3-nitrotyrosine/tyrosine ratio has been acknowledged as a biomarker of nitro-oxidative stress and its elevated level in biological fluids is associated with a wide range of clinical conditions. Despite the importance of 3-nitrotyrosine as a marker of oxidative stress the optimal method for high throughput analysis of clinical samples has yet to be developed. Due to its nanomolar concentration, quantitation of 3-nitrotyrosine is quite challenging. Nowadays, there are many analytical assays available for determination of 3-nitrotyrosine, but the obtained values vary widely and their reliability are still controversial. The purpose of this research was to develop an optimal analytical procedure for simultaneous determination of plasma free 3-nitrotyrosine and tyrosine by gas chromatography (GC) coupled to single stage mass spectrometry (MS) with negative chemical ionization (NCI). The procedure would comprise amino acid isolation by solid phase extraction (SPE), chemical derivatization and gas chromatography/mass spectrometry (GC/MS) analysis. The emphasis was given to the chemical reactions with formation of fluorinated derivatives, which would provide low detection limits of 3-nitrotyrosine. Two types of extraction cartridges were used for the amino acid isolation and four strategies for the chemical derivatization were evaluated in the present paper. The cartridges with enhanced polar and aromatic selectivity showed higher recovery of the amino acids compared to the cation exchangers. The successful derivatization based on ethyl chloroformate (EtCF)/heptafluorobutanol (HFBOH) was extensively studied and attempts were made to optimize it. The one-step derivatization with pentafluorobenzyl bromide (PFBBr) did not yield any stable derivatives. Although easy to perform, derivatization with EtCF/HFBOH or PFBBr did not provide sufficiently low detection limits. Acceptable limits of detection (0.11µg/L) and quantitation (0.13µg/L) were achieved only during the four-step derivatization of 3-nitrotyrosine calibration standard with heptafluorobutyric anhydride/trimethylsilyldiazomethane. In subsequent experiments with human plasma these values proved to be insufficient because of a negative plasma matrix effect on the reaction yield.

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Abbreviations

d_3-NY	3-nitro-*L*-tyrosine-d_3
d_4-HBA	4-hydroxy[2.3.5.6-$^2H_4$]benzoic acid
d_4-Y	*L*-4-hydroxy-phenyl-d_4-alanine or tyrosine-d_4
DIPEA	diisopropylethylamine
EI	electron impact ionization
EtCF	ethyl chloroformate
GC	gas chromatography
HBA	4-hydroxy($^2H_4$)benzoic acid
HCl	hydrochloric acid
HFBA	heptafluorobutyric anhydride
HFBOH	heptafluorobutanol
HPLC	high performance liquid chromatography
LC	liquid chromatography
LLOD	lower limit of detection
LLOQ	lower limit of quantification
MC	mass chromatogram
MS	mass spectrometry
MW	molecular weight
m/z	mass-to-charge ratio of an ion
NCI	negative chemical ionization
NY	3-nitro-*L*-tyrosine
PFB	pentafluorobenzyl group
PFBBr	pentafluorobenzyl bromide
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT	room temperature
SD	standard deviation
SIM	selected ion monitoring
S/N ratio	signal to noise ratio
SPE	solid phase extraction
TFA	trifluoroacetic acid
TIC	total-ion chromatogram
TMSD	trimethylsilyl diazomethane
t_R	retention time
Y	*L*-Tyrosine

Definitions

Blank – a specimen without analyte, yet containing the same solvent in which the analyte is usually dissolved (Skoog, Holler et al. 2007, p. 13).

Derivatization – converting an analyte to its volatile and structurally stable derivative in order to enable chromatographic separations (Snyder and Kirkland et al. 1979, p. 2).

Gas chromatography (GC) - a separation technique in which a volatile sample is carried by an inert gas (mobile phase) into a narrow tube (GC column) that contains a liquid stationary phase through which the mobile phase is forced under pressure. The column is located in an oven which maintains a high temperature. Compounds in the sample separate efficiently due to their different partition between gas and liquid (Skoog, Holler et al. 2007; Sheehan 2009; Snyder, Kirkland et al. 2010)

GC/MS – an instrumental technique in which a gas chromatograph (GC) coupled to a mass spectrometer (MS) in order to separate, identify and quantitate complex mixtures of chemicals (Skoog, Holler et al. 2007, p. 582).

Internal standard – a substance that is added to all samples, blanks and calibration standards in an analysis to compensate for several random and systematic errors.

Isoelectric point (pI) – the pH at which an amino acid has no net charge and is electrically neutral (McKee and McKee 2009, p. 121)**.**

Mass spectrometry (MS) – an analytical technique in which an analyte forms gaseous ions during ionization, the formed ions are sorted by mass-to-charge ratio and displayed in a form of mass spectrum. Depending on the chosen ionization method, it is possible to determine a molecular mass for an intact chemical, deduce its structure after the fragmentation pattern and perform a quantitative analysis of the sample (Skoog, Holler et al. 2007, pp. 550-551).

Mass-to-charge-ratio of an ion (m/z)- the unitless ratio of ion mass number (m) to the number of fundamental charges z on the ion (Skoog, Holler et al. 2007, p. 282).

Lower limit of detection (LLOD) - the minimum concentration of mass of analyte that can be

detected at a known confidence level (Skoog, Holler et al. 2007, p. 20).

Lower limit of quantitation (LLOQ) – the lowest concentration at which quantitative measurement can be made or the concentration at which the calibration curve departs from the linearity more than 5% (Skoog, Holler et al. 2007, p. 21).

Retention time (t_R) - time taken from injection to the top of the peak for each particular analyte (Snyder and Kirkland et al. 1979, p. 21**).**

Sensitivity of an analytical method or equipment – an ability to discriminate between small differences in analyte concentration (Skoog, Holler et al. 2007, p. 19)**.**

Spiking is adding a known amount of analyte to sample aliquots (Skoog, Holler et al. 2007, p. 14).

1. Introduction

3-Nitrotyrosine (NY) is a product of nitration of the amino acid *L*-tyrosine (Y) by reactive nitrogen species (RNS) such as nitric oxide ($NO^{\bullet}$), peroxynitrite ($ONOO^{-}$), nitrogen dioxide ($NO_2^{\bullet}$), dinitrogen trioxide (N_2O_3), nitryl chloride (NO_2Cl), nitrous acid (ONOH) and nitric acid (ONO_2H) (Tsikas and Duncan 2014). Highly toxic RNS as well as reactive oxygen species (ROS) are generated in large amounts in macrophages, neutrophils and other phagocytic cells during antimicrobial and tumoricidal immune responses under the respiratory burst (Fig. 1.1). Other sources for ROS are leakages of electrons from the electron transport pathways in mitochondria and endoplasmic reticulum. The synthesis of RNS is often facilitated by O_2 and ROS; therefore, in some literature RNS are often classified also as ROS (McKee and McKee 2009, pp.330-332). ROS are an integral part of the oxidative metabolism in human cells. Besides their crucial role in protecting a human body against invading microbes and viruses (Wang, Noel et al. 2012), ROS communicate important information to the cell's genetic machinery by altering the redox status of the target proteins (McCord 2000). In living organisms, an antioxidant defence mechanism keeps ROS and RNS formation at a minimum level in order to prevent damage to healthy cells. But if this mechanism is inadequate, ROS and RNS levels increase causing oxidative stress. The main consequence of oxidative stress is the subsequent damage of biomolecules (mostly amino acids and proteins due to their high abundance in biological systems) that theoretically may contribute to the disease process (McKee and McKee 2009, pp.332-334). In plasma NY exists as a free amino acid and as a constituent amino acid within a protein backbone. In his review, Duncan (2002) cited numerous studies where elevated levels of NY were associated with inflammatory disease processes including atherosclerosis, multiple sclerosis, Alzheimer's disease, Parkinson's disease, cystic fibrosis, asthma, other lung diseases, myocardial dysfunction, stroke, trauma, organ transplant rejection, amyotrophic lateral sclerosis, inflamed human colonic epithelium, chronic hepatitis, cirrhosis experimental osteoarthritis, diabetes and a list of other disorders. Thus, NY is recognized as a stable biomarker of RNS-derived oxidants (Dalle-Donne, Rossi et al. 2006). Despite the biological importance of NY a simple, sensitive, reliable and selective method for high throughput analysis of clinical samples has yet to be developed. To date advanced techniques have been required due to the small traces of NY in biological. Such a method if developed, would help to assess the degree of nitro/oxidative stress, indicate a disease or disease progression and evaluate the effect of antioxidant therapy. Currently there are many analytical assays available for determination of NY such as immunoassay, HPLC with a variety of detection methods,

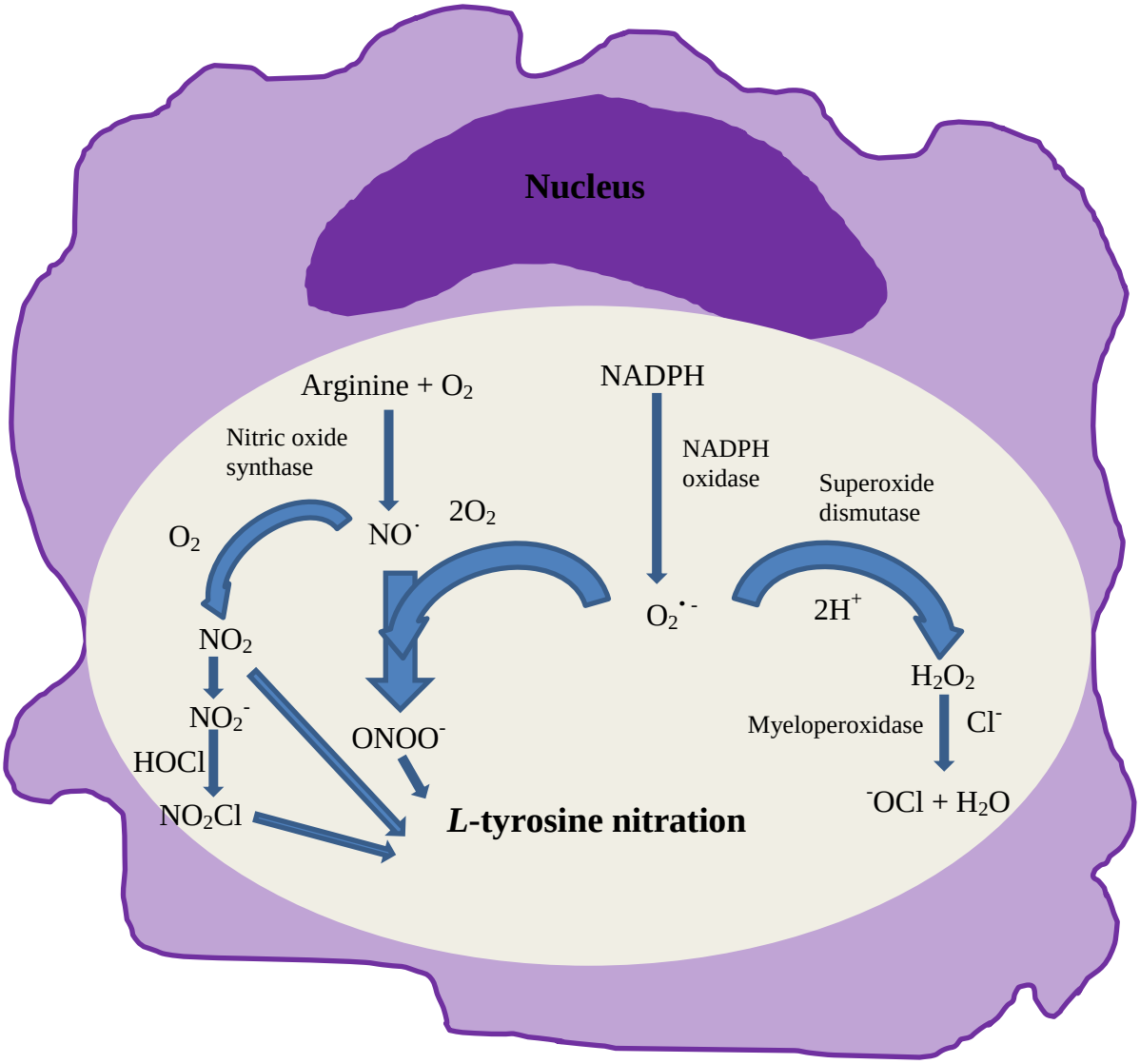


Figure 1.1. Synthesis of RNS and ROS during the respiratory burst in phagocyte. Arginine reacts with oxygen producing nitric oxide. Further reactions with oxygen and superoxide lead to formation of peroxynitrite ($ONOO^-$), nitrogen dioxide ($NO_2^{\cdot}$) and nitryl chloride (NO_2Cl). Due to their destructive properties foreign, damaged and cancerous cells are being eliminated. Excess of RNS react with biomolecules including *L*-tyrosine, leading to *L*-tyrosine nitration (McKee and McKee 2009).

and gas or liquid chromatography coupled to either single stage or tandem mass spectrometry (Duncan 2003). According to the reported results, free NY levels in human plasma vary at least 100-fold among these assays (Tsikas and Duncan 2014). In their reviews, Duncan (2003) and Dalle-Donne, Rossi et al. (2006) consider antibody-based quantitative methods and HPLC with ultraviolet detection as semi-quantitative because of low selectivity, sensitivity and precision. Other analytical techniques such as

HPLC with electrochemical detection, liquid chromatography coupled to mass spectrometry (LC-MS/(MS)), and gas chromatography coupled to mass spectrometry (GC-MS/(MS)) operated in electron capture negative ion mode (NCI) are capable to provide trustworthy results. In particular, GC-MS methods are able to separate and analyse complex mixtures at nano- and picomolar concentrations, however, the analyte must be volatile within 200-300 °C and thermally stable. The purpose of this research was to work out an optimal analytical procedure for simultaneous determination of plasma free NY and Y by GC/MS. The most important part of any analytical procedure is the sample preparation. With respect to GC/MS analysis, sample preparation comprises amino acids isolation with subsequent derivatization. One of the most applicable tools for amino acid isolation from a liquid biological sample is solid phase extraction (SPE). It is based on the affinity of a target compound to the sorbent with selective retention. The selection of SPE method depends on the analyte properties. NY and Y exhibit basic, acidic, polar and aromatic properties. For isolation of NY and Y from water and plasma samples, SPE tubes with two types of sorbent with various retention capabilities have been studied.

The isolated amino acids cannot be directly subjected to GC/MS due to their low volatility. Therefore NY and Y were converted to compounds suitable for GC/MS analysis by derivatization (chemical modification to more volatile and thermostable compounds with enhanced detectability). Four chemical derivatization strategies were investigated. The emphasis was given to the chemical reactions with formation of fluorinated derivatives, because the electron affinity of fluorine provides ultrahigh sensitivity of the analytical procedure. Development of a single-step derivatization procedure was the first priority, but an alternative method also was considered. The methods studied were systematically optimised to achieve maximal recovery and sensitivity.

2. Theory

2.1 Solid phase extraction

"*SPE is the removal of chemical constituents from a flowing liquid sample via retention on a solid sorbent, and the subsequent recovery of selected constituents by elution from the sorbent*." (Wilson, Adlard et al. 2000, p. 4636).

Isolation of NY and Y from the sample by SPE is a critical step in the sample preparation. A typical SPE procedure comprises five basic steps (Fig.2.1). Before the sample loading the sorbent should be conditioned and equilibrated. Conditioning moisturizes and activates the bonded phases to

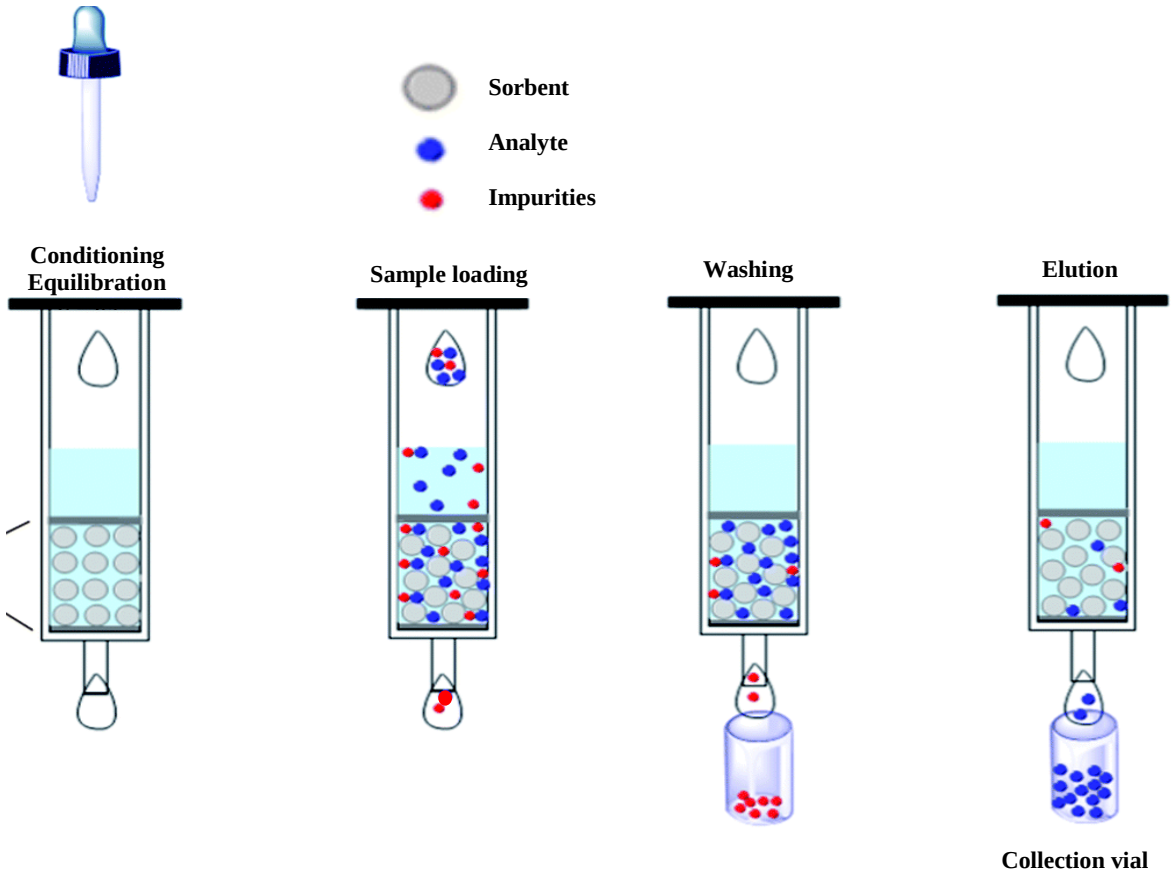


Figure 2.1. Basic steps of SPE (the figure adapted from Su, Wang et al. (2014) with permission of The Royal Society of Chemistry). Conditioning and equilibration steps maximize retention of the analyte. When the sample is loaded, the sorbent retains the analyte, while the impurities pass though the tube. During the washing step the remaining impurities are removed. The elution solvent liberates the analyte into a collection vial.

ensure consistent interaction between the analyte and the sorbent functional groups. Equilibration introduces a solution similar to the sample load in terms of solvent ionic strength and pH in order to maximize retention. One - two tube volumes of buffer (used in the sample pre-treatment) or water are good choices for equilibration. When the sample is loaded into the cartridge, it passes through the sorbent. The sorbent retains the analyte and lets the impurities pass through the tube. The cartridge is then washed to remove remaining impurities. The elution solvent (eluent) disrupts the interactions between the analyte and sorbent functional groups, discharging the analyte into the collection vial (Sigma Aldrich 2013). The choice of sorbent and SPE type is strictly dependent on the analyte properties. Both NY and Y are polar aromatic amino acids (Fig. 2.2). Both contain one carboxyl group (pKa =2.20), one amino group (pKa=9.21) and one hydroxyl group (McKee and McKee 2009).

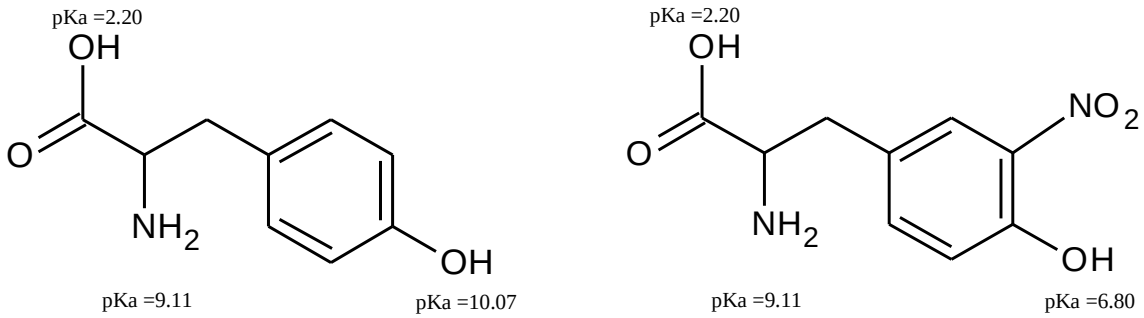


Figure 2.2. Acid dissociation constants **(**pKa) of NY and Y. The left structure is Y molecule; the right structure is NY molecule.

The hydroxyl group of NY and Y has pKa = 6.8 and 10.07 respectively (De Filippis, Frasson et al. 2006). So, the pH of the solution determines if the amino acids are anionic, cationic or neutral. The isoelectric points (pI) of Y and NY are at about pH = 5.65 and 4.5 respectively and the overall charge is zero. At pH< 5.7 Y molecules have the net charge either positive or neutral, but for NY this state is observed at pH< 4.5. The amount of positively charged molecules increases as pH decreases and the carboxylic groups become neutral (Fig. 2.3). During the extraction therefore, the amino acids should be in similar ionic states (pH ranges from 1 to 3, Fig.2.3) to ensure the best recovery of both NY and Y. According to these findings, there are two types of SPE methods that are the most suitable for NY and Y extraction: reversed-phase and cation exchange/reversed-phase. Strata SPE-X tubes for reversed-phase method contain sorbent with superior H-bonding and π-π bonding capabilities for enhanced retention of polar and aromatic analytes (Phenomenex 2007). The neutral form of the analyte

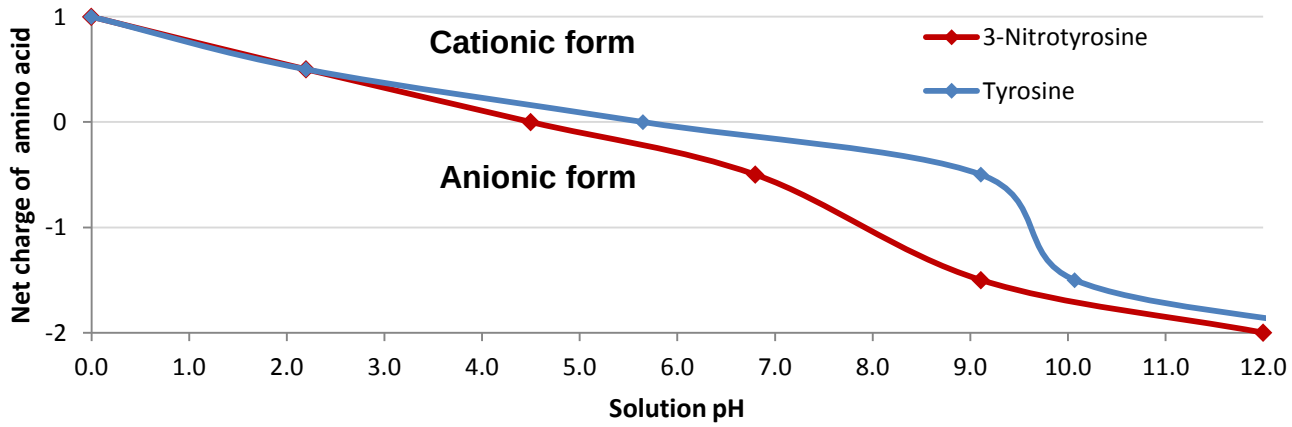


Figure 2.3. Ionic state of NY and Y versus solution pH.

strengthens the retention to the reversed phase sorbent, so the pH of the Y and NY solutions should be two units lower than their pIs in order to interact with the sorbent (Sigma-Aldrich 2013). Therefore, the sample pH before entering the tube should be about 3 as well as the pH of the washing solution to keep the analyte bound to the sorbent. For elution 50% methanol in water is usually used as the eluent. Strata-X-C SPE tubes utilize cation exchange/reversed-phase properties. The retention mechanism is based on electrostatic attraction of charged functional groups of the analyte(s) to oppositely charged functional groups on the sorbent. In the cationic form NY and Y have a positively charged amino group and a neutral carboxyl group. This ionic state can be achieved at pH < 2. The eluent usually contains cations which could effectively replace the analyte, for example, 2-5% ammonium hydroxide dissolved in 50% methanol, pH=10 (Phenomenex 2007). These basic principles were taken into consideration in order to develop a SPE procedure for simultaneous extraction of NY and Y from plasma samples.

2.2 Derivatization

Prior to GC-MS analysis, NY and Y should be subjected to derivatization, which is a chemical conversion to more volatile and thermostable compounds with enhanced detectability. Nanomolar amounts of free NY in plasma samples requires the development of a highly sensitive analytical method. GC/MS analysis in NCI mode should provide sufficient selectivity and sensitivity if NY and Y form polyfluorinated derivatives. Moreover, it is highly desirable that NY and Y would form derivatives simultaneously and preferably in one step. There are more criteria for a hypothetical, ideal derivatization procedure of amino acids, such as simple sample handling with one reagent or few reagents, very rapid reaction speed at room temperature and in aqueous medium, picomolar or nanomolar limits of detection of the analyte, and low reagent and instrumental cost (Husek 1991). The realization of all these requirements is unrealistic. But there are a few criteria that the present research aims to achieve, whereof the two most important are: 1) the formation of a stable single derivative of NY and, 2) nanomolar limits of detection of the derivative in plasma matrix.

A wide range of chemical derivatization strategies has been reviewed by Tsikas and Duncan (2014). It was highly recommended to convert NY to 3-aminotyrosine in the first step in order to prevent artifactual NY formation. Söderling, Ryberg et al. (2003) developed such a procedure, which showed high sensitivity and stability of the NY derivative. The derivatization pathway had four steps including NY reduction in the first step. Due to its high reactivity, heptafluorobutyric anhydride (HFBA) readily reacts with carboxyl, hydroxyl and amino groups in aprotic solvent (Wade 2014, pp. 1038-1039), forming tetra-heptafluorobutyryl (HFBO) derivative of NY and tri-HFBO derivative of Y. During the extraction step in water/dichloromethane under moderate acidic conditions and excess of water, the ester and anhydride bonds hydrolyse, while amides are left intact due to their higher stability (Wade 2014, p. 1047). In the last step, NY and Y are converted to their methylated derivatives by trimethylsilyldiazomethane (TMSD), forming methyl ethers and methyl esters with previously liberated hydroxyl and carboxyl groups respectively. Söderling, Ryberg et al. (2003) had not applied this derivatization strategy to Y, and therefore it became a subject of the present study. Y was expected to form a derivative in the same manner as NY but only with one heptafluorobutyryl (HFBO) group (Fig. 2.4). However, a single-step derivatization approach would also be considered here. The derivatization with pentafluorobenzyl bromide (PFBBr) described by Jiang and Balazy (1998) was reported as a sensitive and quantitative single-step method in terms of NY determination. The reaction with PFBBr was mediated by diisopropylethylamine (DIPEA) to obtain a di-*O*-PFB-*N*-PFB derivative

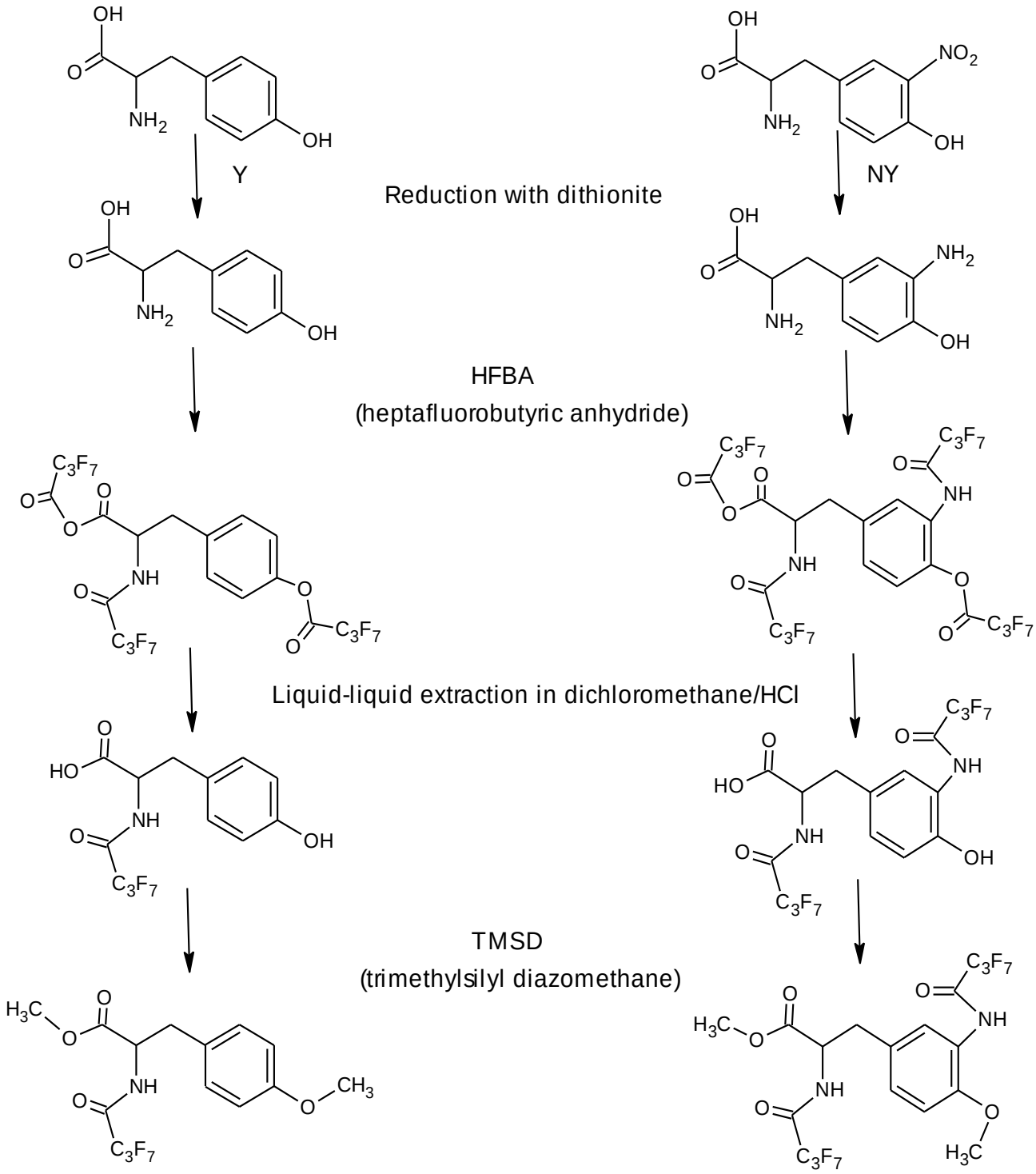


Figure 2.4. Derivatization pathway of Y and NY with HFBA/TMSD. Y forms methyl *O*-methyl-*N*-heptafluorobutyryl- tyrosinate, NY forms methyl *O*-methyl-*N*,*N*-diheptafluorobutyryl-3-aminotyrosinate.

of NY during second-order nucleophilic substitution (Fig. 2.5):

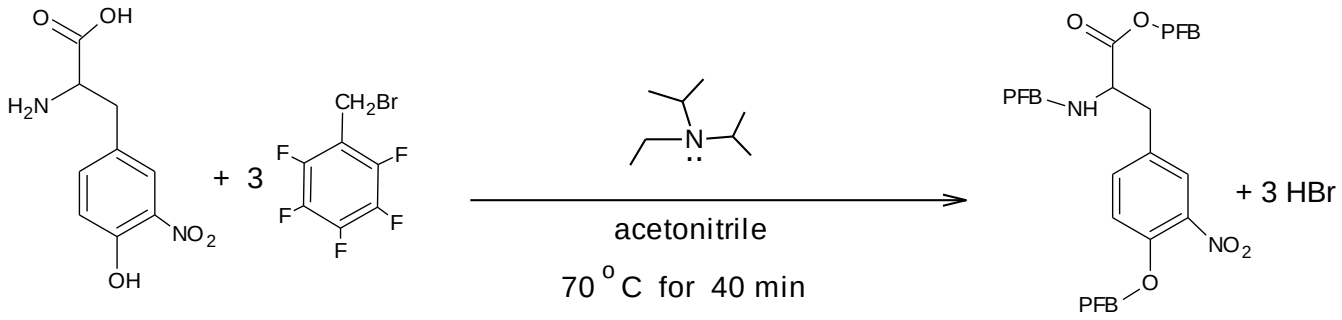


Figure 2.5. Derivatization of NY with PFBBr mediated by DIPEA. During this reaction NY forms tri-PFB derivative or PFB *N,O*-diPFB-3-nitrotyrosinate.

DIPEA is a poor nucleophile due to its bulky structure, but is a good base, because it can easily accommodate a proton. During derivatization, DIPEA deprotonates carboxyl and hydroxyl groups of NY, enhancing their nucleophilic properties, and neutralizes the large quantities of hydrobromic acid formed. Assuming that Y forms its derivative in a similar way, this method can be used in simultaneous sample preparation and GC/NCI-MS analysis of NY and Y.

Davis (1977) successfully tested derivatization of carboxylic acids and phenols with PFBBr mediated by crown ether. The chemical equation is the same as in Fig. 2.5, but the reaction is catalysed by crown ether (18-crown-6) and potassium carbonate instead of DIPEA. 18-crown-6 forms a complex with the potassium cation in the center of the ring (Fig. 2.6) (Wade 2014, p. 653).

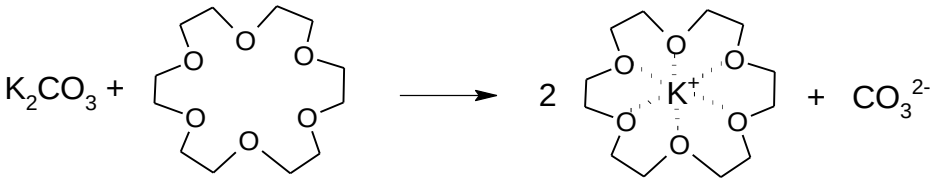


Figure 2.6. Complexation of the potassium cation by crown ether. 18-crown-6 is a large cyclic polyether that specifically solvates polar inorganic potassium carbonate in non-polar organic solvents such as acetonitrile and ethyl acetate.18-crown-6 complexes the potassium cation in the center of the ring, leaving the carbonate anion bare and highly reactive (Wade 2014, p.653).

This approach can also be applied to NY and Y, because the uncomplexed carbonate ion is a strong base and can deprotonate carboxyl and hydroxyl groups of NY and Y, forwarding the nucleophilic

substitution of the leaving bromide ion. After the derivatization the sample does not require any further treatment and can be directly subjected to GC/MS analysis, which is a major advantage over other methods.

There is one additional very interesting single–step chemical derivatization strategy recently described by Pavlovich, Biondi et al. (2012). They extended the approach reported by Husek (1991), who described the treatment of aqueous amino acid solutions with ethyl chloroformate (EtCF)/ ethanol in the presence of pyridine. As a result, the carboxyl group of amino acids were converted to ethyl esters, while hydroxyl and amino groups reacted with EtCF substituting chlorine. Pavlovich et al. (2012) applied this reaction to NY and Y in terms of preparation of fluorinated derivatives. For this purpose they used alkyl chloroformate/perfluoroalcohol combination and obtained the best results with isobutyl chloroformate/trifluoroethanol and EtCF/heptafluorobutanol (HFBOH). The latter combination was applied to prepare NY and Y derivatives in the present study and the applicability of this derivatization procedure for determination of plasma free NY and Y was assessed.

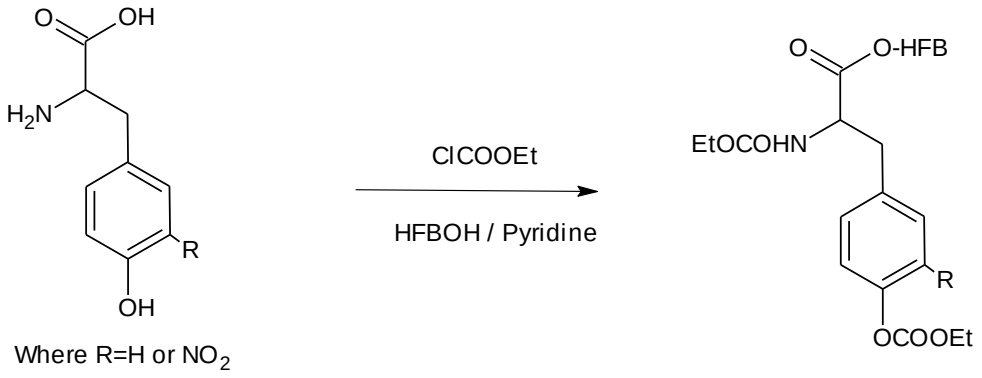


Figure 2.7. Derivatization of NY and Y with EtCF/HFBOH. The products of the reaction, *N,O*-diethoxycarbonyl heptafluorobutyl esters of NY or Y, extracted with *n*-hexane prior to GC/MS analysis.

This method has many advantages. Both NY and Y had previously been derivatized and both amino acids had formed the derivatives. The procedure is time saving, about 15 min, and uses an aqueous medium.

Thus, preliminary investigation of the derivatization methods discussed above potentially could help determine and develop the most suitable derivatization procedure for GC/MS analysis of plasma free NY and Y.

2.3 Principles of GC/MS analysis

GC/MS is a combination of gas chromatography (GC) and mass spectrometry (MS). The main purpose of this analytical tool is to separate and identify the components of complex mixtures in relatively short times. GC is a column chromatography, in which a sample is injected into a capillary column (Fig.2.8). During the injection the sample evaporates as the injector is heated up to 200-300 °C. The column is located in a thermostated oven and the optimal column temperature depends on the boiling points of the compounds in the sample. Control of the temperature is a critical factor, because the correct temperature gradient may ensure a high degree of separation. It is common to employ temperature programming with continuous or stepwise temperature rise. The column is covered with

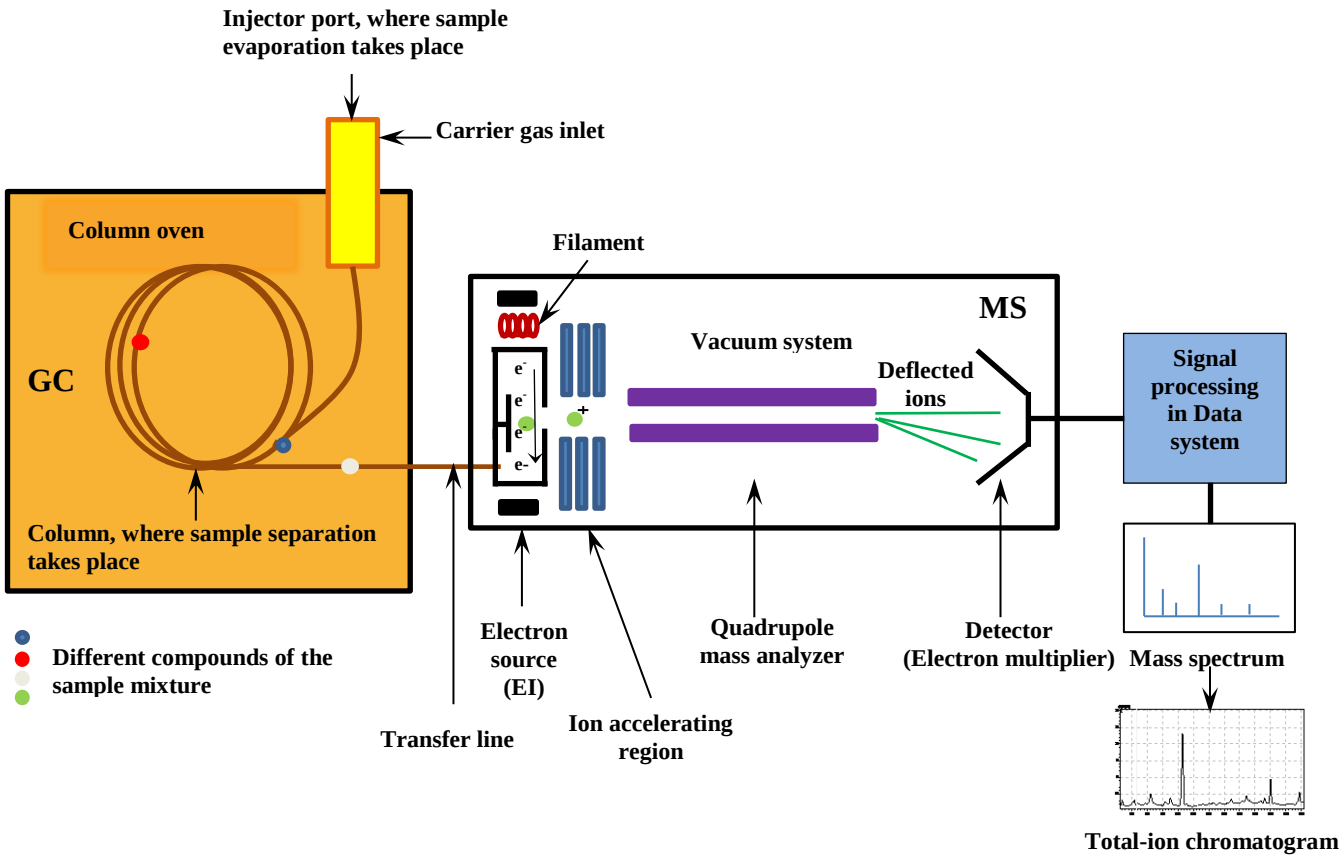


Figure 2.8. Main components of GC/MS system. The sample separated on the GC column moves through the transfer line into the inlet of mass spectrometer, where the molecules are ionized, fragmented, analysed and detected. An electrical signal from the detector is processed in the data system and interpreted into a mass spectrum. The summary of the ion abundances in each spectrum plotted against time gives a total-ion chromatogram (Skoog, Holler et al. 2007; Sheehan 2009).

a thermostable, chemically inert, low volatile immobilized liquid (stationary phase). The evaporated sample is carried by an inert gas mobile phase (carrier gas) throughout the column. The components of the sample mixture separate by their boiling points. Compounds with a low boiling point are eluted first or have shorter retention times (t_R) (Skoog, Holler et al. 2007; Snyder, Kirkland et al. 2010). After GC separation, the carrier gas with the sample passes the transfer line that connects the GC column outlet and the vacuum system of the mass spectrometer. The mass spectrometer has three main compartments that are operated under a high vacuum: ion source, mass analyser and detector. The sample enters the ion source as a vapour. The heated filament emits electrons which move in an electrical field of 70 volts. This potential generates a beam of energetic (70 eV) electrons. The diffusing sample molecules collide with electrons, becoming positively or negatively charged ions depending on the ionization mode. The ions are accelerated and then separated according their mass-to-charge (m/z) ratio in the mass analyzer, where the ions deflect in the electric field according their masses. The vacuum system ensures the gaseous phase of the ions and prevents collisions with residual gas and carrier gas molecules. The ions separated in the mass analyzer are detected and converted into an electrical signal by the detector (Sheehan 2009). The m/z ratios are finally displayed as a mass spectrum, a plot of relative ion abundance against its m/z. The plot is normalized to the peak with the highest intensity (the base peak) assumed as 100%. The obtained spectra are summarized and plotted as a function of time in form of a total-ion chromatogram (TIC) (Skoog, Holler et al. 2007).

The present research has employed two types of ionization sources: 1) electron impact ionization source (EI), and 2) negative chemical ionization source (NCI). EI is classified as hard ionization, because it generates highly energetic 70 eV-electrons which break up the molecular bonds producing a large number of single charged cations of different masses (Fig. 2.9). The obtained complex mass spectrum helps to understand the chemical structure of the compound. The molecular ion corresponds to the molecular mass of the target molecule. The base peak is formed by the most abundant ion and frequently used as a target-molecule identifier at a specific retention time when inspecting an ion chromatogram (Skoog, Holler et al. 2007). EI can be applied to a wide range of molecules suitable for GC/MS (Shimadzu 2015).

NCI is classified as a soft ionization source, where reagent gas (methane, isobutane or ammonia) is an essential part of the process (Skoog, Holler et al. 2007). Colliding with 70 eV-electrons (Shimadzu 2015), reagent gas molecules ionize while the electrons lose their energy and can be captured by the analyte molecules producing single-charged anions of molecular species such as M^- or $[M-H]^-$ or cause little fragmentation (Fig. 2.10) (Hoffmann and Stroobant 2007).

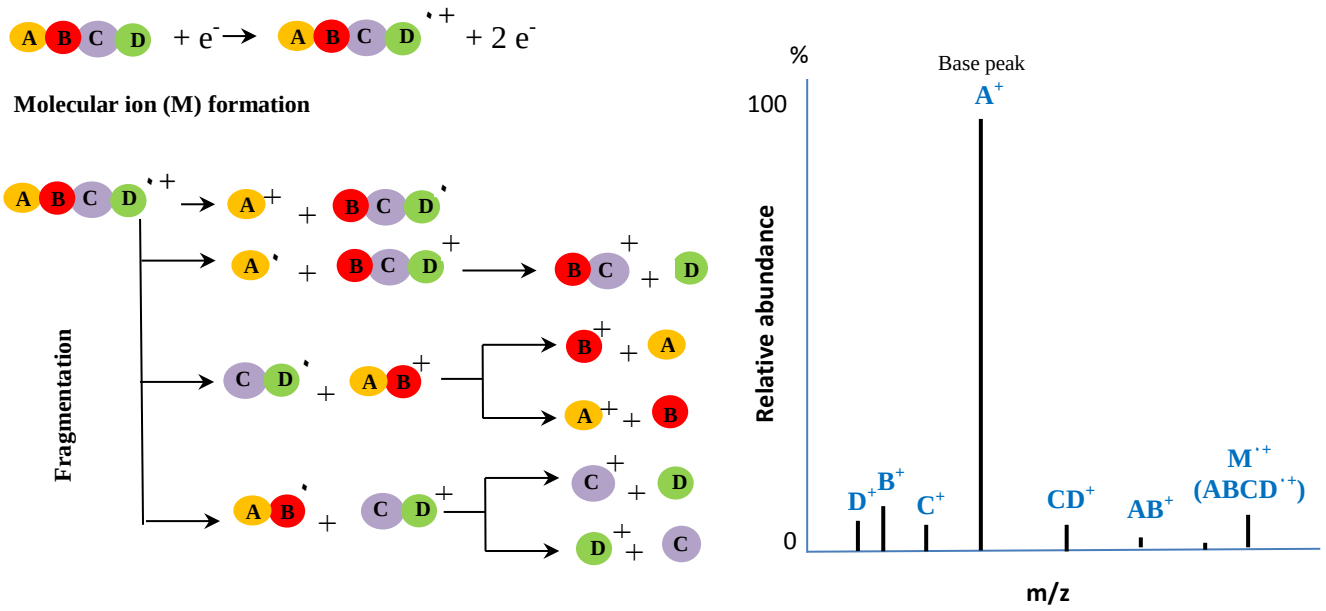


Figure 2.9. Typical reactions in an EI source (left). During electron-impact ionization electrons collide with the analyte molecules, leaving them in a highly exited state and, thus, mediating formation of molecular ions and fragment ions. The molecular ion ($M^{\cdot+}$) lacks one electron and has the same mass as the original compound. Relaxation of the molecular ion causes rupture of bonds, producing fragment ions. **Mass spectrum with EI (right).** The spectrum of m/z ratios plotted against the relative ion abundance. All peaks have intensity in a range 0-100%. The base peak has the highest intensity and equals 100%. The spectrum includes peaks produced by molecular ions and fragment ions (Skoog, Holler et al. 2007; Sheehan 2009).

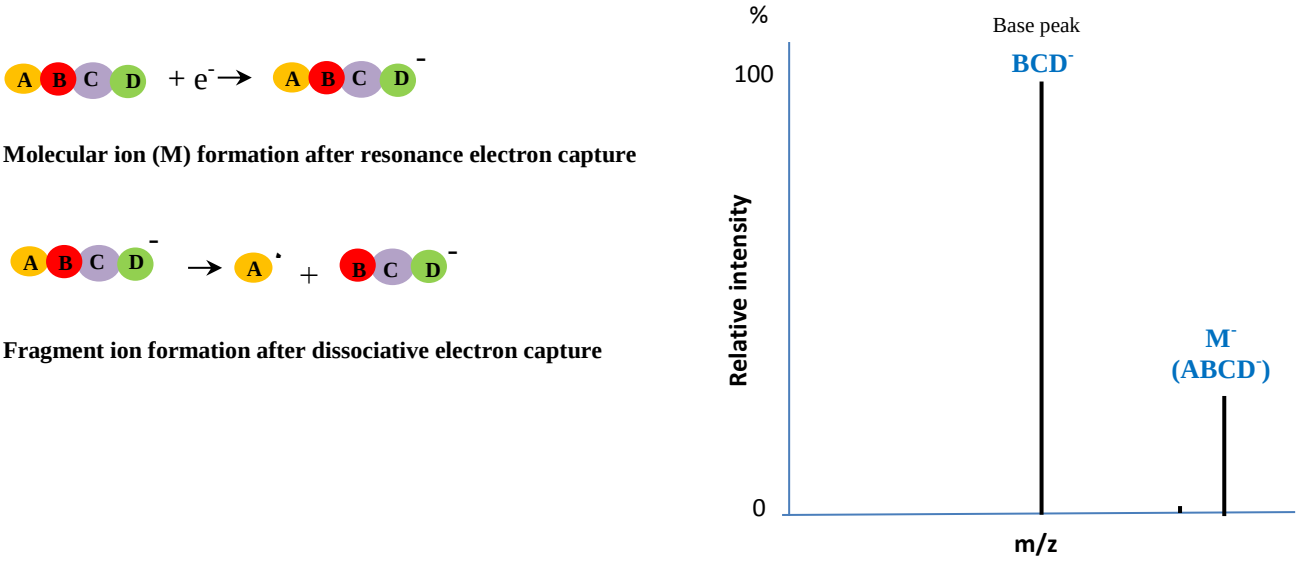


Figure 2.10. Typical reactions in an NCI source (left). During NCI the energy of electrons is reduced by collisions with reagent gas molecules. Analyte molecules capture the electrons and become negatively charged molecular ions frequently with the same mass as the original compound. Some of the electrons have enough energy to produce fragment ions (Shimadzu 2015). The fragmentation is not extensive, as a rule one or two types of fragment ions are formed. **Mass spectrum with NCI (right).** Due to restricted fragmentation the mass spectrum is simpler compared to EI.

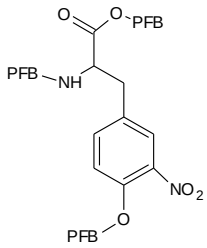
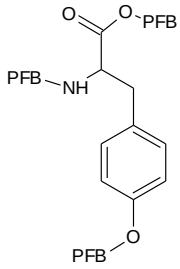
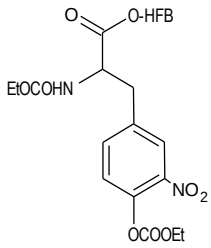
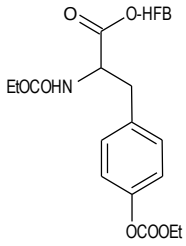
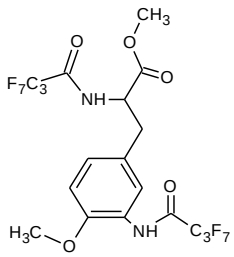
Therefore, NCI produces a simple mass spectrum compared to EI. GC/NCI-MS methods detect primarily halogenated analytes capable of capturing electrons. This feature makes GC/NCI-MS more selective and sensitive compared to GC/EI-MS (Shimadzu 2015; Skoog, Holler et al. 2007).

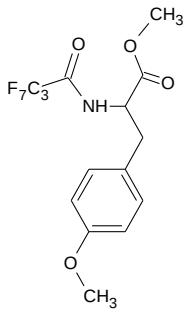
The MS detector can acquire data for analysis in two modes: scanning (scan) or selected ion monitoring (SIM). In scan mode all ions in a defined m/z range that reach the detector are registered. This mode therefore provides useful information about the molecular structure. A large library of mass spectra acquired in EI at 70 eV enables identification of hundreds of thousands of organic compounds by a similarity search (Wiley and NIST mass spectral libraries). The obtained MS data can be displayed either as a total-ion chromatogram which includes all masses acquired, or as a mass chromatogram (MC) which includes one or several specific m/z. The background noise is generally significantly reduced in the MC compared to the TIC (Shimadzu 2015). The noise has a chemical and instrumental nature; it is always present in an analytical measurement. The sensitivity of MS can be improved by increasing the scan time or decreasing the number of masses. When the analyte spectral characteristics are known, the mass spectrometer can be set at suitable m/z values in SIM mode. SIM gives maximum sensitivity, because the range of masses is significantly limited. The sensitivity may increase up to 10- 100 times compared to the scan mode by monitoring only a few characteristic ions (Hoffman and Stroobant 2007; Shimadzu 2015). As a rule, SIM is applied for quantitation and for determination of the lower detection limit (LLOD) and the lower quantitation limit (LLOQ) of an analyte. For the evaluation of these limits such an important parameter as a signal-to-noise ratio (S/N) is often used. In most measurements the level of noise is constant and independent of the signal strength, but its effect on the relative error becomes perceptible when the concentration of an analyte decreases in magnitude (Skoog, Holler et al 2007). In this regard, since some noise is still present even in SIM mode, Snyder, Kirkland et al. (2010) defined LLOD as S/N = 3 and LLOQ as S/N = 10. At S/N less than 3 it is impossible to distinguish signal from noise (Skoog, Holler et al. 2007).

Some of the NY and Y derivatives discussed in Section 2.2 had been studied previously and their spectral characteristics had been determined. A summary of the experimental and theoretical information about these compounds is listed in Table 2.1. This information will be used for identifying the NY and Y derivatives in TIC as well as for developing SIM settings.

To ensure precise and accurate MS-based quantitation of an analyte, an internal-standard method is widely used. An internal standard is defined as a compound that should be absent from the sample matrix, has the same physical and chemical properties as the analyte and provide a signal that is similar to the analyte signal but distinguishable for the analytical instrument. A constant amount of the internal

Table 2.1. Structural and spectral characteristics of Y and NY derivatives

Derivatization reagents	Derivative name	Derivative chemical structure	EI m/z			NCI m/z		
			Target ion	Characte-ristic ions	Molecular ion	Target ion	Characte-ristic ions	Molecular ion
PFBBr , reaction mediated either by DIPEA or crown ether and K_2CO_3	PFB *N*,*O*-diPFB-3-nitrotyrosinate		To be studied	To be studied	766	585[a]	196[a]	766
	PFB *N*,*O*-diPFB - tyrosinate		To be studied	To be studied	721	360, 540	To be studied	541, 721
EtCF/HFBOH	HFB *N*,*O*-diethoxycarbonyl - 3-nitrotyrosinate		152[b]	135,256,391, 462[b]	552[b]	To be studied	To be studied	552
	HFB *N*,*O*-diethoxycarbonyl-tyrosinate		107[b]	135,179,280, 346,434[b]	507[b]	To be studied	To be studied	507
HFAA/TMSD	Methyl *O*-methyl-*N*,*N* diheptafluorobutyryl-3-aminotyrosinate		To be studied	To be studied	616	576[c]	596[c]	616

Derivatization reagents	Derivative name	Derivative chemical structure	EI m/z			NCI m/z		
			Target ion	Characte-ristic ions	Molecular ion	Target ion	Characte-ristic ions	Molecular ion
HFAA/TMSD	Methyl *O*-methyl-*N*-heptafluorobutyryl-tyrosinate		To be studied	To be studied	405	To be studied	To be studied	405

[a] (Jiang and Balazy 1998)
[b] (Pavlovic, Biondi et al. 2012)
[c] (Soderling, Ryberg et al. 2003)

standard is added to all samples and calibration standards to be analyzed. When the internal standard is chosen correctly, it will undergo the same loss in the extraction step, influenced in the same way by the matrix effect, have the same chemical transformation during the derivatization and respond identically to random instrumental and method fluctuations (Hoffmann and Stroobant 2007). Since both compounds behave nearly identical during the entire analytical procedure, the ratio of their signals is not affected. Thus, the calibration with an internal standard "*involves plotting the ratio of the analyte signal to the internal-standard signal as a function of the analyte concentration of the standards*" (Skoog, Holler et al., 2007, p.17). The internal-standard method can, therefore, compensate for several types of errors (Hoffmann and Stroobant 2007; Skoog, Holler et al. 2007). The use of isotopic analogues of NY and Y as internal standards in MS-based quantitation promotes high precision of the MS measurements (Tsikas and Duncan 2014).

3. Experimental

3.1 Chemicals and equipment

Organic solvents: Acetonitrile and methanol (GC/MS grade) from Fisher Chemical, UK;

Dichloromethane (GC grade) from Fluka Chemie GmbH**,** Buchs, Germany;

Toluene (100%), *tert*-butyl methyl ether (min. 99.5%) and ethyl acetate (99.9%) from VWR International, France;

n-Hexane (min 97%) and pyridine (99.8%) from Sigma Aldrich, Schnelldorf, Germany.

Inorganic and organic acids : Trifluoroacetic acid (TFA, 98.%) from Merk ,Darmstadt, Germany;

Hydrochloric acid (HCl, 37%) from VWR International, France.

Derivatization reagents: Pentafluorobenzyl bromide (PFBBr, min 99%), diisopropylethylamine (DIPEA, min 99%), heptafluorobutanol (HFBOH, 98%), ethyl chloroformate (EtCF, min 98%), potassium carbonate (99%), dithionite (sodium hydrosulfite, 85%), disodium phosphate (99%), heptafluorobutyric anhydride (HFBA, 99%) and trimethylsilyl diazomethane solution (TMSD, 2M in hexane) from Sigma Aldrich, Steinheim, Germany;

18-Crown-6 or crown ether (1,4,7,10,13,16-hexaoxacyclooctadecane, min 99%) from Fluka Chemie GmbH, Buchs, Germany.

Amino acids: *L*-tyrosine,(min 99%) from Merck, Darmstadt, Germany;

3-Nitro-*L*-tyrosine (min 98%) from Roth, Karlsruhe, Germany.

Isotopic compounds: 4-hydrobenzoic 2,3,5,6-d_4 acid (98,8 % atom D) and L-4-hydroxy-phenyl-d_4-alanine (tyrosine-d_4, 99.1% atom D) from CDN ISOTOPES , Quebec, Canada;

3-Nitro-*L*-tyrosine-d_3 from TRC (Toronto research chemicals), Toronto, Canada.

Solid phase extraction (SPE) cartridges: Strata-X-C 33 um 60 mg/ 3 mL SPE tubes and Strata-X 33u 60 mg/3 mL SPE tubes from Phenomenex, USA.

GC/MS equipment and software: GC- microvials 0.3 mL and screw caps PP transparent 9mm silic.whi./PTFE from VWR, Oslo, Norway.

The GC separation was carried out on Rxi-5Sil (30m x 0.25mm x 0.25 um) MS column from RESTEK, Lisses, France.

The GC/MS analysis was performed on GCMS-QP2010 Ultra gas chromatograph/mass detector coupled with auto injector/auto sampler AOC-20i and controlled by GCMSsolution, Ver.2.6 from Shimadzu Corporation, Kyoto, Japan.

3.2 Sample preparation

3.2.1 3-Nitrotyrosine and tyrosine standard solutions

Stock solutions of NY and Y were prepared by dissolving 0.2 mg of each amino acid in 10 mL of distilled water. The stock solution was used to make a dilution series with concentration of 10, 1, 0.1, 0.01, 0.001, 0.0001 mg/L to determine the lower limit of detection (LLOD) of NY and Y derivatives. The amino acids were subjected to derivatization as single compounds or mixed together in a volume ratio 1:1. Stock solutions of d_3-NY and d_4-Y were prepared by dissolving 0.1 mg of d_3-NY in 3 mL and 0.05 mg of d_4-Y in 4 mL of distilled water. The stock solution of d_4-Y was diluted to 0.1 mg/L and used as an internal standard for determination of Y derivatives (Section 4.1.4). The stock solution of d_3-NY was diluted to 1 mg/L and used in experiments with plasma samples (Section 4.2) as an internal standard.

3.2.2 Plasma samples preparation

Blood from one healthy person was collected in 9 mL EDTA blood collection tubes and centrifuged at 2500 G at 4 °C for 15 min. Then plasma was homogenized and aliquoted into 600 µL vials and stored at - 80 °C before analysis.

3.2.3 Extraction of amino acids

Two solid phase extraction methods were used to obtain purified plasma NY and Y. The optimal pH values for retention of NY and Y for each type of SPE column were determined experimentally by measuring UV absorbance on eluting phases at 190-500 nm. NY and Y were extracted from water solution with a concentration of 20 mg/L. All eluting fractions after loading the sample were collected and analyzed by spectrophotometer. The most relevant results were used in the SPE methods described below.

SPE with Srata-X-C tubes: 200 µL of EDTA plasma were added 1000 µL of 1% (v/v) TFA aqueous solution (pH 1). NY and Y were extracted on reverse-phase column Strata-X-C that had been pre-washed with 2 mL methanol, 2 mL water and preconditioned with 2 mL of 1% (v/v) TFA aqueous solution. After loading the sample, the column was washed with 3 mL of 1% (v/v) TFA in

methanol/water (1:4). NY and Y were eluted with 1 mL of 5% (v/v) ammonium hydroxide in methanol/water (1:1) (Phenomenex 2014). The eluent was evaporated under vacuum at 50 °C and the residue was dissolved in 100 µL of water prior to derivatization.

SPE with Strata – C tubes: 200 µL of EDTA plasma were added 800 µL of 0.6% (v/v) TFA aqueous solution (pH 3). NY and Y were extracted on reverse-phase column Strata-X that had been pre-washed with 2 mL methanol, 2 mL water and preconditioned with 2 ml of 0.1% (v/v) TFA aqueous solution, (pH 2). After loading the sample, the column was washed with 1.5 mL of 0.1% (v/v) TFA in water. NY and Y were eluted with 1 mL of methanol/water (1:1) (Phenomenex 2014). The eluent was evaporated under vacuum at 50 °C and the residue dissolved in 100 µL of water prior to derivatization.

Comparison of SPE tubes: the following samples were prepared prior the derivatization to estimate the recovery of NY:

- Plasma samples spiked with 1 mg/L of NY standard before SPE
- Plasma samples spiked with 1 mg/L of NY standard after SPE
- Standard solutions with 1 mg/L of NY subjected to SPE
- Standard solution with 1 mg/L of NY standard not subjected to SPE as a 100% recovery control.

All samples were spiked with 1 mg/L of NY-d_3 used as an internal standard.

3.3 Derivatization procedures

3.3.1 Reaction with pentafluorobenzyl bromide catalysed by diisopropylethylamine

The general guidelines for NY and Y derivatization with PFBBr were adopted from the research of Jiang, H. and M. Balazy (1998). 6-12 µg of dry amino acids were added to 200 µL of acetonitrile, 40 µL of 10% (v/v) PFBBr and 40 µL of 10% (v/v) DIPEA in acetonitrile, mixed vigorously, and heated at 70 ˚C for 40, 60, 90 and 120 minutes. The reaction mixture was then dried under vacuum and dissolved in 1 mL of solvent. MTBE, ethyl acetate or n-hexane were used as solvents. The samples were then analysed by GC/NCI-MS.

3.3.2 Reaction with pentafluorobenzyl bromide catalysed by potassium carbonate and crown ether

NY and Y were subjected to derivatization as described by Davis (1977). In order to derivatize up to 10 µg of carboxylic acids and/or phenols, 50 µL of PFBBr solution is needed. To prepare the PFBBr solution, 37 µL of PFBBr and 37 mg of crown ether were dissolved in 12.4 mL of ethyl acetate or acetonitrile. Reaction vials, containing dry NY (4; 0.4; 0.04 µg), a mixture of NY (4; 0.4; 0.04 µg) and Y (4; 0.4; 0.04 µg), were added 1 mL of the PFBBr solution and a few grains of powdered potassium carbonate. The reaction mixture was shaken occasionally during heating. The following temperature and time conditions were tested: in ethyl acetate at 70 °C for 30, 60, 90 and 120 min and at 25 °C for 2 hours; in acetonitrile at 40 °C for 1 hour. After cooling down to 25 °C the reaction mixture was centrifuged for 1 min. at 15000 rpm. The samples prepared in ethyl acetate were analyzed by GC/NCI-MS, and the samples prepared in acetonitrile were analyzed by GC/EI-MS. In the case of acetonitrile, 4-hydroxy [2.3.5.6-$^{2}H_4$] benzoic acid (d_4-HBA) (5; 0.5; 0.05 µg) was subjected to derivatization as well to verify if this derivatization method is applicable to carboxylic acids and phenols as Davis (1977) reported.

3.3.3 Reaction with ethyl chloroformate and heptafluorobutanol

NY and Y were subjected to derivatization following the protocol described by Pavlovich, Biondi et al. (2012). 100 µL of the amino acids standard solution was added 100 µL of HFBOH/pyridine solution in a volume ratio 80:20, mixed well and heated for 10 min at 60 °C. Then 12.5 µL of EtCF was added and the reaction mixture was vortexed for 30 seconds. The derivatives were extracted with 150 µL n-hexane containing 2% (v/v) EtCF by shaking vigorously or vortexing for 15-30 seconds. 60 µL of the sample from the lower phase was pipetted into a GC-vial and analyzed by GC/EI-MS and GC/NCI-MS.

Evaluation of the method sensitivity: known amounts of NY and Y standards (20, 10, 1, 0.1, 0.01, 0.001, 0.0001 and 0 mg/L) were subjected to derivatization and analysed by GC/EI-MS and GC/NCI-MS.

3.3.4 Reaction with heptafluorobutyric anhydride and trimethylsilyldiazomethane

The method was adopted from the assay of Soderling, A. S., H. Ryberg, et al. (2003). 100 µL of the calibration standard or the plasma sample obtained after extraction was added 20 µL of 50 mM dithionite solution in 50 mM di-sodium hydrogen phosphate buffer (pH 9). The mixture was vortexed for 5-10 seconds and evaporated to dryness under vacuum at 60 °C for 1 hour. The residue was dissolved in 300 µL of 10% HFBA in acetonitrile, sonicated for 30 min and evaporated to dryness under vacuum at 60 °C for 1 hour. The sample was added 800 µL of dichloromethane and 200 µL of 0.5 M HCl and rotated for 30 min at 40 rpm. After centrifugation at 3000 rpm for 1 min the organic phase was transferred into a new tube and evaporated to dryness under vacuum at 40 °C for 30 min. The residue was reconstituted in 100 µL of methanol-toluene solution in ratio of 4 to 1 (v/v). 20 µL of TMSD was added to the sample, mixed well and the reaction was stopped after 10 min by evaporation of excess solvent and reagent at 40 °C for 45min. The derivatives were dissolved in 100 µL of acetonitrile and analysed by GC/EI-MS and GC/NCI-MS.

Standard curve: standard solutions of NY and Y (10.0, 1.0, 0.1, 0.01, 0.001, 0.0001 and 0 mg/L) were subjected to derivatization and analysed by GC/NCI-MS in SIM acqusition mode. The signal intensities were plotted against the concentrations.

3.4 GC/MS methods for analysis of derivatives

3.4.1 Pentafluorobenzyl derivatives

3.4.1.1 Diisopropylethylamine mediated reaction

The mass spectrometer was operated in NCI mode with methane as a reagent gas; the ion source temperature was 200 °C, the interface temperature was set at 250 °C. 1 µL of a sample was automatically injected into the GC column. The injector was operated in splitless mode at 250 °C. The column oven temperature was set at 50 °C for 1 min, increased to 120 °C at 25 °C/min and finally increased to 330 °C at 12 °C/min (a detailed description of the method is shown in **Appendix I** under **GC/MS method 1)**. The derivatization products were monitored in scan mode. The formation of peaks were studied at m/z= 360 $[M-2PFB]^-$, m/z = 540 $[M-PFB]^-$, m/z=721 $[M]^-$ for PFB derivative of Y and at m/z= 405 $[M-2PFB]^-$, m/z = 585 $[M-PFB]^-$, m/z=766 $[M]^-$ for PFB derivative of NY, where the molecular ion (M) corresponds to the tris-substituted amino acid derivative (Table 2.1).

3.4.1.2 Crown ether and potassium carbonate mediated reaction

The GC/ MS analysis of NY and Y PFB derivatives formed in ethyl acetate was conducted as described in Section 3.4.1.1. For analysis of the derivatives formed in acetonitrile the mass spectrometer was operated in EI mode; the ion source temperature was 200 °C and the interface temperature was set at 250 °C. 1 µL of a sample was automatically injected into the GC column. The injector was operated in splitless mode at 250 °C. The column oven temperature was set at 95 °C for 1 min, increased to 120 °C at 25 °C/min and finally increased to 330 °C at 12 °C/min (a detailed description of the method is shown in **Appendix I** under **GC/MS method 2**). The derivatization products were monitored in scan mode. The sample TIC was compared to a blank TIC in order to trace extra peak formation with molecular ions at m/z = 721, 766 and 502 for PFB derivatives of Y, NY and d_4-HBA respectively.

3.4.2 *N,O* –diethoxycarbonyl heptafluorobutyl esters

Initially, *N,O* –diethoxycarbonyl heptafluorobutyl esters of Y and NY extracted with *n*-hexane, ethyl acetate and dichloromethane were analysed with EI. The ion source temperature was 210 °C; the interface temperature was set at 250 °C. 1 µL of a sample was automatically injected into GC column. The injector was operated in splitless mode at 280 °C. The column oven temperature was set at 95 °C for 1 min, increased to 120 °C at 25 °C/min and finally increased to 330 °C at 12 °C/min (a detailed description of the method is shown in **Appendix I** under **GC/MS method 3**). The ions at m/z = 107 for the Y derivative and at m/z= 152 for the NY derivative as well as the characteristic ions listed in Table 2.1 (Pavlovic, Biondi et al. 2012) were monitored in scan and SIM modes.

The derivatives extracted with ethyl acetate were also analyzed in NCI mode with methane as a reagent gas; the ion source temperature was set at 180 °C and the interface temperature set at 260 °C. 1 µL of a sample was automatically injected into the GC column. The injector was operated in splitless mode at 250 °C. The column oven temperature was set at 70 °C for 1 min, increased to 120 °C at 25 °C/min and finally increased to 330 °C at 15 °C/min (a detailed description of the method is shown in **Appendix I** under **GC/MS method 4**). The derivatization products were monitored in scan mode. The TIC chromatograms of the sample and a water blank were compared in order to find an extra peak formation.

3.4.3 Dimethyl - heptafluorobutyryl derivatives

The GC/MS analysis of the derivatives was performed with EI and NCI sources. In EI mode the ion source temperature was 210 °C and the interface temperature was set at 250 °C. 1 µL of a sample was automatically injected into the GC column. The injector was operated in splitless mode at 280 °C. The column oven temperature was set at 95 °C for 1 min, increased to 120 °C at 25 °C/min and finally increased to 330 °C at 12 °C/min (a detailed description of the method is shown in **Appendix I** under **GC/MS method 3**)**.** The derivatization products were monitored in scan acquisition mode. The GC-EI/MS TIC chromatograms of the derivatives and a water blank were compared in order to find extra peak formation.

In NCI mode with methane as reagent gas the ion source temperature was set at 180 °C and the interface temperature was set at 260 °C. 1 µL of a sample was automatically injected into the GC column. The injector was operated in splitless mode at 250 °C. The column oven temperature was set at 70 °C for 1 min, increased to 120 °C at 25 °C/min and finally increased to 330 °C at 15 °C/min (a

detailed description of the method is shown in **Appendix I** under **GC/MS method 5**). The derivatization products were monitored in scan and SIM modes at m/z = 371, 443 for Y and at m/z = 576 and 596 for NY (Soderling, Ryberg et al. 2003) and at m/z = 375, 447 and 579, 599 for d_4-Y and d_3-NY respectively.

4. Results and discussion

4.1 Preliminary evaluation of the derivatization methods

4.1.1 Derivatization with pentafluorobenzyl bromide catalysed by diisopropylethylamine

Y and NY were subjected to derivatization following the protocol from Section 3.3.1 with subsequent GC-NCI/MS analysis described in Section 3.4.1.1. Several attempts to obtain the pentafluorobenzyl derivatives of Y and NY catalysed by DIPEA in acetonitrile were not successful. The main drawback, which came up during the derivatization procedure, was the low solubility of the amino acids (in particular Y) in acetonitrile. Changes of reaction time (from 45 to 60, 90 and 120 min) did not lead to formation of the derivatization products. Different solvents such as MTBE, ethyl acetate and n-hexane were used to dissolve the derivatives anticipated after evaporation of the excess reagents with no results. In light of this, the derivatization method reported by Jiang and Balazy (1998) was modified. Since the basic conditions were supposed to favour the formation of PFB derivative, pyridine with its slightly basic properties (Wade 2014, p. 510) was considered to be a good alternative to acetonitrile. The method was altered as follows: Dry Y and NY were dissolved in pyridine (73.5 and 83.5 mg/L respectively). This solution was used as a stock solution in further experiments. 30 µL of DIPEA and 30 µL of PFBBr were added to 1 mL of the stock solution. The reaction was performed at 25 °C, 40 °C and 70 °C for 60 min. After cooling down to 25 °C the reaction products were analysed by GC/NCI-MS as previously. The putative NY derivatives were obtained in only one out of all derivatization experiments performed at 40 °C for 60 min. The derivatives of Y were not revealed in any conditions. The GC/NCI-MS analysis showed that NY formed diPFB derivative as a major product leaving the phenol group of NY underivatized and triPFB derivative as a minor product (Fig. 4.1). The mass spectrum of the NY bis-derivative contained the molecular ion at m/z= 586 and the base peak at m/z = 405, which corresponded to $[M\text{-}PBF]^-$ (Fig. 4.1 B). The mass spectrum of the NY tris derivative contained the molecular ion at m/z= 766 and the base peak at m/z = 585, which corresponded to $[M\text{-}PBF]^-$ (Fig. 4.1 C). The big disadvantage with this method was formation of by-products with viscous consistency which created technical problems during GC/MS analysis such as GC column degradation and injection needle bending. Since subsequent attempts to obtain the NY and Y derivatives were unsuccessful, the current derivatization procedure was rejected.

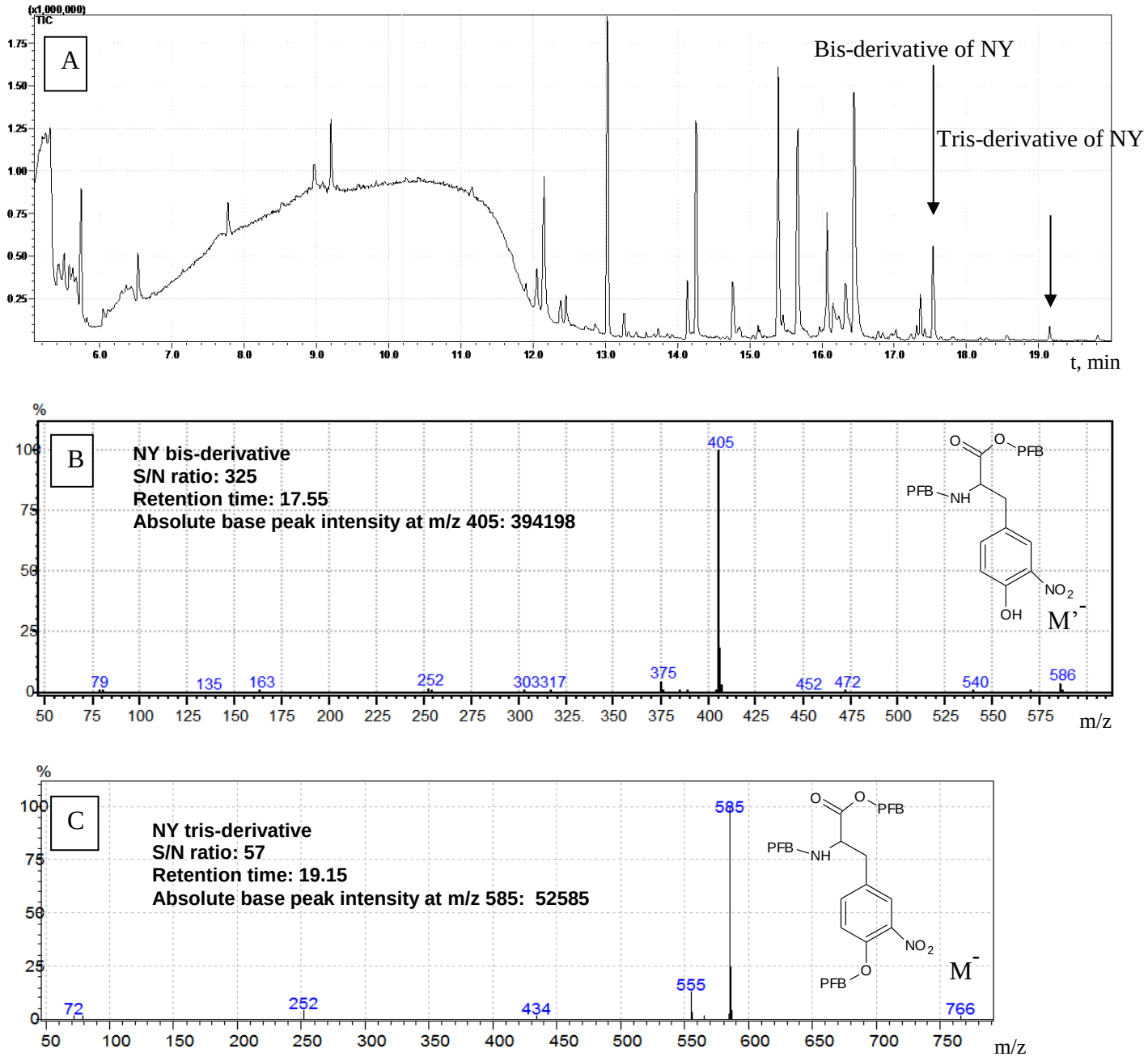


Figure 4.1. GC/NCI-MS scan analysis of Y and NY as there PFB derivatives. Concentration of Y and NY was 73.5 mg/L and 83.5 mg/L respectively.

(A) GC/NCI-MS TIC of Y and NY treated with PFBBr and DIPEA in pyridine at 40 °C for 1 hour. In the chromatogram the arrows indicate peaks that correspond to the NY derivatives.

(B) Ion mass spectrum of diPFB derivative of NY. The molecular ion $(M')^-$ is detected at m/z = 586. The most abundant ion corresponds to $[M' - PFB]^-$ at m/z = 405.

(C) Ion mass spectrum of triPFB derivative of NY. The molecular ion (M^-) is detected at m/z = 766. The most abundant ion corresponds to $[M - PFB]^-$ at m/z = 585.

4.1.2 Derivatization with pentafluorobenzyl bromide catalysed by potassium carbonate and crown ether

Initially, NY and Y were subjected to derivatization in ethyl acetate as described in Section 3.3.2 with subsequent GC/NCI-MS analysis described in Section 3.4.1.1. Since neither NY nor Y derivatives were revealed, ethyl acetate was substituted with acetonitrile as a reaction medium and GC/EI-MS analysis was performed as described in Section 3.4.1.2. Along with NY and Y, d_4- HBA was also subjected to derivatization to verify if this method was applicable for phenols and carboxylic acids as Davis (1978) stated. The GC/EI-MS analysis confirmed the formation of diPFB derivative of d_4-HBA (Fig. 4.2A). The peak appeared in the chromatogram at t_R =16.835 min.

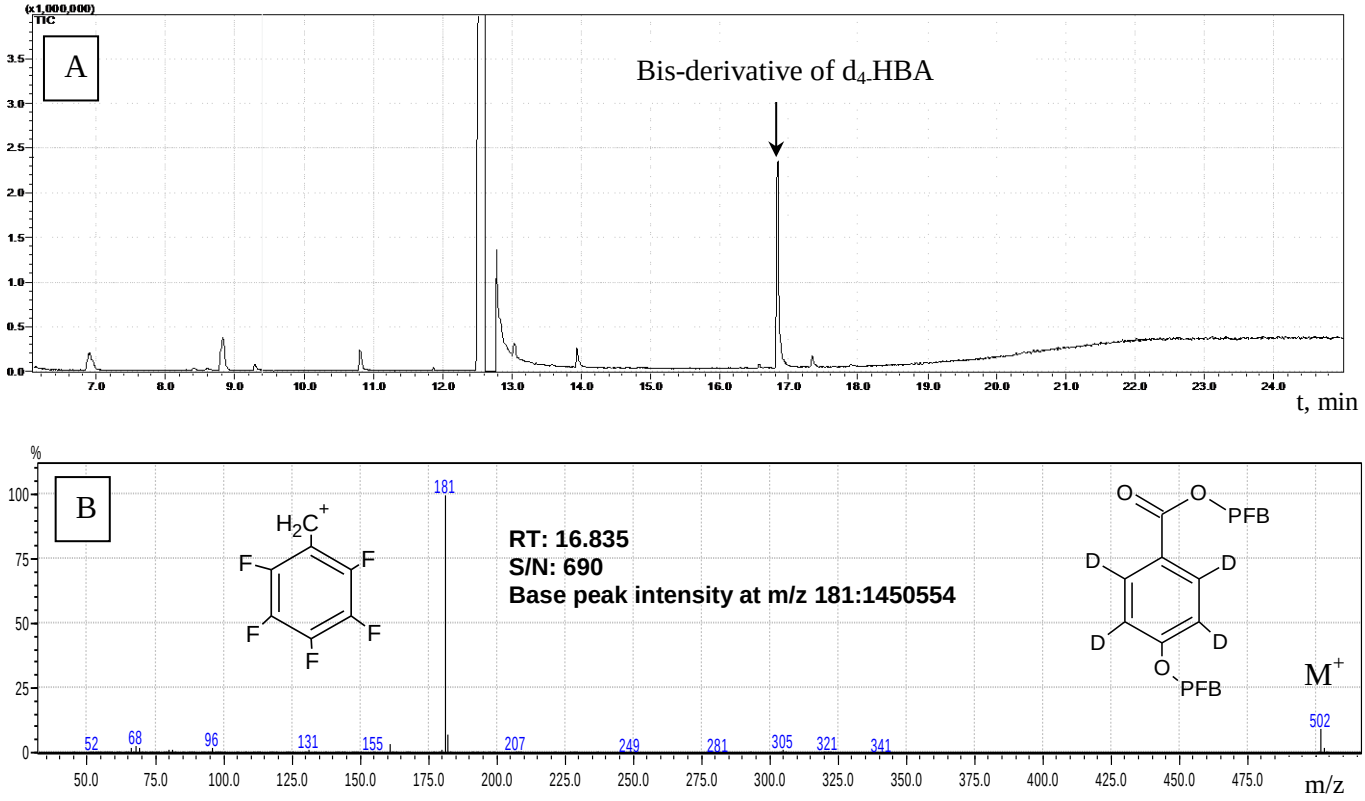


Figure 4.2. GC/EI-MS scan analysis of d_4-HBA as its diPFB derivative. Concentration of d_4-HBA was 5 mg/L.

(A) GC/EI-MS TIC of d_4-HBA treated with PFBBr, crown ether and K_2CO_3 in acetonitrile at 40 °C for one hour. In the chromatogram the arrow indicates a peak that corresponds to the d_4-HBA derivative.

(B) Ion mass spectrum of d_4-HBA derivative. The molecular ion marked with M^+ is detected at m/z = 502. The most abundant ion at m/z = 181 corresponds to PFB-group.

The molecular ion M^+ found at m/z = 502 corresponded to MW of the diPFB derivative of d_4-HBA and the base peak at m/z = 181 represented the PFB-fragment (Fig. 4.2B). A plausible explanation for

the unsuccessful derivatization of NY and Y may be their poor solubility in organic solvents. Indeed, d_4-HBA is readily soluble in many organic solvents while NY and Y have good solubility only in water (according the product information from Sigma-Aldrich). Therefore, it was necessary to work out a derivatization method which could be performed in an aqueous solution. Such methods were described by Pavlovich, Biondi et al. (2012) and Soderling, A. S., H. Ryberg, et al. (2003). The result from the application of these methods is described below.

4.1.3 Derivatization with ethyl chloroformate and heptafluorobutanol

An aqueous solution of Y and NY with a concentration of 10 mg/L of each amino acid was subjected to derivatization following the protocol in Section 3.2.1. After the extraction step the reaction mixture formed three phases, see Fig. 4.3 (A): two organic and one aqueous phase. According to Pavlovich, Biondi et al. (2012) the derivatives were supposed to be found in the organic phase, therefore, the abundance of *N,O* –diethoxycarbonyl heptafluorobutyl esters of NY and Y were studied in both organic phases by GC/EI-MS as described in Section 3.4.2. The GC/EI-MS analysis showed that the highest concentration of the NY and Y derivatives was found in the lower organic phase (Fig. 4.3 B). Consequenly, further analysis was performed with aliquots taken from this phase.

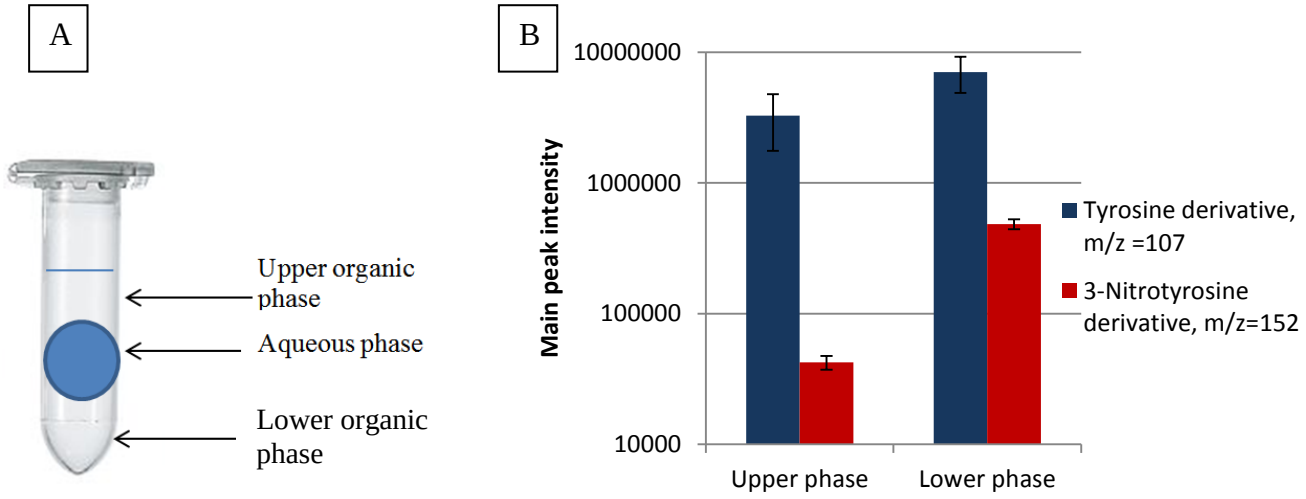


Figure 4.3. (A) Reaction mixture after the extraction step. (B) Abundance of the NY and Y derivatives in the upper and lower organic phase of the extraction mixture. Diagram shows mean values (± SD, n = 4) of the signal strength.

Peak formation of the Y and NY derivatives was detected at t_R = 14.825 and 16.72 respectively (Fig. 4.4 A). The mass spectra of the derivatives (Fig. 4.4 B and C) contained the expected target ions such as 4-hydroxybenzyl cation at m/z = 107 as the base peak (Fig. 4.4 B) in combination with the characteristic ions at m/z = 135, 179, 280, 346, 434 for *N,O* –diethoxycarbonyl heptafluorobutyl ester of Y and 3-nitro-4-hydroxybenzyl cation at m/z = 152 as the base peak (Fig. 4.4 C) with the characteristic ions at m/z = 135, 256, 391, 462 (Pavlovic, Biondi et al. 2012). At a concentration of 10 mg/L the S/N for Y and NY derivatives was calculated to be 2534 and 423 respectively (Fig. 4.4 B

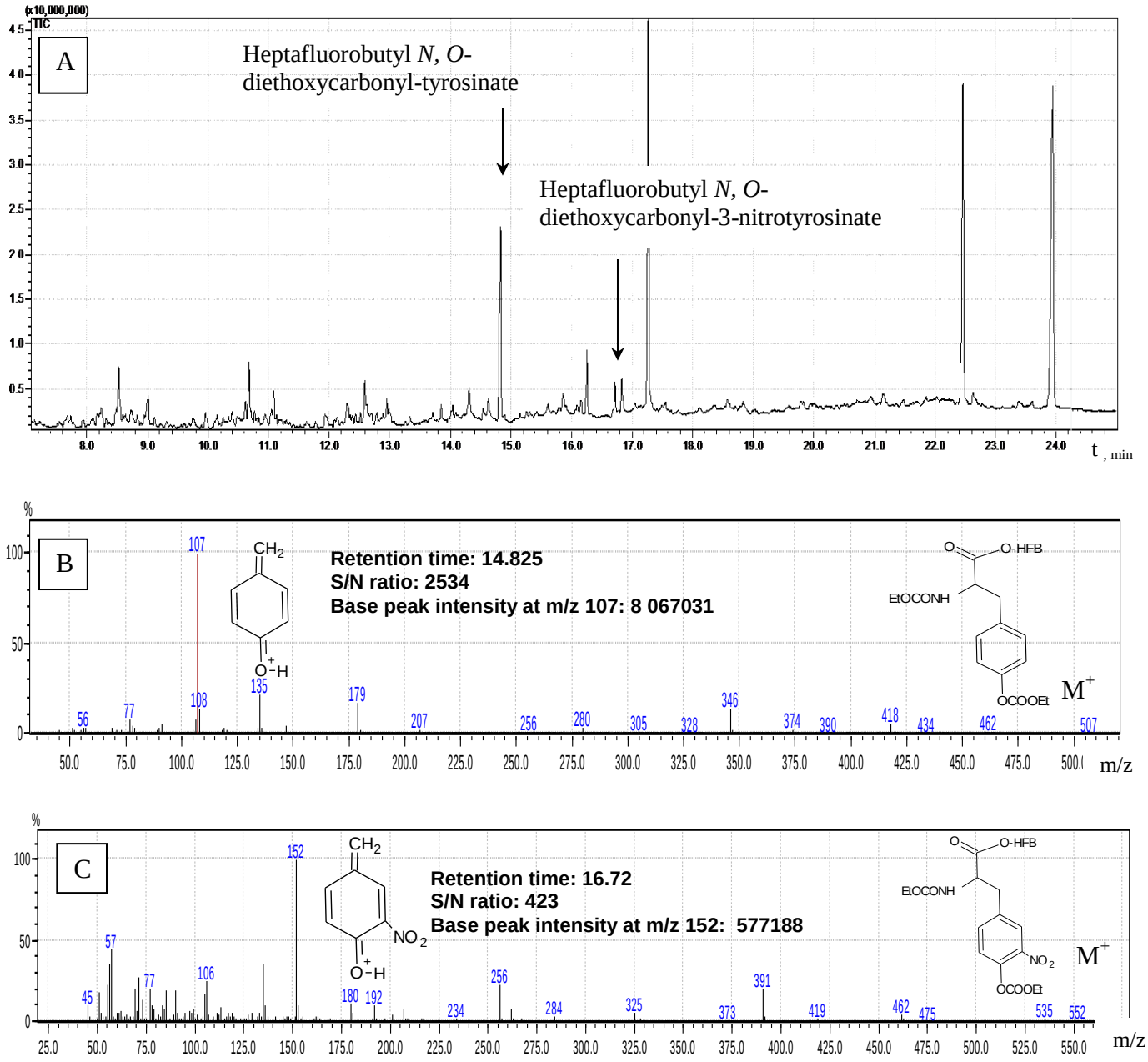


Figure 4.4. GC/EI-MS scan analysis of the *N,O*-diethoxycarbonyl heptafluorobutyl esters of NY and Y. Concentration of NY and Y was 10 mg/L.

(A) GC-MS TIC of the derivatization products. In the chromatogram the arrows indicate peaks that correspond to the NY and Y derivatives.

(B) Mass spectrum of heptafluorobutyl *N, O*-diethoxycarbonyl-tyrosinate. The molecular ion (M^+) is detected at m/z = 507. The most abundant ion at m/z = 107 corresponds to 4-hydroxybenzyl cation (Pavlovic, Biondi et al. 2012).

(C) Mass spectrum of heptafluorobutyl *N, O*-diethoxycarbonyl-3-nitrotyrosinate. The molecular ion (M^+) is detected at m/z = 552. The most abundant ion at m/z = 152 corresponds to 3-nitro-4-hydroxybenzyl cation (Pavlovic, Biondi et al. 2012).

and C). Assuming that the signal response positively correlates with the concentration of the derivatives, at S/N = 3 the limit of detection in scan mode lies roughly around 0.1 - 0.01 mg/L for Y and 0.1 mg/L for NY. To evaluate these limits experimentally, standard solutions of NY and Y with a series of dilutions were prepared as described in Section 3.3.3, subjected to derivatization and analysed by GC/EI-MS in scan mode as described in Section 3.4.2. The signal intensities were plotted against the concentration as shown in Fig. 4.5. The experimental values of the detection limits of Y and NY were found to be 0.1 mg/L and 10 mg/L respectively. These findings stand in agreement with the expected detection limit of Y and will allow quantification of plasma free Y, earlier reported to be 12 mg/L (Frost, Halliwell et al. 2000). The detection limit of NY was found to be surprisingly high (10 mg/L) and not sufficiently low to quantitate plasma free NY which has been reported to range from 0.16 to14 µg/L (Tsikas and Duncan 2014).

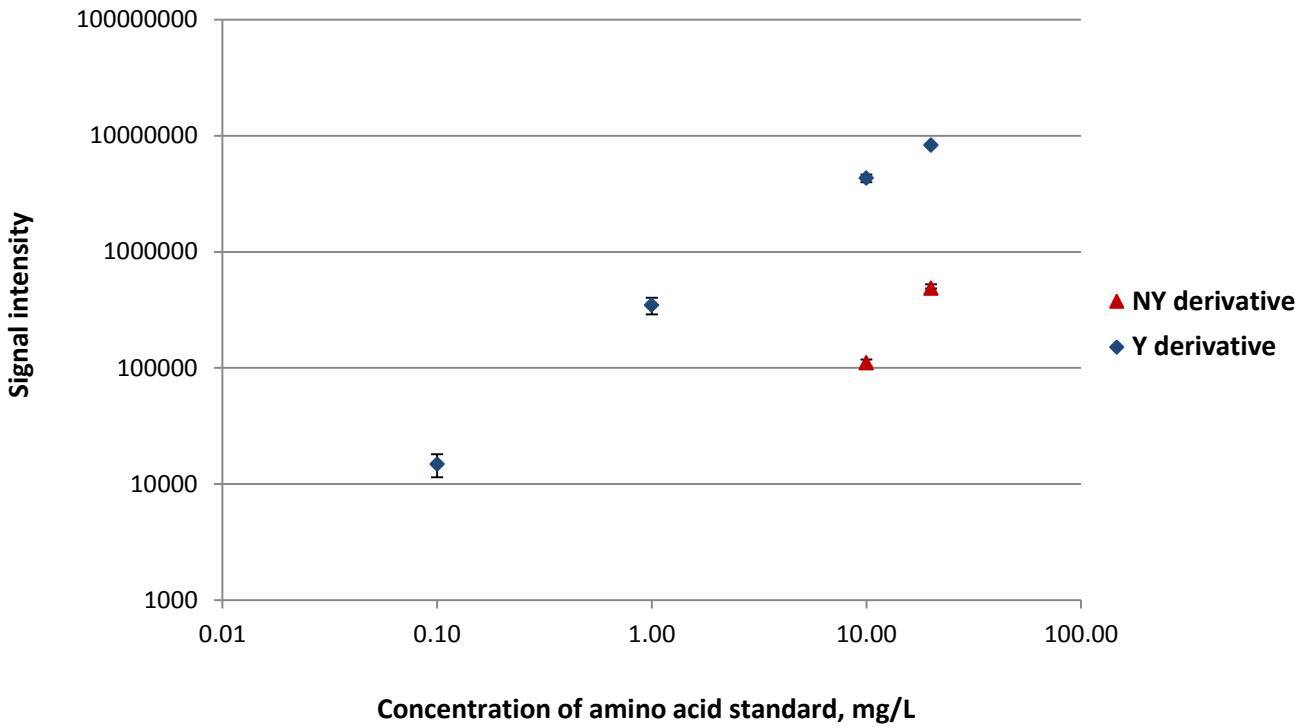


Figure 4.5. Evaluation of the detection limits of NY and Y dissolved in water. Mean values (± SD, n = 4) of signal intensities at m/z = 107 for Y and m/z = 152 for NY plotted against the concentration of Y and NY standards.

From these results, the question arises whether the NY derivative decomposes with time in the extract? To investigate this hypothesis and to evaluate stability of the derivatives in *n*-hexane the volumes of the reactants and extraction solvent were increased four times during the derivatization. The extract were portioned equally between four GC-vials and analyzed by GC/EI-MS as previously described

with two replicate injections. The aliquots of the extract were injected with four hours intervals. The GC/EI-MS analysis showed that the *N*,*O*-diethoxycarbonyl heptafluorobutyl esters of NY and Y were stable for at least for 12 hours at RT (Fig. 4.6).

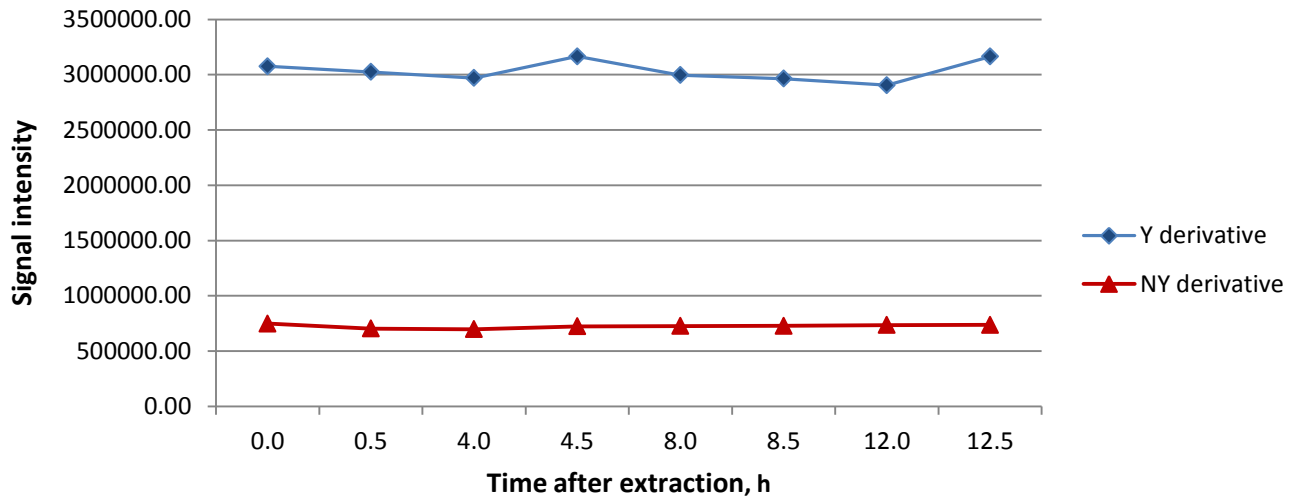


Figure 4.6. Evaluation of stability of the NY and Y derivatives in the extract. Concentration of standards was 10 mg/L. Signal intensities were measured at m/z = 107 and m/z = 157 for the Y and NY derivatives respectively.

To elucidate if decreased pH when dissolving NY and Y would increase derivatization yield, thereby increasing detection limits, the amino acids were dissolved in 0.1 M HCl instead of water with the same dilution series prior to derivatization and GC/EI-MS scan analysis. The signal intensities were plotted against the concentration (Fig. 4.7). The GC/EI-MS scan analysis showed that the decreased pH resulted in a 10-fold increase of the Y detection limit. The detection limit of NY was not altered. However, the SD of the analyte signal rose five times and the chromatograms contained numerous additional peaks. Thus, the decreased pH had a rather negative effect on the analysis of the NY derivative. Application of SIM mode during GC/EI-MS analysis provided the same detection limits as found above. Moreover, during the current research it was observed that injection of the derivatives right after the extraction without further purification had many drawbacks. Firstly, after relatively few (n = 50) injections of the derivatization products, the injection needle was broken and the GC column was contaminated, resulting in peak tailing and decreased sensitivity. The gradual degradation of the GC column would eventually influence the reliability of the whole analytical procedure. Attempts to evaporate excess solvent and reagents under vacuum after the derivatization and then reconstitute the derivatives in acetonitrile before GC/MS analysis lowered the signal intensity

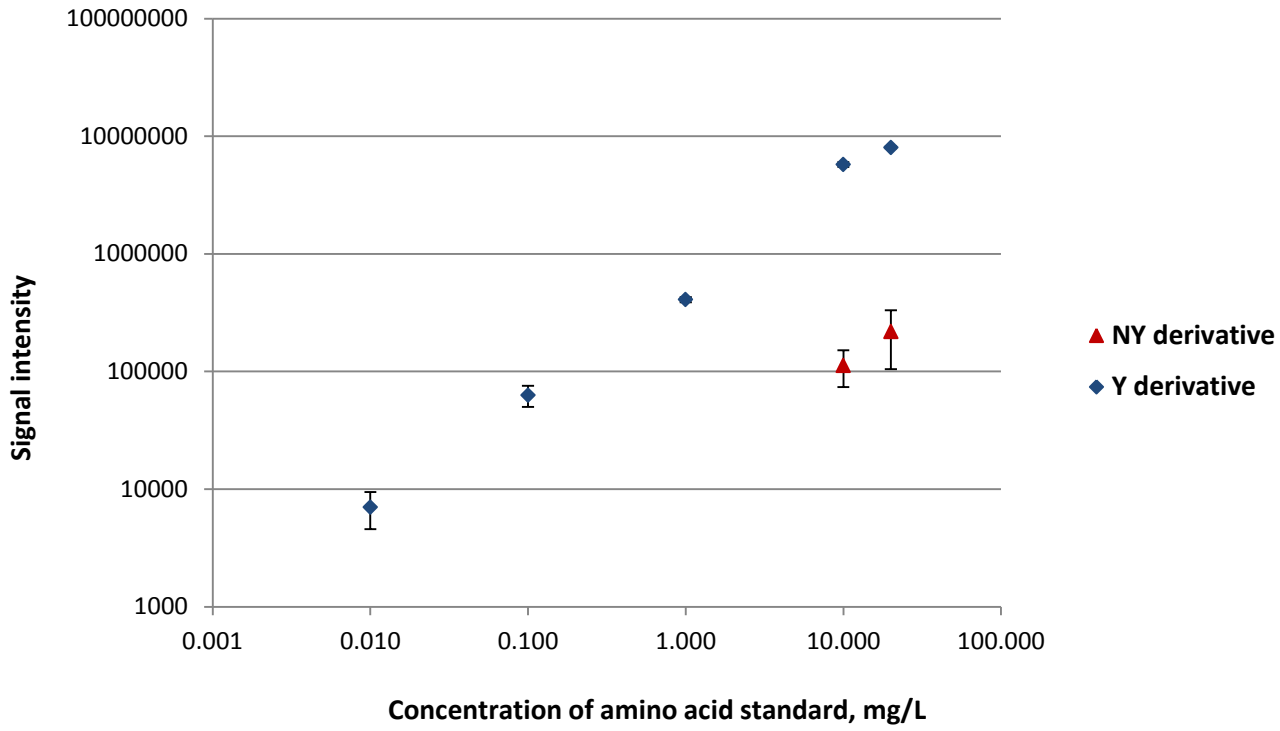


Figure 4.7. Evaluation of the detection limits of NY and Y dissolved in 0.1 HCL. Mean values (± SD, n = 4) of signal intensities at m/z = 107 for the Y derivative and m/z = 152 for the NY derivative plotted against the concentration of Y and NY standards.

to a tenth. Secondly, pipetting the sample from the lower phase (Fig. 4.3 A) might contaminate the organic phase with small amounts of water and distort the chromatographic separation. So, further application of the current derivatization method was questionable unless a better extraction solvent was found. The use of dichloromethane or ethyl acetate as a solvent could be a good alternative to *n*-hexane. Dichloromethane has a higher density than water and the extract should form only two phases: aqueous on the top and organic at the bottom. Ethyl acetate has much in common with dichloromethane in terms of solvent properties (similar polarity index and immiscible with water) (J.T. Baker Inc., 1988), but has lower density than water forming the organic phase in the upper layer of the reaction mixture. Consequently, an aqueous mixture of NY and Y standards with a concentration of 10 mg/L of each amino acid was subjected to derivatization as previously: one aliquot was extracted with dichloromethane; the other aliquot was extracted with ethyl acetate. The reaction mixtures formed only two phases in both cases. However, in the case of dichloromethane the organic phase was observed at the bottom and in the case of ethyl acetate it was formed at the top of the extraction mixture as expected.

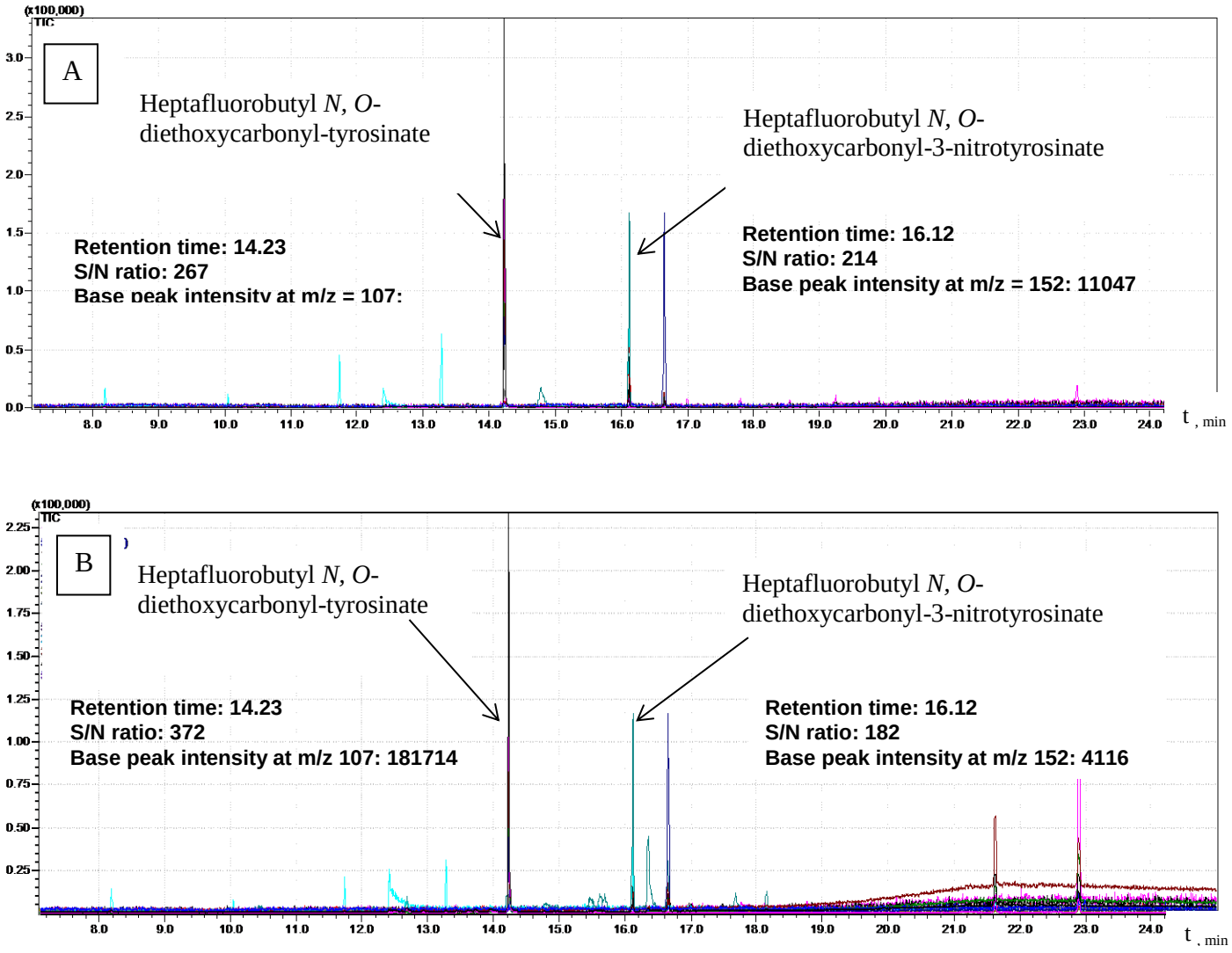


Figure 4.8. GC/EI-MS SIM comparative analysis of NY and Y as their *N,O*-diethoxycarbonyl heptafluorobutyl esters extracted with different solvents. Concentration of NY and Y was 10 mg/L. The arrows indicate peaks that correspond to the NY and Y derivatives.

(A) GC-MS SIM chromatogram of the *N,O*-diethoxycarbonyl heptafluorobutyl esters extracted with ethyl acetate.

(B) GC-MS SIM chromatogram of the *N,O*-diethoxycarbonyl heptafluorobutyl esters extracted with dichloromethane.

Comparison of GC/EI-MS signal responses for the Y and NY derivatives in SIM mode extracted by three different solvents showed decreasing response for *n*-hexane, ethyl acetate and dichloromethane, respectively (Fig. 4.8 and Fig. 4.9). The signal intensities in ethyl acetate were slightly higher than in dichloromethane (Fig. 4.9). Both S/N and total signal intensities for Y and NY derivatives were clearly improved in *n*-hexane compared to ethyl acetate and dichloromethane (Fig. 4.8 and 4.9).

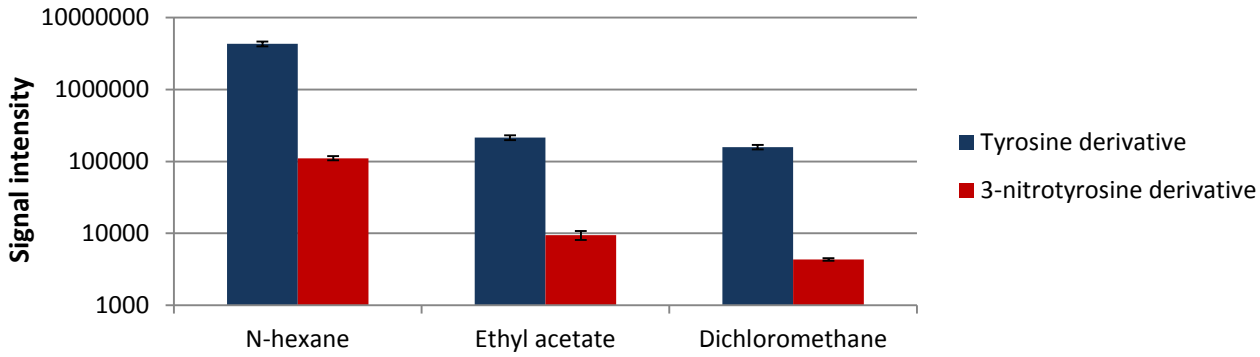


Figure 4.9. Comparison of the signal responses of the Y and NY derivatives in different extraction solvents (± SD, n = 4).

These results suggested that use of a new solvent would not provide better sensitivity of the method. It followed that the more sensitive GC/MS analysis usually achieved by negative chemical ionization (GC/NCI-MS) was considered worth trying since the derivatives contained several fluorine atoms. Thus, NY and Y standard solutions with concentrations 10, 1 and 0.1 mg/L were subjected to derivatization following the protocol in Section 3.3.3, however, ethyl acetate was used as an extraction solvent instead of *n*-hexane. The derivatization products extracted with ethyl acetate might cause less contamination of the GC column. The GC/NCI-MS analysis of the NY and Y derivatives was conducted as described in Section 3.4.2. The GC/NCI-MS TIC of the standards at a concentration of 10 mg/L had two peaks at t_R = 11.49 and 12.95 min (Fig. 4.10 A), which were not observed in the water blank. The peak at t_R = 11.49 min was identified as the derivatized Y product from the mass spectrum (Fig. 4.10 B) with base peak at m/z = 418 which corresponds to $[M\text{-}OCOOEt]^-$. The peak at t_R = 12.95 was identified as the derivatized NY product (Fig. 4.10 A). In the mass spectrum (Fig 4.10 C) the peak at m/z = 479 corresponds to $[M\text{-}OCOEt]^-$, but the ion that formed the base peak at m/z = 418 was not identified. The GC/NCI-MS TIC of standard solutions with 1 mg/L of NY and Y had one peak at t_R = 12.95 (apparently the NY derivative) and no peaks at a concentration of 0.1 mg/L. Unfortunately, further research was cancelled due to ongoing degradation of the GC column and increasing tailing of the peaks formed by the derivatives. On any account, the GC/MS scan analysis in NCI mode proved to be ten times more sensitive than in EI mode in terms of NY determination. NY was detected at 1 mg/L compared to 10 mg/L in EI mode. For Y the result was just the opposite. The Y derivative was not detected at a concentration lower than 10 mg/L. Lack of peaks at a concentration of 0.1 mg/L (while 0.0001 mg/L was required for NY) suggested that this derivatization method would not be sensitive enough in terms of quantification of free NY and Y in human plasma.

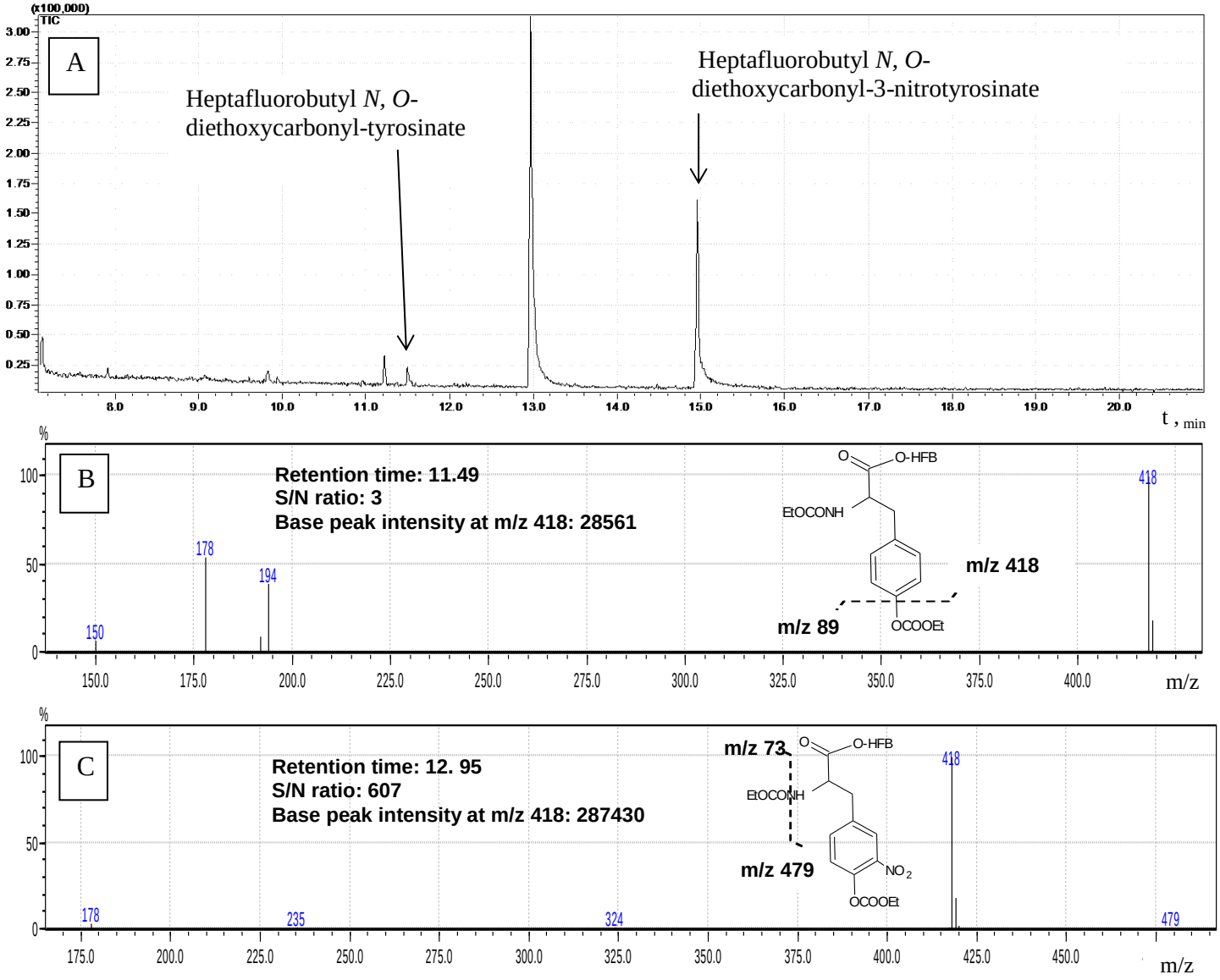


Figure 4.10. GC/NCI-MS scan analysis of the *N,O*-diethoxycarbonyl heptafluorobutyl esters of NY and Y. Concentration of NY and Y was 10 mg/L.

(A) GC-MS TIC of the derivatization products. The arrows indicate peaks that correspond to the NY and Y derivatives.

(B) Mass spectrum at t_R = 11.49 of heptafluorobutyl *N, O*-diethoxycarbonyl-tyrosinate. The base peak corresponds to [M - OCOOEt]$^-$. Molecular ion (M) of the Y derivative was not observed. The dotted line indicates a probable site of fragmentation.

(C) Mass spectrum at t_R = 12.95 of heptafluorobutyl *N, O*-diethoxycarbonyl 3-nitrotyrosinate. The ion at m/z = 479 corresponds to [M - OCOEt]$^-$. Molecular ion (M) of the NY derivative was not observed. The dotted line indicates a probable site of fragmentation.

4.1.4 Derivatization with heptafluorobutyric anhydride and trimethylsilyldiazomethane

Standard solutions containing 10 mg/L of NY and Y were prepared as described in Section 3.2.1 and subjected to derivatization following the protocol in Section 3.3.4. GC/EI-MS analysis was conducted as described in section 3.4.3 for EI mode. EI mode was applied to obtain structural information about the NY and Y derivatization products from the fragmentation pattern in the mass spectrum. The GC/EI-MS analysis of the standards confirmed formation of the NY and Y derivatives. The GC/EI-MS scan chromatogram contained three peaks at t_R = 10.20; 10.915 and t_R = 12.08 (Fig. 4.11 A). According to the mass spectra in Fig. 4.11 B and C, Y formed two major products: the expected methyl *O*-methyl-*N*-heptafluorobutyryl - tyrosinate (DMHFB derivative of Y) (Fig. 4.11 C) and one more product, identified as methyl *O*-trimethylsilyl-*N*-heptafluorobutyryl tyrosinate (MTMSHFB derivative of Y) (Fig. 4.11 B). After fragmentation the MTMSHFB derivative of Y gave rise to 4-hydroxybenzyl cation as the base peak at m/z = 107 (Fig. 4.11 B) discussed earlier in Section 4.1.3 (Fig. 4.4 B). The DMHFB derivative of Y produced presumedly a 4-methoxybenzyl cation with the base peak at m/z = 121 (Fig. 4.11 C). NY formed only the expected methyl *O*-methyl-*N*,*N*-diheptafluorobutyryl-3-aminotyrosinate (DMHFB derivative of NY) as a major product and produced a 3-heptafluorobutyramido-4-methoxybenzyl cation with the base peak at m/z = 332 (Fig. 4.11 D). The peaks were observed at concentrations of 10 mg/l and 1 mg/L of NY and Y. The peak formation at a concentration of 0.1 mg/L and lower was not detected in the scan mode. To evaluate the detection limits of NY and Y, GC-MS analysis was performed in NCI mode as described in Section 3.4.3 for NCI mode. The GC/NCI-MS TIC chromatogram contained two peaks at t_R = 10.3 and t_R = 11.2 for Y and NY derivative respectively (Fig. 4.12 A). The GC/NCI-MS MC of the Y derivative at t_R =10.3 at m/z = 371 and 443 revealed an overlap of two peaks. The formation of two Y derivatives confirmed the findings during GC/EI-MS analysis discussed in this section above. The fragmentation patterns of the Y derivatives confirmed the formation of the DMHFB derivative with the base peak at m/z = 371 $[M\text{-}HF\text{-}CH_2]^-$ and the MTMSHFB derivative with the base peak at m/z=443 $[M\text{-}HF]^-$ (Søderling, Ryberg et al. 2003) (Fig. 4.12 C and D). The mass spectrum of the NY derivative confirmed formation of the DMHFB derivative as a major derivatization product, previously reported by Søderling, Ryberg et al. (2003) (Fig. 4.12 E).

To clarify if Y indeed formed two derivatives, a standard solution containing 1 mg/L of Y was added 0.1 mg/L of d_4-Y as an internal standard before derivatization. Subsequent GC-/NCI-MS analysis

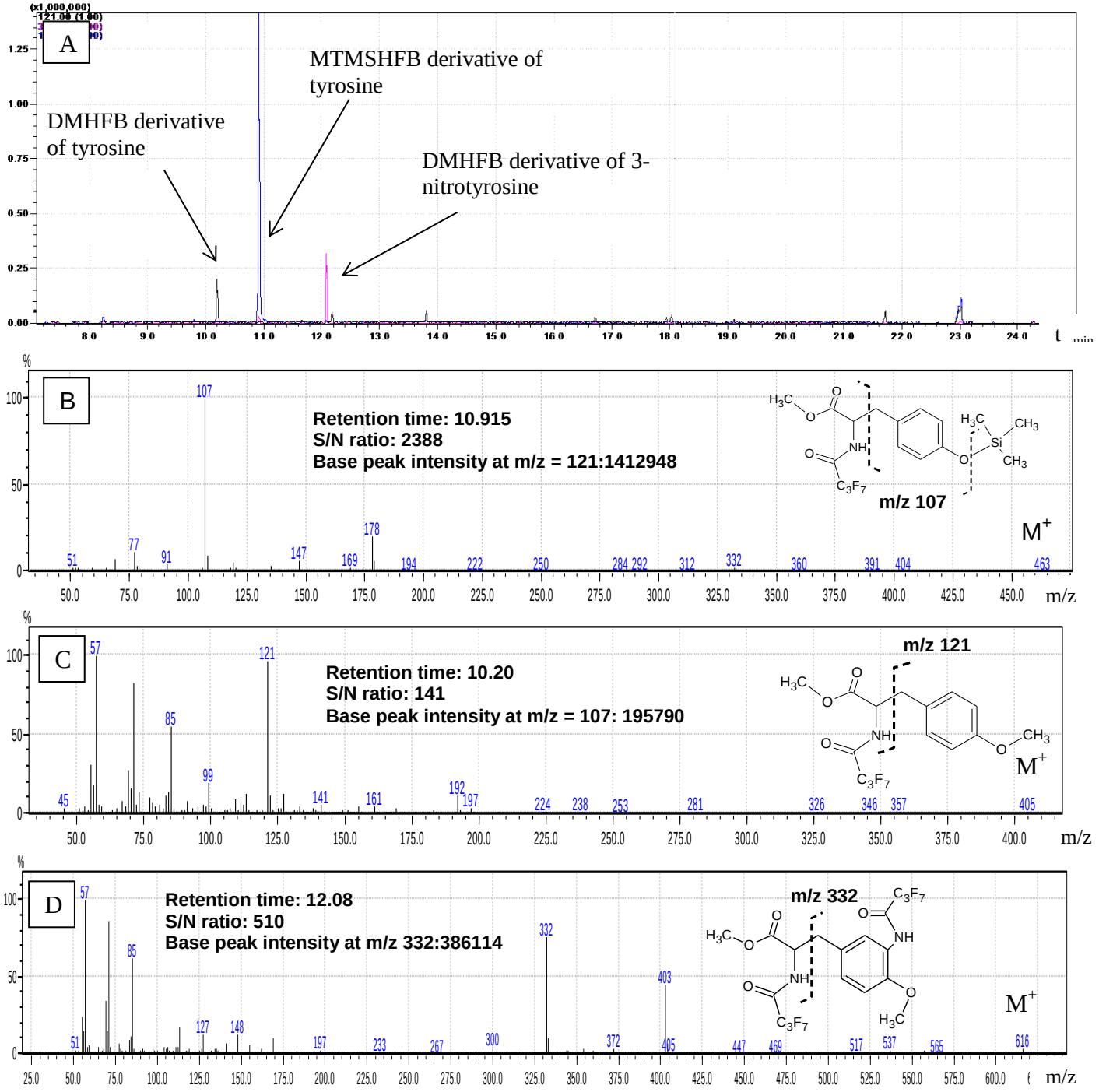


Figure 4.11. GC/EI-MS scan analysis of NY and Y derivatives. Concentration of NY and Y was 10 mg/L. The molecular ion is marked by M; the dashed line crossing the molecular structures shows putative sites of fragmentation.

(A) GC/EI-MS MC at m/z = 107, 121, 332. The arrows indicate peaks that correspond to the NY and Y derivatives.

(B) Mass spectrum of MTMSHFB derivative of Y at t_R = 10.915.

(C) Mass spectrum of DMHFB derivative of Y at t_R = 10.20.

(D) Mass spectrum of DMHFB derivative of NY at t_R = 12.08.

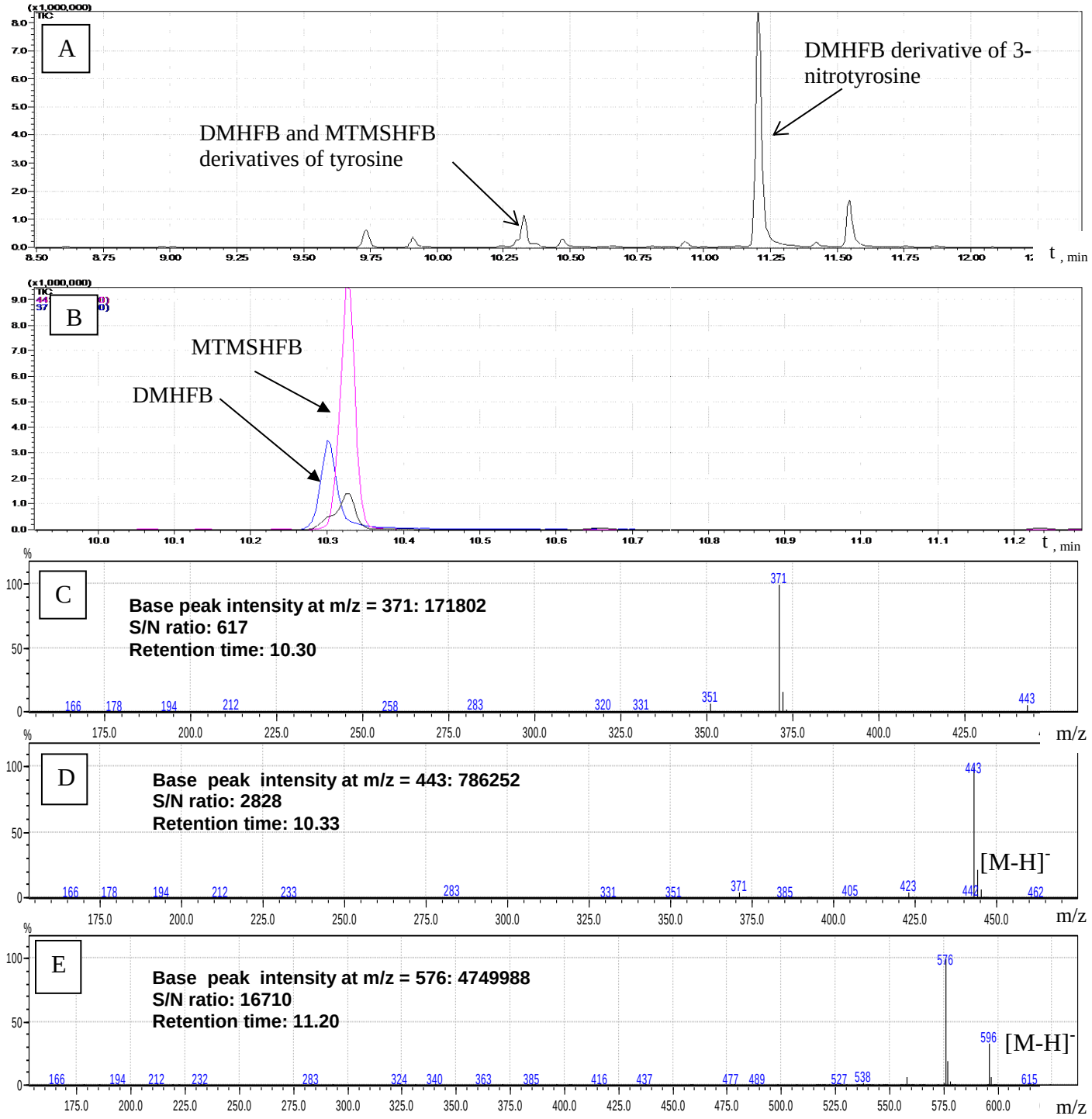


Figure 4.12. GC/NCI-MS scan analysis of the derivatization products. Concentration of NY and Y was 10 mg/L. The molecular ion is marked by M.

(A) GC/NCI-MS TIC of the derivatization products. The arrows indicate peaks that correspond to NY and Y derivatives.

(B) GC/NCI-MS MC at m/z = 371 and 443. The arrows indicate peaks that correspond to Y derivatives.

(C) Mass spectrum of the DMHFB derivative of Y at t_R = 10.30.

(D) Mass spectrum of the MTMSHFB derivative of Y at t_R = 10.33.

(E) Mass spectrum of the DMHFB derivative of NY at t_R = 11.20.

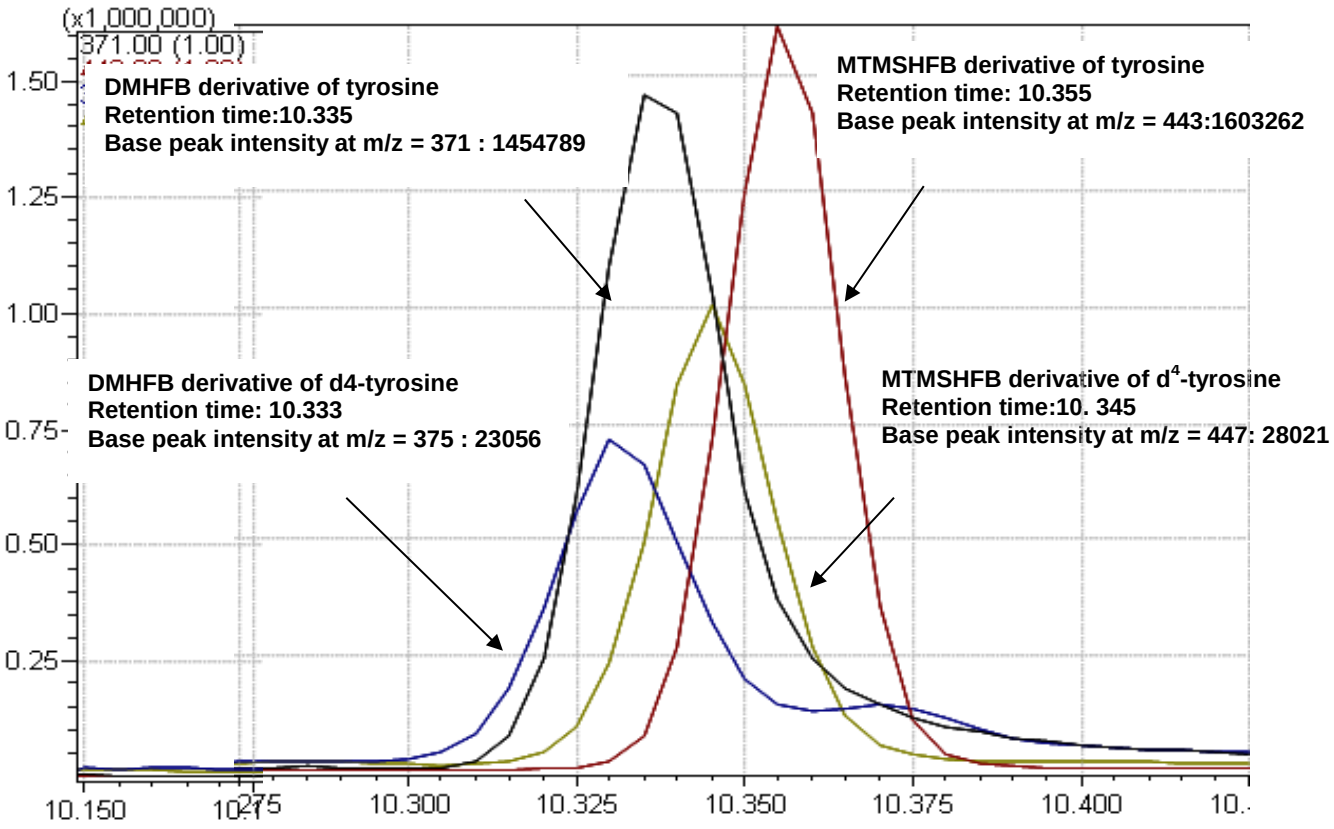


Figure 4.13. GC/NCI-MS SIM analysis of the Y derivatives. Concentration of Y and d_4-Y was 1 and 0.1 mg/L respectively.

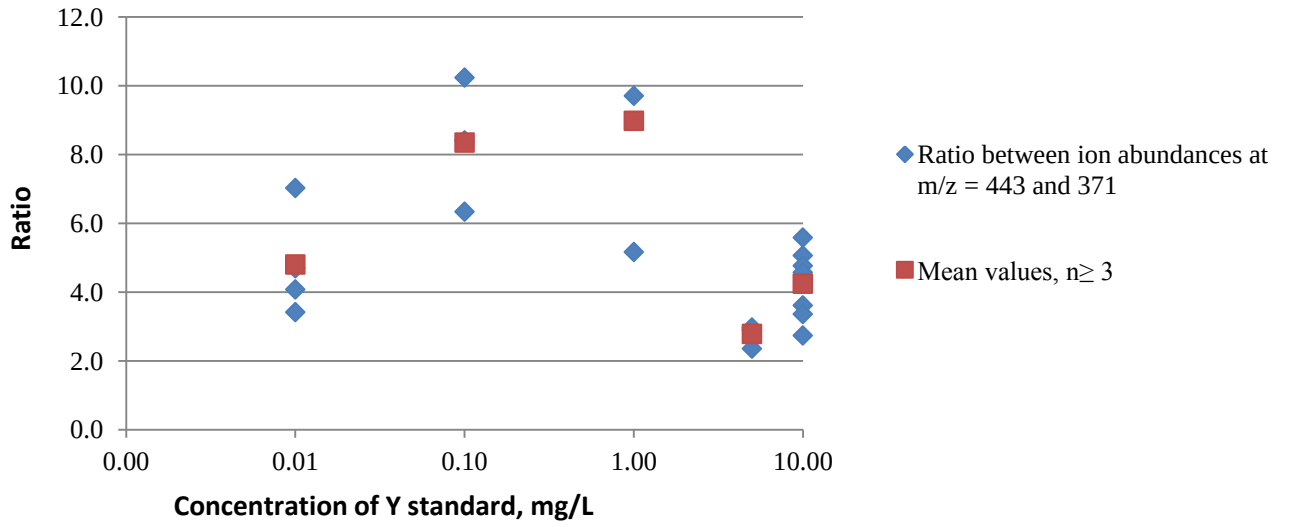


Figure 4.14. Ratio between signals of the two Y derivatives versus Y concentration. Values of the signal intensities were taken from GC/NCI-MS SIM analyses.

confirmed formation of two Y derivatives (Fig. 4.13). The MTMSHFB derivatives of both Y and d_4-Y had the stronger signals than their DMHFB derivatives. Thus, the MTMSHFB derivative with the base peak at m/z =443 could represent Y in the GC/NCI-MS analysis, provided a ratio between signals at m/z =371 and 443 was constant. The ratio was plotted against the concentration of Y (Fig. 4.14). However, its extensive variation would not allow the use of only one derivative for Y quantification. Accordingly, the standard curve of Y was plotted as a sum of the signal intensities versus different concentrations of Y (Fig. 4.15). The standard curve had good linearity in the range 10 - 0.01 mg/L, where R^2= 0.9971. At a concentration of 0.001 mg/L and less, the signal was constant and had the same value as in the blank.

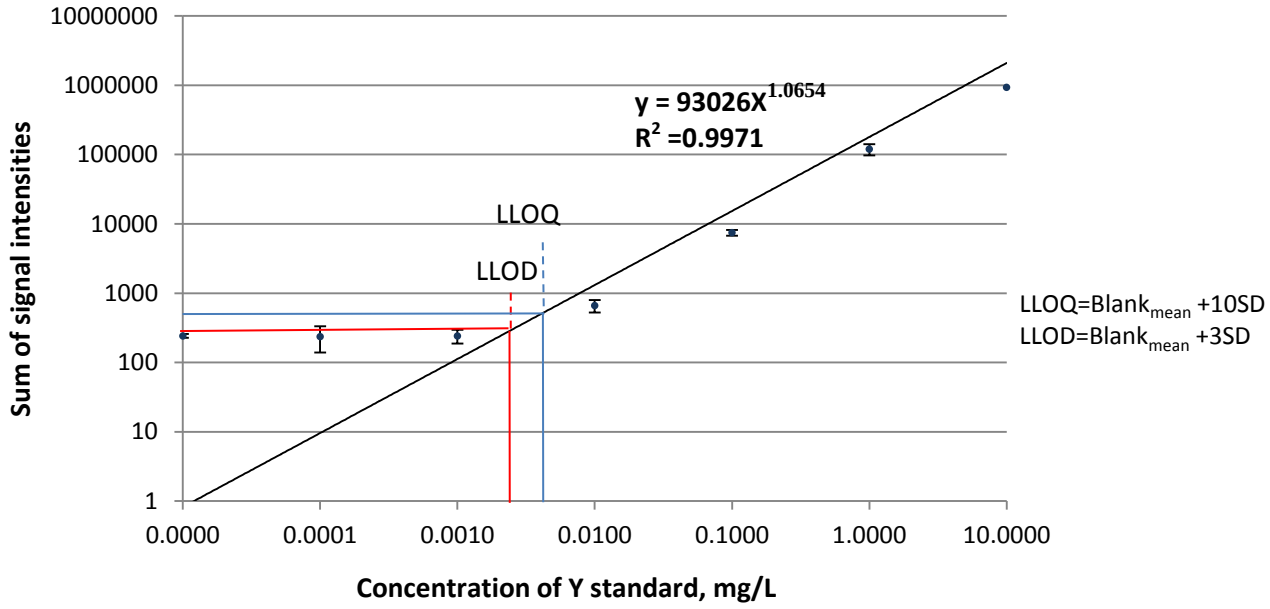


Figure 4.15. Standard curve of Y. Mean values (± SD, n = 4) of the sum of signal intensities of the Y derivatives at m/z = 371 and 443 plotted versus the concentration of Y standard.

According to Skoog, Holler et al. (2007, p.21), when a signal in a blank is different from 0 the minimum distinguishable analytical signal can be assumed as the sum of the mean blank signal plus 3 times the SD of the blank. The calculated value of the minimum distinguishable analytical signal substituted into the trendline equation gives LLOD. LLOQ is usually taken as the concentration of the analyte when the signal equals the mean blank signal plus 10 times the SD of the blank (Fig. 4.15) (Skoog, Holler et al. 2007) and then calculated similarly to LLOD.

The standard curve of NY (Fig. 4.16) was plotted following the description in Section 3.3.4. The standard curve had good linearity (R^2 = 0.9903) in the range 1 - 0.0001 mg/L. At a concentration

of 10mg/L the signal was too high and could not be measured adequately. It was therefore not included. The LLOD and LLOQ of NY were calculated in the same manner as for Y, due to

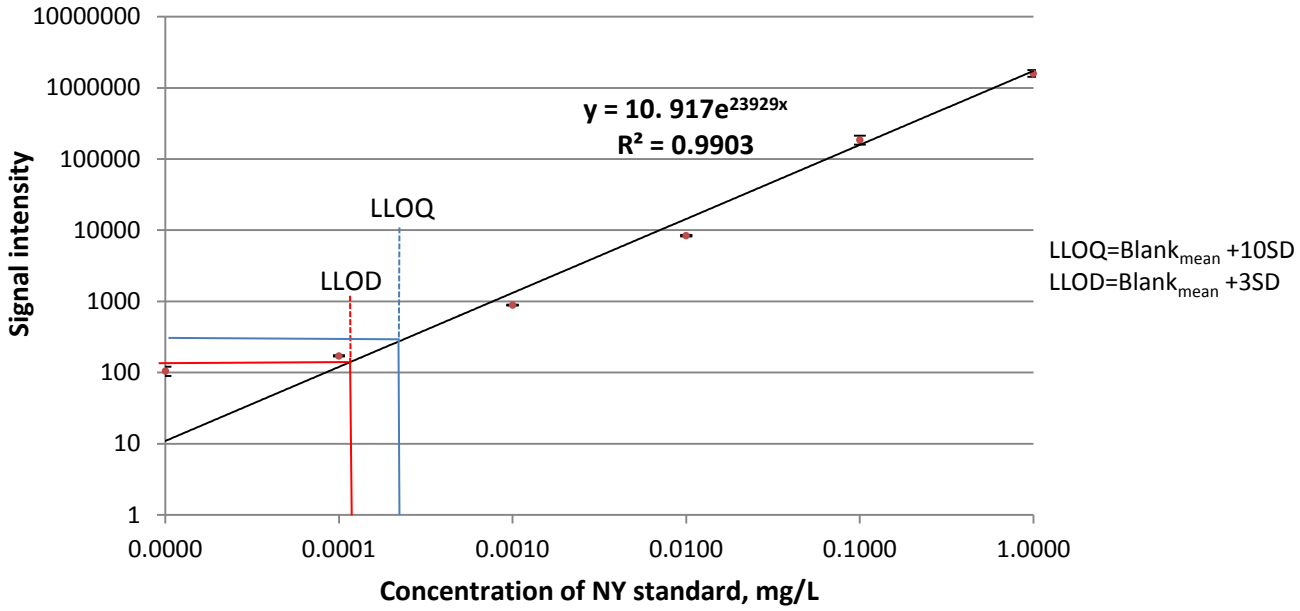


Figure 4.16. Standard curve of NY. Mean values (± SD, n = 4) of signal intensities at m/z = 576 plotted versus the concentration of NY standard.

the background signal at m/z= 152 when analyzing the blank control. The LLOD and LLOQ of NY and Y are summarised in Table 4.1.

Table 4.1. Important analytical values for tyrosine and 3-nitrotyrosine

Name of analyte	LLOD//LLOQ calculated	Reported level in plasma	Reference
Tyrosine	4 µg/L // 6 µg/L	12 mg/L	Frost, Halliwell et al. (2000)
3-Nitrotyrosine	0.11 µg/L // 0.13 µg/L	14 - 0.16 µg/L	Tsikas and Dunkan (2014)

Even though the LLOQ of Y is fully acceptable for its quantitation in plasma, the formation of two derivatives whose peaks overlap each other makes this derivatization method less than optimal. In addition, it may influence the accuracy of Y determination by GC/NCI-MS analysis. The LLOQ of NY in the current method is hundred times below the upper limit and almost at the lower limit of the

reported free NY concentrations in human plasma (Table 4.1). This fact may put into question the application of this method. However, adjusting the temperature gradient in the GC-MS program may improve separation of the peaks, lower the noise level, allow detection and possibly quantification of plasma free NY. The application of this derivatization method to human plasma is discussed in the following section.

4.2 Determination of free 3-nitrotyrosine in human plasma

This section focuses mainly on NY analysis since the derivatization step did not provide a single Y derivative suitable for GC/NCI-MS analysis. Prior to derivatization, plasma free NY was extracted using SPE (Section 3.2.3). A comparative analysis of Strata-X and Starata-X-C tubes was conducted in order to choose the optimal tubes for NY isolation. The samples for the analysis were prepared following the description in Section 3.2.3. under **Comparison of SPE tubes**, then subjected to derivatization and analysed by GC/NCI-MS as previously described in Section 3.4.3. Before discussing the results shown in Fig. 4.17 A and B, two assumptions should be made: 1) the signal

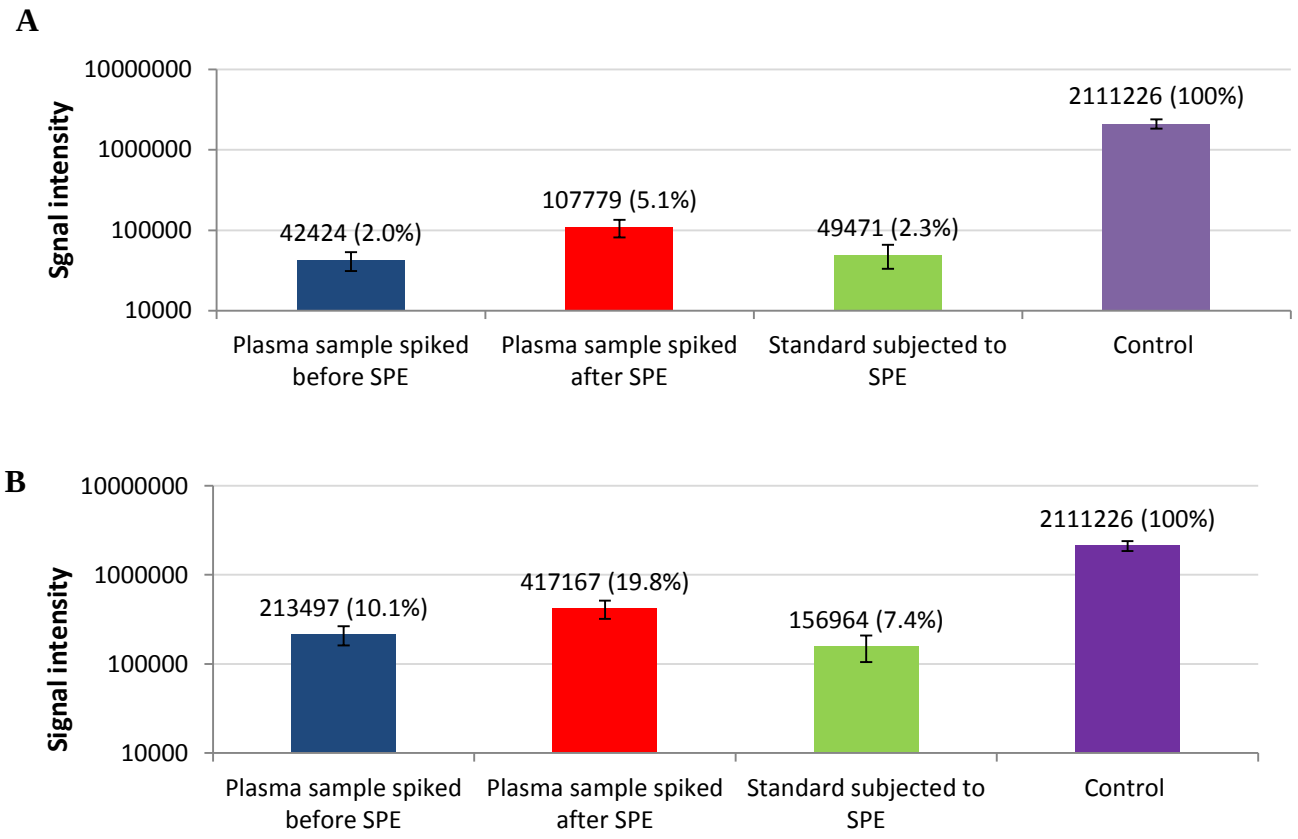


Figure 4.17. Comparison of SPE tubes. Plasma samples were spiked with 1 mg/L of NY, the concentration of NY in standard was 1 mg/L. The standard not subjected to extraction used as a control. The signal intensity of the NY derivative was measured at m/z = 576.

(A) Samples subjected to extraction with Strata-X-C tubes. The diagram shows the mean signal intensity (± SD, n = 4) at m/z = 576.

(B) Samples subjected to extraction with Strata-X tubes. The diagram shows the mean signal intensity (± SD, n = 3) at m/z = 576.

intensity of the control is equal to 100%, and 2) the concentration of endogenous plasma NY (16 - 0.14 µg/L (Tsikas and Dunkan 2014)) is negligible compared to a spike of 1mg/L. Given these assumptions the overall recovery of NY in plasma samples and standards was expected to be at least 50% (considering loss of NY during extraction and the matrix effect). The overall recovery of NY and the matrix effect on NY recovery was defined by Equations 1 and 2 respectively:

$$\%RE_{total} = \frac{Responce\ of\ extracted\ spiked\ plasma\ sample/standard\ solution}{Responce\ of\ a\ control} \text{ x } 100 \quad (1)$$

$$\%ME = \frac{Responce\ of\ \ plasma\ sample\ spiked\ after\ extraction}{Responce\ of\ a\ control} \text{ x } 100 \quad (2)$$

A comparison of SPE cartridges showed that the plasma samples and the standard solution loaded into Strata-C-X tubes had unexpectedly low signals compared to the control (Fig. 4.17 A). The overall recovery of NY in the plasma samples was 2.0%, while in the standard solution this value was 2.3% (Fig. 4.17 A). The recovery of NY with respect to the matrix effect was 5.1 %. Hence, the extraction with Strata-X-C tubes in combination with the derivatization reaction yielded an insufficient amount of the derivatization product. It is apparent that the strong cationic exchange mechanism of the Strata-X-C sorbent uses ammonium hydroxide as an eluent. A significant amount of ammonium hydroxide is always present in the elution fraction and may react with NY, influencing the derivatization reaction yield, thereby lowering the signal strength. Neutralization of ammonium hydroxide by adding hydrochloric acid in the eluate prior to evaporation resulted in the formation of an overabundance of salt crystals, making derivatization impossible to perform. The application of Strata-X-C tubes was therefore rejected.

The overall recovery of NY in the plasma samples and the standard solution subjected to extraction with Strata-X tubes was 10.1% and 7.4% respectively. The recovery of NY with respect to the matrix effect was 19.8 % (Fig. 4.17 B) indicating that the plasma matrix reduced the derivatization reaction yield approximately five times. Nevertheless, the overall recovery of NY in the plasma samples extracted with Strata-X tubes was five times higher (10.1%) compared to Strata-X-C tubes (2.0%). This finding made Strata-X tubes more suitable for NY determination, facilitated by the current derivatization reaction. However, further experiments showed that water blanks subjected to extraction with these tubes prior to derivatization and GC/NCI-MS analysis produced a significant signal at the

same m/z = 576 and t_R as the NY derivative. The signal interfered with the analyte signal and influenced the accuracy of the measurement. Attempts to minimize or eliminate this interference by varying a temperature gradient (Δt = 15 °C/min, 10 °C/min, 5 °C/min) in the GC program had no effect, while in the water blanks not subjected to extraction the interfering signal decreased by 72% when Δt was changed from 15 °C/min to 10 °C/min. This artefact can be associated with the Strata-X sorbent properties making this SPE tool not applicable for NY isolation at micro- and nanogram levels. To test the sensitivity of Strata-X sorbent, water blanks and plasma samples spiked with 0.0, 0.1, 0.5 and 1.0 µg/L of NY were subjected to extraction with subsequent derivatization and analyzed by GC/NCI-MS. Water blanks not subjected to extraction were used as a control (Fig. 4.18).

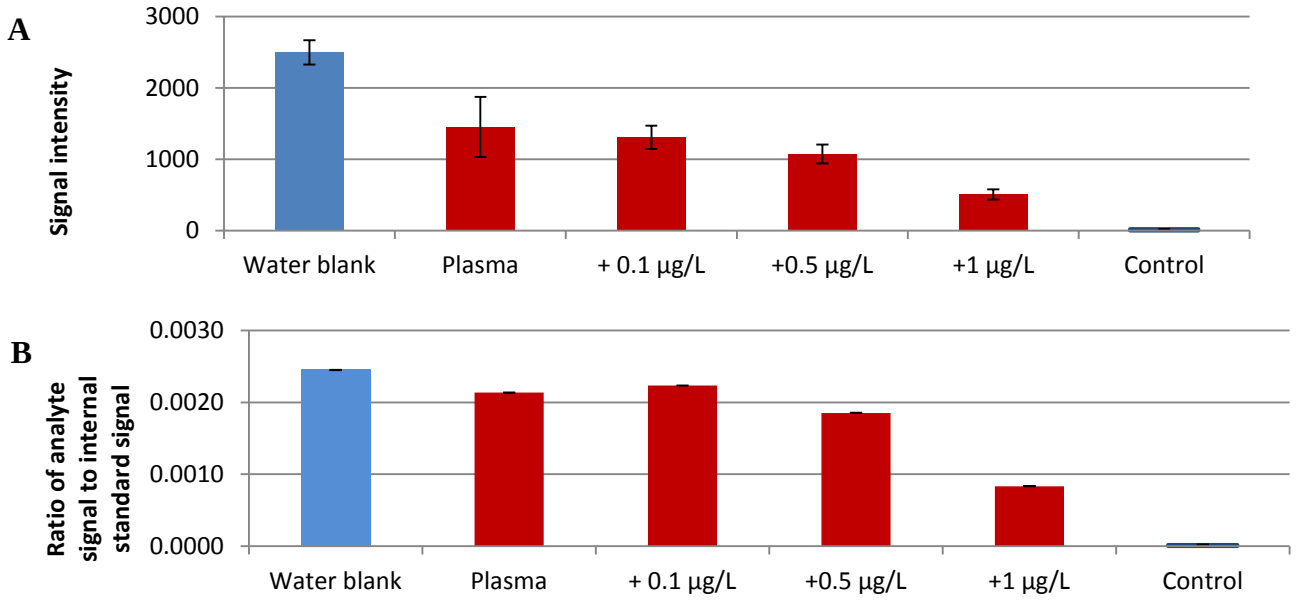


Figure 4.18. Analysis of sensitivity of Strata-X tubes. Water blanks and plasma samples spiked with 0.0, 0.1, 0.5 and 1.0 µg/L of NY were subjected to extraction, derivatization and analysed by GC/NCI-MS using Δt = 10 °C/min. Water blanks not subjected to extraction were used as a control. The plasma samples and the water blanks were spiked with 1 mg/L of d_3-NY as an internal standard.

(A) The mean signal intensity (± SD, n = 4) at m/z = 576.

(B) The mean ratio (± SD, n = 4) of the analyte signal at m/z = 576 to the internal standard signal at m/z = 579.

The GC/NCI-MS analysis did not reveal any logical correlation between added NY and signal strength (Fig. 4.18 A), and the spiked samples did not show an increase in signal as expected. Addition of an internal standard confirmed these findings (Fig. 4.18 B). Moreover, the water blanks subjected to extraction had the highest signal (while the opposite was expected). These findings imply that the

analyte signal at concentrations between 0.1 and 1 µg/L of NY cannot be distinguished, and that isolation of NY from human plasma with Strata-X tubes does not provide sufficient sensitivity of the analytical procedure.

5. Concluding summary

Four methods of derivatization of NY and Y were investigated in both EI and NCI modes during the development of an optimal procedure for simultaneous determination of free NY and Y in human plasma by GC/NCI-MS. Three one-step and one four-step derivatization strategy were evaluated:

1) Derivatization with PFBBr mediated by DIPEA with formation of triPFB derivatives of NY and Y;
2) Derivatization with PFBBr mediated by crown ether and K_2CO_3 with formation of triPFB derivatives of NY and Y;
3) Derivatization with EtCF and HFBOH leading to formation of *N,O*-diethoxycarbonyl heptafluorobutyl esters of NY and Y;
4) Four-step method based on reduction of the nitro group of NY by dithionite with subsequent derivatization with HFBA and TMSD and formation of methyl *O*-methyl-*N,N*-diheptafluorobutyryl-3-aminotyrosinate and two Y derivatives: methyl *O*-methyl-*N*-heptafluorobutyryl-tyrosinate and methyl *O*-trimethylsilyl-*N*-heptafluorobutyryl-tyrosinate.

Evaluation of the derivatization reactions indicated that the first two procedures failed to form NY and Y derivatives. The reaction with PFBBr was performed in organic solvents, and both NY and Y dissolved poorly in the available organic solvents. The last two procedures were performed in an aqueous phase, which probably ensured formation of the derivatives. The derivatization reaction with EtCF/HFBOH gave stable derivatives. Both their structures and fragmentation patterns were studied in EI scan mode and were in agreement with former findings (Pavlovich, Biondi et al. 2012). The approximate limits of detection of NY and Y were estimated as 10 mg/L and 0.01 mg/L in EI scan mode and 1 mg/L and 10 mg/L in NCI scan mode respectively. Analysis in EI SIM mode did not improve the detection limits. The GC/MS scan analysis in NCI mode provided limits of detection only for comparison with EI scan analyses. GC/NCI-MS analysis in SIM mode was not conducted due to GC column degradation after injection of the derivatization products. Although many attempts to optimise the derivatization procedure were made, the findings indicated that it is not possible to improve the limits of detection as long as the derivatization products gradually degrade the GC column, thereby decreasing its sensitivity. The method was therefore considered as unsuitable for determination of plasma free NY.

The derivatization products obtained in the four-step derivatization were at first studied by GC/MS in EI mode. It was revealed that NY formed a single derivative, while Y formed two derivatives. The NY derivative and Y derivatives possessed good stability when stored in acetonitrile at 4 °C. Further GC/MS analysis in NCI mode showed that the ratio between the responses of the Y derivatives was random. Moreover, the peaks produced by these derivatives overlapped each other, making the quantitation of Y inaccurate even if a sum of the signals was used. Nevertheless, LLOD and LLOQ were evaluated for both amino acids. The LLOD and LLOQ of NY were calculated to be 0.11 µg/L and 0.13 µg/L respectively. These values indicated that the given derivatization procedure could be used in determination of plasma free NY by GC/NCI-MS. LLOD and LLOQ of Y were calculated to be 4 µg/L and 6 µg/L respectively. Even though these limits are fully acceptable in terms of determination of plasma free Y, the precise quantitation requires either a single derivatization product or at least a constant and concentration independent relationship between the two products.

Isolation of NY turned out to be a crucial step for the whole analytical procedure. Two SPE methods were tested to ensure the maximum recovery of NY. Cation exchangers (Strata–X-C), based on retention of basic compound, were previously reported to have an excellent retention capacity in terms of amino acid isolation. UV-spectroscopy tests with standard solutions of NY and Y confirmed this fact. Nevertheless, after application of these cartridges before NY derivatization a significant drawback was discovered. Release of ammonium hydroxide during the elution of NY and its presence in the derivatization mixture reduced the reaction yield, and thus, the analyte signal. The overall recovery of NY in plasma therefore was only 2.0%. The other type of SPE tubes (Strata-X) with enhanced polar and aromatic selectivity showed five times higher recovery of NY in plasma samples (10.1%), but still insufficient for NY quantitation at micro- and nanogram levels. It was also ascertained that the plasma matrix decreased the analyte signal to one fifth.

Even though it was a challenging endeavour to establish a new analytical procedure based on GC/NCI-MS analysis, a great deal was discovered about the physicochemical properties of NY and Y. Their derivatization products were carefully examined using advanced equipment and techniques. The very latest SPE tubes were tested on NY and Y standards and plasma samples. Some results were in accordance with the previously reported studies (Pavlovich, Biondi et al. 2012; Söderling, Ryberg et al. 2003). The analysis of *N,O*-diethoxycarbonyl heptafluorobutyl esters of NY and Y by GC/NCI-MS (Section 4.1.3) as well as the determination of the chemical structures of the Y derivatives obtained in the reaction with HFBA/TMSD (Section 4.1.4) however, are presented in this report for the first time. Although the method development for determination of NY and Y has not been concluded, the research within this project has revealed several interesting ideas regarding the further simplification

and optimization of the analysis of NY and Y. Derivatization of NY with trifluoroacetic anhydride (TFAA)/trifluoroethanol (TFEOH) (Pavlovich, Biondi et al. 2012) prior to methylation with TMSD may be of particular interest. The reaction with TFAA/TFEOH yields a product with two perfluorinated substituents, ensuring high detectability of the NY derivative (Pavlovich, Biondi et al. 2012). Methylation with TMSD protects a free phenolic group, making the derivatization product more suitable for GC/MS analysis (Söderling, Ryberg et al. 2003). Application of SPE tubes based on the weak anion exchange mechanism to NY and Y isolation from human plasma may also be considered. The weak anion exchanging properties of the Strata-X-AW sorbent provides selective retention of compounds at pH 6-7. At this pH plasma free NY is already in its anionic state (Fig. 2.3). Since elution of NY is conducted with acidic solvents, the eluate evaporates easily under vacuum and the residue contains no chemicals that may hamper the subsequent derivatization reaction.

6. References

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Appendix I. GC/MS methods

GC/MS method 1. **GC/NCI-MS analysis of PFB- derivatives of NY and Y**

Column oven temperature	50 °C
Injector temperature	250 °C
Injector mode	splitless
Sampling time	1 min
Carrier gas	Helium
Flow control mode	Linear velocity
Pressure	26.7 kPa
Total flow	10.5 mL/min
Column flow	0.68 mL/min
Linear velocity	30.0 cm/sec
Purge Flow	3.0 mL/min
Split Ratio	10.0

Column oven temperature program

Step, nr	Rate, °C	Final temperature, °C	Hold, min
0	-	50.00	1.00
1	25.0	120.00	0.00
2	12.0	330.00	10.00

Total program time: 31.3 min

Column: Rxi-5Sil (30m x 0.25mm x 0.25 um) MS

Film thickness: 0.25 um

Length: 30 m

Diameter: 0.25 mm

MS data acquisition mode	scan m/z: 45-800
Ion source temperature	200 °C
Interface temperature	250 °C
Solvent cut time	5 min
Ionization mode	NCI
Reagent gas	methane

GC/MS method 2. **GC/EI-MS analysis of crown ether catalysed PFB derivatives of NY and Y**

Column oven temperature	95 °C
Injector temperature	250 °C
Injector mode	splitless
Sampling time	1 min
Carrier gas	Helium
Flow control mode	Linear velocity
Pressure	38.0 kPa
Total flow	10.2 mL/min
Column flow	0.65 mL/min
Linear velocity	30.0 cm/sec
Purge Flow	3.0 mL/min
Split Ratio	10.0
MS data acquisition mode	scan m/z: 45-800
Ion source temperature	200 °C
Interface temperature	250 °C
Solvent cut time	7 min
Ionization mode	EI

Column oven temperature program

Step, nr	Rate, °C	Final temperature, °C	Hold, min
0	-	95.00	1.00
1	25.0	120.00	1.00
2	12.0	330.00	5.00

Total program time: 25.5 min

Column:Rxi-5Sil (30m x 0.25mm x 0.25 um) MS
Film thickness: 0.25 um
Length :30.0 m
Diameter: 0.25mm

GC/MS method 3. GC/EI-MS analysis of *N,O* – diethoxycarbonyl heptafluorobutyl esters of NY and Y

Column oven temperature	95 °C
Injector temperature	280 °C
Injector mode	splitless
Sampling time	1 min
Carrier gas	Helium
Flow control mode	Linear velocity
Pressure	38.0 kPa
Total flow	10.2 mL/min
Column flow	0.65 mL/min
Linear velocity	30.0 cm/sec
Purge Flow	3.0 mL/min
Split Ratio	10.0

Column oven temperature program

Step, nr	Rate, °C	Final temperature, °C	Hold, min
0	-	95.00	1.00
1	25.0	120.00	1.00
2	12.0	330.00	5.00

Total program time: 25.5 min

Column :Rxi-5Sil (30m x 0.25mm x 0.25 um) MS

Film thickness: 0.25 um
Length: 30.0 m
Diameter: 0.25mm

MS data acquisition mode	scan : m/z: 45-800
Ion source temperature	210 °C
Interface temperature	280 °C
Solvent cut time	7 min
Ionization mode	EI

SIM :
Target ions at m/z:

Tyrosine	**107,179,280, 346, 434, 507**
Nitrotyrosine	**152 ,256 ,391 462, 552**

GC/MS method 4. **GC/NCI-MS analysis of *N,O* – diethoxycarbonyl heptafluorobutyl esters NY and Y**

Column oven temperature	70 °C
Injector temperature	250 °C
Injector mode	splitless
Sampling time	1 min
Carrier gas	Helium
Flow control mode	Linear velocity
Pressure	31.8 kPa
Total flow	10.4 mL/min
Column flow	0.67 mL/min
Linear velocity	30.0 cm/sec
Purge Flow	3.0 mL/min
Split Ratio	10.0

Column oven temperature program

Step, nr	Rate, °C	Final temperature, °C	Hold, min
0	-	70.00	1.00
1	25.0	120.00	0.00
2	15.0	320.00	5.00

Total program time: 21.33 min

Column Rxi-5Sil (30m x 0.25mm x 0.25 um) MS

Film thickness: 0.25 um

Length: 30.0 m

Diameter:0.25 mm

MS data acquisition mode	scan m/z: 45-800
Ion source temperature	180 °C
Interface temperature	260 °C
Solvent cut time	7 min
Ionization mode	NCI
Reagent gas	methane

GC/MS method 5. GC/NCI-MS analysis of methyl-heptafluorobutyryl derivatives of Y and NY

Column oven temperature	70 °C
Injector temperature	250 °C
Injector mode	splitless
Sampling time	1 min
Carrier gas	Helium
Flow control mode	Linear velocity
Pressure	31.8 kPa
Total flow	10.4 mL/min
Column flow	0.67 mL/min
Linear velocity	30.0 cm/sec
Purge Flow	3.0 mL/min
Split Ratio	10.0

Column oven temperature program

Step, nr	Rate, °C	Final temperature, °C	Hold, min
0	-	70.00	1.00
1	25.0	120.00	0.00
2	15.0	320.00	5.00

Total program time: 21.33 min

Column Rxi-5Sil (30m x 0.25mm x 0.25 um) MS

Film thickness: 0.25 um

Length: 30.0 m

Diameter:0.25 mm

MS data acquisition mode	scan m/z:	45-800	SIM	Target ions at	m/z:
Ion source temperature	180 °C				
Interface temperature	260 °C			Tyrosine	**371, 443**
Solvent cut time	7 min			d_4-Tyrosine	**375, 447**
Ionization mode	NCI			Nitrotyrosine	**576, 596**
Reagent gas	methane			d_3-Nitrotyrosine	**579, 599**

Appendix II. Tables containing data to the figures

Table 1. Abundance of the NY and Y derivatives in the upper and lower organic phase of the extraction mixture (Fig. 4.3 B).

Attempt number	Base peak intensity at m/z =107 for tyrosine derivative		Base peak intensity at m/z= 152 for 3-nitrotyrosine derivative	
	Upper phase	Lower phase	Upper phase	Lower phase
1	3666273	8390535	42760	542814
2	3885745	8387368	49916	474998
3	4737524	8067031	40857	495028
4	746658	3287612	35765	425605
Average	3259050	7033137	42325	484611
Standard deviation (SD)	1504707	2166470	5074	42042
Coefficient of variation (CV), %	46	31	12	9

Table 2. Evaluation of the detection limits of NY and Y dissolved in water (Fig. 4.5).

Nitrotyrosine concentration, mg/L	Base peak intensity at m/z = 152 for 3-nitrotyrosine derivative				Mean	SD	CV, %
	Attempt 1	Attempt 2	Attempt 3	Attempt 4			
0.00	0	0	0	0	0	0	0
0.01	0	0	0	0	0	0	0
0.10	0	0	0	0	0	0	0
1.00	0	0	0	0	0	0	0
10.00	118607	98742	113819	112130	110825	7369	7
20.00	474988	495028	542814	425605	484609	42043	9
Tyrosine concentration, mg/L	**Base peak intensity at m/z = 107 for tyrosine derivative**				**Mean**	**SD**	**CV, %**
	Attempt 1	**Attempt 2**	**Attempt 3**	**Attempt 4**			
0.00	0	0	0	0	0	0	0
0.01	0	0	0	0	0	0	0
0.10	17550	15983	16242	9132	14727	3284	22
1.00	295582	281492	399076	407200	345838	57588	16
10.00	4737524	3885745	4143180	4475613	4310516	323274	8
20.00	8390535	8387368	8067031	-	8281645	151760	2

Table 4. Evaluation of stability of the NY and Y derivatives in the extract (Fig. 4.6).

Time after extraction, h	Signal intensity	
	Tyrosine derivative	3-Nitrotyrosine derivative
	m/z = 107	m/z = 152
0.0	3075142	749554
0.5	3024913	703752
4.0	2969916	698919
4.5	3164494	724872
8.0	2995478	727446
8.5	2964031	728079
12.0	2904926	735473
12.5	3163558	736879

Table 5. Evaluation of the detection limits of NY and Y dissolved in 0.1 M HCl (Fig. 4.7).

Nitrotyrosine concentration, mg/L	Signal intensity at m/z = 152 for 3-nitrotyrosine derivative				Mean	SD	CV, %
	Attempt 1	Attempt 2	Attempt 3	Attempt 4			
0.00	0	0	0	0	0	0	0
0.01	0	0	0	0	0	0	0
0.10	0	0	0	0	0	0	0
1.00	0	0	0	0	0	0	0
10.00	98945	154388	141364	55750	112612	38704	34
20.00	403456	211414	152226	105835	218233	113296	52
Tyrosine concentration, mg/L	**Signal intensity at m/z = 107 for tyrosine derivative**				**Mean**	**SD**	**CV, %**
	Attempt 1	Attempt 2	Attempt 3	Attempt 4			
0.00	0	0	0	0	0		
0.01	4716	5316	11006	7039	7019	2455	35
0.10	63459	80372	44335	63383	62887	12752	20
1.00	550805	517482	566841	-	408782	20559	5
10.00	5369676	5976277	5915940	-	5753964	272847	5
20.00	8008665.00	8245743.00	7999241.00	7841798.00	8023862	144236	2

Table 6. Compasicson of the signal response of the Y and NY derivatives in different extraction solvents (Fig. 4.9).

Extraction solvent	Tyrosine derivative, m/z =107						
	Attempt 1	**Attempt 2**	**Attempt 3**	**Attempt 4**	**Mean**	**SD**	**CV, %**
N-hexane	4737524	3885745	4143180	4475613	4310516	323274	7
Ethyl acetate	221963	234005	210885	189331	214046	16446	8
Dichloromethane	173025	160640	155998	141285	157737	11356	7
	3-Nitrotyrosine derivative, m/z=152						
N-hexane	118607	98742	113819	112130	110825	7369	7
Ethyl acetate	8152	10468	11047	8102	9442	1331	14
Dichloromethane	4116	4240	4310	4644	4328	195	5

Table 7. The ratio between signals of the two Y derivatives (Fig. 4.14).

Tyrosine concentration, mg/L	Signal intensity at m/z = 371	Signal intensity at m/z = 443	Ratio between signals
0.01	135	634	4.7
0.01	118	404	3.4
0.01	163	665	4.1
0.01	66	464	7.0
Mean			**4.8**
SD			**1.4**
CV, %			**28**
0.10	586	6001	10
0.10	772	6494	8
0.10	1171	7421	6
0.10	798	6710	8
Mean			**8**
SD			**1**
CV,%			**17**
1.00	13302	68799	5.2
1.00	10150	122515	12.1
1.00	12714	123450	9.7
Mean			**9.0**
SD			**2.9**
CV,%			**32**
5.00	126859	299896	2.4
5.00	113210	328274	2.9
5.00	129083	375867	2.9
5.00	125907	374941	3.0
Mean			**2.8**
SD			**0.25**
CV,%			**9**

Tyrosine concentration, mg/L	Signal intensity at m/z = 371	Signal intensity at m/z = 443	Ratio between signals
10.00	108276	604706	5.6
10.00	146486	742235	5.1
10.00	162932	777426	4.8
10.00	171802	786252	4.6
10.00	347841	953927	2.7
10.00	305298	1102683	3.6
10.00	257851	867058	3.4
Mean			**4.2**
SD			**0.9**
CV,%			**22**

Table 8. Standard curve of Y (Fig. 4.15).

Tyrosine concentration, mg/L	Sum of signal intensities of tyrosine derivatives at m/z = 443 and 371				Mean	SD	CV, %
	Attempt 1	Attempt 2	Attempt 3	Attempt 4			
0.0000	262	233	249	222	242	15	6
0.0001	140	305	358	141	236	97	41
0.0010	229	283	298	157	242	55	23
0.0100	769	522	828	530	662	138	21
0.1000	6587	7266	8592	7508	7488	721	10
1.0000	82101	132665	136164	126036	119242	21749	18
10.000	853571	882968	1009900	1001696	937019	69624	7

Table 9. Standard curve of NY (Fig. 4.16).

Nitrotyrosine concentration, mg/L	Base ion peak intensity at m/z = 576				Mean	SD	CV, %
	Attempt 1	Attempt 2	Attempt 3	Attempt 4			
0.0000	88	117	95	119	105	16	15
0.0001	175	168	158	99	172	4	2
0.0010	877	903	834	599	890	13	1
0.0100	8696	8281	8262	3585	8413	208	2
0.1000	207706	149197	201507	-	186137	26243	14
1.0000	1371709	1576942	1608587	1855776	1603254	171829	11

Table 10. Comparison of SPE tubes (Fig. 4.17 A).

Type of tubes: Strata-X-C	Plasma sample spiked before SPE	Plasma sample spiked after SPE	Standard subjected to SPE	Control
	Signal intensity of NY derivative at m/z =576			
Attempt 1	54802	124129	24593	1792883
Attempt 2	41812	142132	45154	2453976
Attempt 3	48340	89158	62793	2086820
Attempt 4	24742	75695	65344	-
Mean	42424	107779	49471	2111226
SD	11194	26569	16332	270441
CV, %	26	25	33	13

Table 11. Comparison of SPE tubes (Fig. 4.17 B).

Type of tubes: Strata-X	Plasma sample spiked before SPE	Plasma sample spiked after SPE	Standard subjected to SPE	Control
	Signal intensity of NY derivative at m/z =576			
Attempt 1	149475	290594	223788	1792883
Attempt 2	275886	521337	149737	2453976
Attempt 3	215129	439569	97368	2086820
Mean	213497	417167	156964	2111226
SD	51620	95523	51863	270441
CV, %	24	23	33	13

Table 12. Analysis of sensitivity of Strata-X tubes (Fig. 4.18 A).

Signal intensity at m/z = 576	Water blank	Plasma	+ 0.1 µg/L	+0.5 µg/L	+1 µg/L	Control
Attempt 1	2587	1942	1268	1018	577	19
Attempt 2	2203	1752	1520	1287	528	20
Attempt 3	2623	1233	1370	1065	532	19
Attempt 4	2574	875	1066	923	386	20
Mean	2497	1451	1306	1073	506	20
SD	171	422	165	134	72	1
CV, %	7	29	13	12	14	3

Table 13. Test of sensitivity of SPE-X tubes (Fig. 4.18 B).

Ratio of the NY derivative signal to the internal-standard signal	Water blank	Plasma	+ 0.1 µg/L	+0.5 µg/L	+1 µg/L	Control
Attempt 1	0.0027	0.0021	0.0022	0.0018	0.0009	0.000020
Attempt 2	0.0023	0.0022	0.0023	0.0019	0.0009	0.000021
Attempt 3	0.0024	0.0021	0.0021	0.0019	0.0008	0.000017
Attempt 4	0.0024	0.0021	0.0023	0.0018	0.0008	0.000018
Mean	0.0025	0.0021	0.0022	0.0019	0.0008	0.000019
SD	0.00002	0.00005	0.00007	0.00005	0.00004	0.000001
CV, %	7	2	3	3	5	7