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Different behaviour of 3-nitrotyrosine and tyrosine toward perfluorinated reagents suitable for the one-step preparation of volatile derivatives

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Abstract: With the view of developing a gas-chromatographic (GC) determination of the 3-nitrotyrosine (NY)/tyrosine (Y) ratio as a marker of nitro-oxidative stress, different reagents were tested with the objective of obtaining a single volatile fluorinated product for each amino acid in a one-step derivatisation procedure. The heptafluorobutyric anhydride (HFBA)/heptafluorobutanol (HFBOH) mixture proved unsuccessful for the simultaneous analysis of NY and Y. The reaction with different chloroformates, isobutyl chlorofomate (*i*BuCF) and ethyl chloroformate (EtCF) in the presence of different perfluorinated alcohols, such as trifluoroethanol (TFEOH) and HFBOH, was investigated. A combination EtCF/fluorinated alcohols yielded derivatives of NY and Y as single peaks suitable for the GC determination of the NY/Y ratio. The different behaviours of the two amino acids in the employed reaction mixtures and the parameters influencing the results are discussed.

Keywords: perfluorinated derivatives; tyrosine; 3-nitrotyrosine; GC–MS.

INTRODUCTION

Nitration of tyrosine Y (**1**) (Scheme 1), under particular conditions that are generally termed “nitro-oxidative stress”, may impair cell function by altering the protein conformation, solubility and susceptibility to aggregation, and could be responsible for increased protein degradation. This is especially relevant if enzymes of patho-physiological significance are involved.[1–3] 3-Nitrotyrosine (NY, **2**) is formed under the above conditions and, therefore, its detection and quanti-

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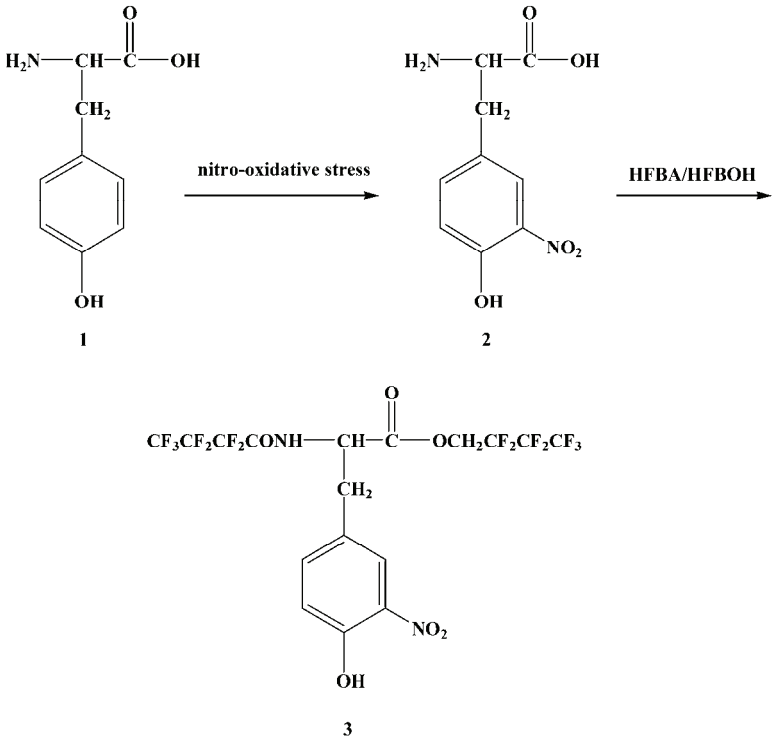
fication has been used as an indicator of the nitro-oxidative stress in biological systems.[4–10]



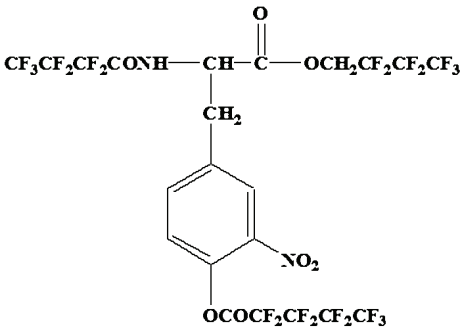
Scheme 1. Nitration of Y and structure of the derivative obtained from NY by treatment with a HFBA/HFBOH mixture.

Recently, the preparation of derivative (**3**), obtained with a heptafluorobutyric anhydride (HFBA)/heptafluorobutanol (HFBOH) mixture, was described for the evaluation of the NY content in the plasma of human volunteers by gas chromatography with an electron capture detector (GC–ECD).[11] This method was considered as a significant contribution to 3-nitrotyrosine quantification.[12]

In the above paper,[11] a combination of HFBA/HFBOH was selected in order to prepare in a one-step procedure a derivative with the highest possible content of fluorine atoms, since GC–ECD sensitivity is strongly influenced by the fluorine content of the derivative to be analyzed*.[13]

* Another procedure has been reported for the preparation of a volatile derivative for GC/ /negative chemical ionization (NCI) MS/MS analysis containing fourteen fluorine atoms.[14] However, it should be mentioned that this derivative was obtained in three steps, which included the reduction of NY to the corresponding amino derivative and the use of trimethylsilyl diazomethane for the methylation step.

During the experimental work, several conditions were tried in order to achieve the formation of the tris-substituted derivative **4** (Scheme 2), but with no success. This could be explained by the presence of an electron-withdrawing group at position 3 in NY that should significantly influence the reactivity of the phenolic OH and, consequently, the chemical properties of the NY derivatives that could be prepared.



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Scheme 2. Structure of NY heptafluorobutyl ester reacted with HFBA at both amino and phenol moieties.

The main aim of the present work was to propose a one-step procedure suitable for obtaining volatile derivatives for the simultaneous determination of Y and NY as a useful approach to evaluate protein nitro-oxidative stress. Major efforts were related to the possibility of obtaining the same fluorinated derivative for both compounds, taking into account the different reactivity of NY and Y under the derivatisation conditions.

All procedures described in the present paper were realised on the micromolar scale under conditions that should simulate the analysis of biological samples. Due to potential application as an analytical method, special attention was devoted to the achievement of the completeness of the derivatisation in a simple, one-step procedure to give a single product. After performing preliminary studies with the GC–FID analytical technique, all structures of the obtained derivatives were identified by MS analysis and the fragmentation spectra are presented herein as proof of the chemical structures.

MATERIALS AND METHODS

Chemicals

Y (**1**) and NY (**2**) were purchased from Sigma–Aldrich Italia, as well as derivatisation reagents (trifluoroacetic anhydride (TFAA), heptafluorobutyric anhydride (HFBA), ethyl chloroformate (EtCF), *iso*-butyl chloroformate (*i*BuCF) 2,2,2-trifluoroethanol (TFEOH), heptafluorobutanol (HFBOH), ethanol (EtOH), *iso*-butanol (*i*BuOH), *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and *N*-methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA)).

Preparation of N-*perfluoroacyl perfluoroalkyl esters and their silyl derivatives*

Y and NY were derivatised by conversion to their *N*- or *N*,*O*-perfluoroacyl perfluoroalkyl esters according to a previously reported procedure.[15] Briefly, the two derivatising agents (50 μL perfluoroalcohol and 100 μL of perfluoroalkyl anhydride) were added to the dry amino acids (0.1 μmol each) and heated at 80 °C for 30 min. The alcohols used in the present experiments were TFEOH and HFBOH, while the anhydrides were TFAA and HFBA. Excess reagents were removed under a stream of nitrogen and the residues were dissolved in ethyl acetate. The organic extract was both directly injected or treated with 100 μL of BSTFA or MTBSTFA and heated at 80 °C for 30 min. After cooling to room temperature, 1μL of the resulting mixture was injected into the GC–MS system.

Preparation of N(O)-*alkoxycarbonyl alkyl esters*

Y and NY were derivatised to their N(O)-alkoxycarbonyl alkyl esters and analyzed following the protocol previously described by Husek[16] and modified by Wang *et al.*[17] In brief, amino acids (0.10 μmol each) were dissolved 100 μL of H_2O and treated with 100 μL of alcohol/pyridine in a volume ratio 80:20. The alcohols used in the present experiments were: EtOH, *i*BuOH, TFEOH and HFBOH. Then 12.5 μL of EtCF or *i*BuCF were added and mixed by shaking gently for 30s. The derivatives were extracted with 150 μL of hexane containing 2 % chloroformate. A 1 μL aliquot was taken from the organic layer and injected.

GC–FID

A Trace GC Ultra (Thermo) (Milan, Italy) fitted with an FID was used in the control experiments. This chromatograph was equipped with a capillary column Optima 1701 (15 m× ×0.25 mm, 0.25 μm) (Macherey-Nagel, Duren, Germany). The temperatures of the injector and detector were 250 and 280 °C, respectively. The split injection technique was used with a 1:10 ratio. The column temperature was programmed from 140 to 290 °C at 10 °C min^{-1}. Helium at a flow rate of 0.9 ml min^{-1} was used as the carrier gas.

GC/MS

GC/MS Analyses were performed on a 5972A mass selective detector interfaced to a 5890 gas chromatograph (Agilent, Milano, Italy). The instrument was operated in the electron ionisation (EI) mode with an electron energy of 70 eV. The GC separation was accomplished on a HP-5 MS (30 m×0.25 mm, 0.25 μm) column. The transfer line temperature was 310 °C and the injector temperature was set at 300 °C. The injector was operated in the splitless mode. The column temperature was held at 50 °C for 5 min, then programmed to increase at a rate of 20 °C min^{-1} to 300 °C and finally held for 15 min at the end temperature.

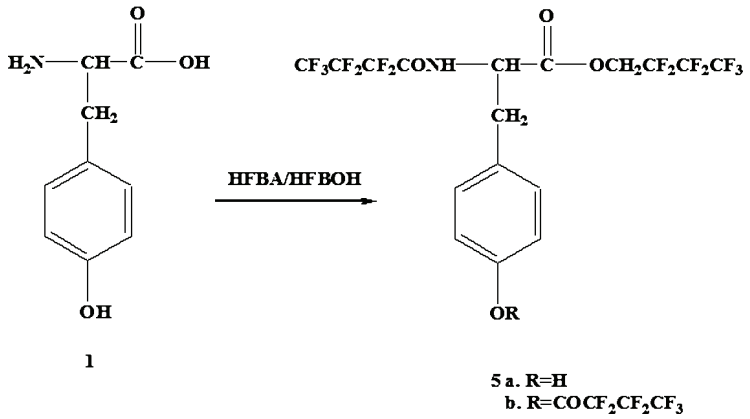
RESULTS AND DISCUSSION

As the objective of this work was the simultaneous derivatisation of Y and NY, the optimal reaction conditions (80 °C, 30 min) that afforded the NY bis-derivative **3** with a HFBAA/HFBOH mixture in a previous study[11] were repeated on the parent proteinogenic amino acid Y. However, the reaction of Y afforded a mixture of tris- and bis-derivatives (Scheme 3, compounds **5a** and **5b**) as shown in Fig. 1.

As a possible explanation of these results, it should be considered that a strong electron-withdrawing group, such as a nitro group, increases the acidity of the phenolic hydroxyl moiety. Consequently, the intermediate *O*-heptafluoro bu-

tanoate could be more susceptible to *in situ* hydrolysis, constantly producing free OH. Interestingly, an internal hydrogen bond between the phenolic OH and the nitro group in NY[18] could lower the OH reactivity.

Since a reliable, quantitative analysis is generally based on unique peaks of the analytes, the simultaneous determination of Y and NY would not be possible using the HFBA/HFBOH mixture.



Scheme 3. Derivatisation of tyrosine by the perfluroacylation/perfluoroesterification procedure.

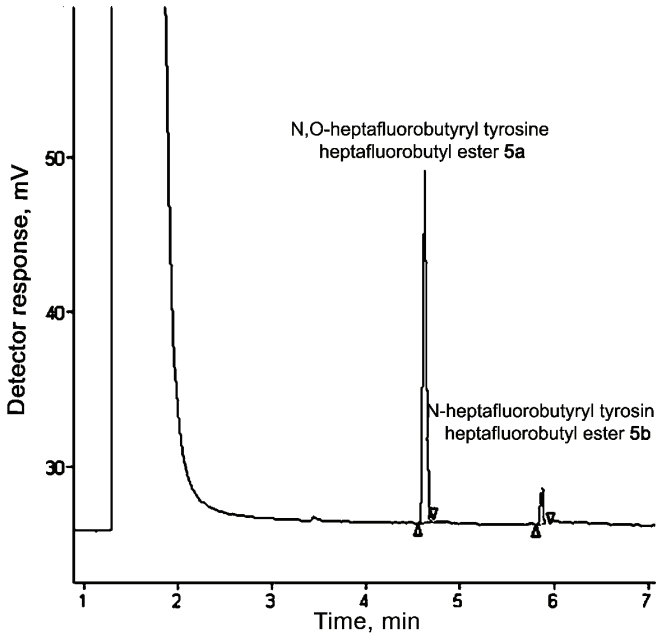
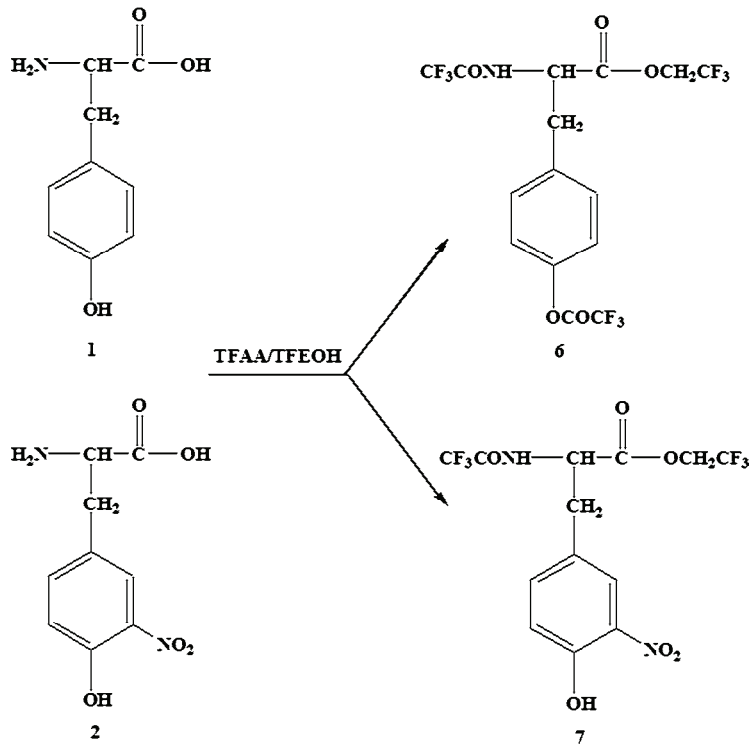


Fig. 1. GC–FID Chromatogram of the products obtained by treatment of Y with HFBA and HFBOH, tris- (first peak) and bis-substituted (second peak) derivatives.

Therefore, the derivatives obtained using the TFAA/TFEOH combination were examined. Both Y and NY afforded unique products, but while Y gave the tris-derivative **6**, NY afforded only the bis-derivative with a free phenolic OH (Scheme 4, compound **7**). The mass spectrometric data of the obtained derivatives are given in Table I. Some results are in the agreement with previously reported information,[19,20] while those regarding **5b** are presented here for the first time. In particular, the fragmentation pattern of the NY derivatives was especially investigated. The main peak for both derivatives was the 4-methylene-2--nitro-cyclohexa-2,5-dienylidene-oxonium cation (3-nitro-4-hydroxybenzyl cation) (*m*/*z* = 152). Important ions were present in both fluorinated derivatives **7** (Fig. 2a) and **3**, such as the ones originating from the cleavage of the C–C bond next to the ester group, with the formation of peaks at *m*/*z* = 277 (M–127) for the TFAA–TFEOH derivative and *m*/*z* = 377 (M–227) for the one obtained with HFBA–HFBOH. Moreover, other peaks in both cases were derived from the fragment produced by the loss of CF_3CONH_2, *m*/*z* = 291 (M–113) for the TFAA–TFEOH derivative or $CF_3(CF_2)_2CONH_2$, *m*/*z* = 391 (M–213) for the one obtained from HFBA–HFBOH.



Scheme 4. Derivatisation of 3-nitrotyrosine by the perfluroacylation/perfluoroesterification procedure.

TABLE I. Characteristic ion peaks in the mass spectra of *N*,(*O*)-perfluoroacyl perfluoroalkyl tyrosine and 3-nitrotyrosine esters

Amino acid	Anhydride/alcohol	Derivative	*m/z*	Main peak, *m/z*	Characteristic peaks, *m/z*
Tyrosine	TFAA/TFEOH	*N*,*O*-trifluoracetyl tyrosine trifluorethyl ester **6**	455[a]	203	231, 243, 328, 342
	HFBA/HFBOH	*N*,*O*-heptafluorobutyryl tyrosine heptafluorobutyl ester **5a**	755[a]	542	275, 303, 343
		N-heptafluorobutyryl tyrosine heptafluorobutyl ester **5b**	559	107	332, 346
3-Nitrotyrosine	TFAA/TFEOH	*N*-trifluoracetyl 3-nitrotyrosine trifluor-ethyl ester **7**	404[a]	152	106, 277, 291, 386
	HFBA/HFBOH	*N*-heptafluorobutyryl 3-nitrotyrosine heptafluorobutyl ester **3**	604	152	106, 377, 391

[a]No molecular ion identified

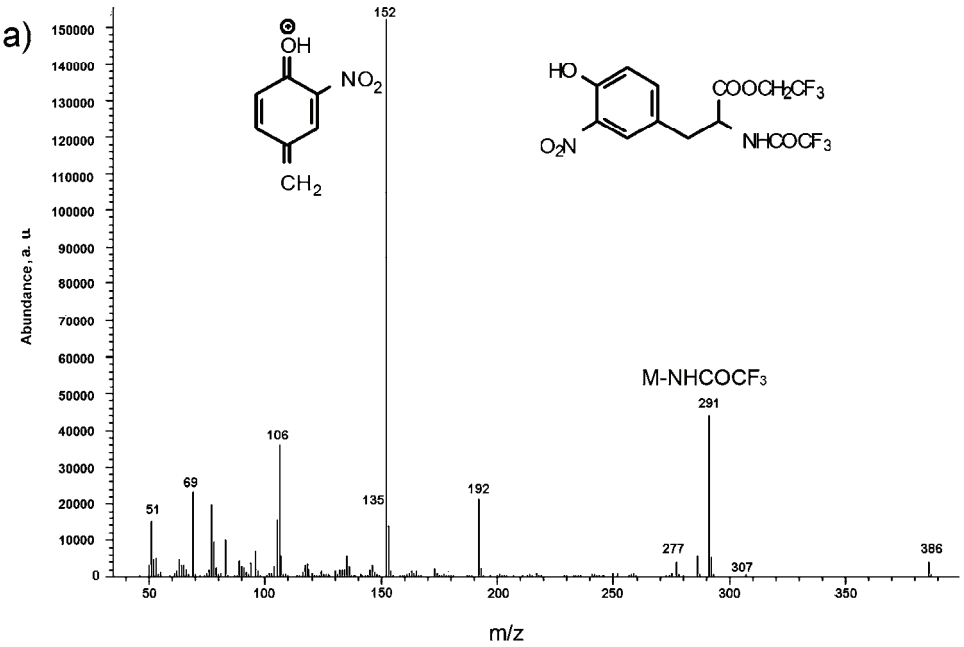


Fig. 2. Mass spectra of NY, *N*-trifluoracetyl trifluorethyl ester and its silyl derivatives: a) compound **6**.

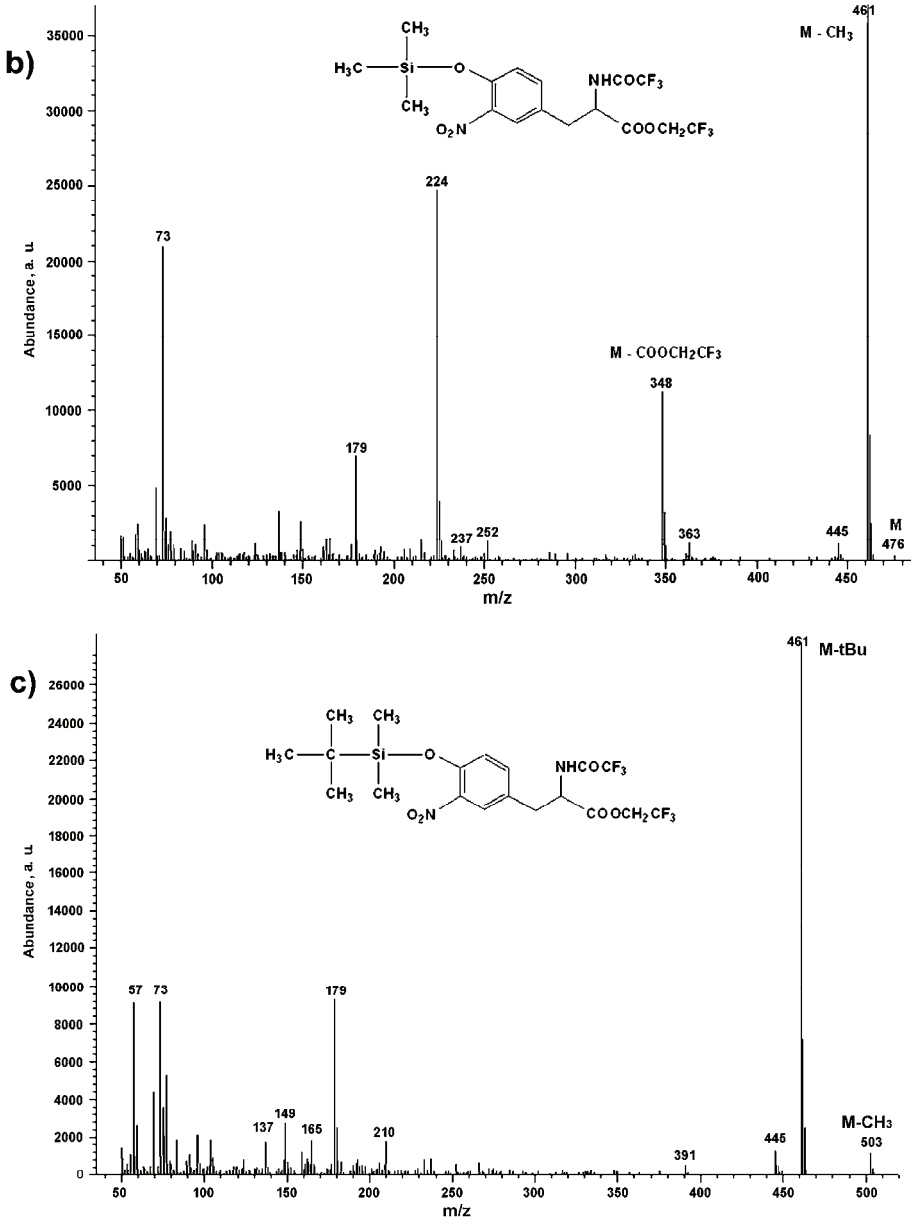
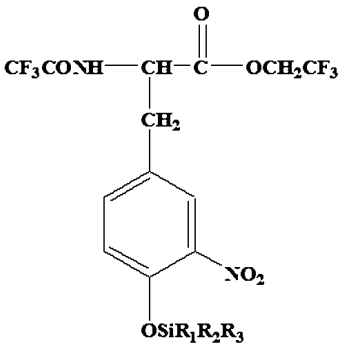


Fig. 2. (Continued) Mass spectra of NY, *N*-trifluoracetyl trifluorethyl ester and its silyl derivatives: b) compound **8a** and c) compound **8b**.

Moreover, additional studies are reported that were performed during a reappraisal of the NY protocol.[11] Namely, it was observed that after numerous injections of standard and biological samples into the same column, the peak

shape of the NY derivatives showed an increasing tailing. It was reasoned that the presence of the free phenolic group could influence the reliability of the analytical procedure, as already observed for other 2-nitrophenols.[21] In order to demonstrate that a derivative of NY with a protected phenolic OH is more suitable for gas-chromatographic analysis, silylation of compound **7** with different silylating agents was performed. Trimethylsilyl (TMS) and *t*-butyldimethylsilyl (TBDMS) derivatives **8a** and **8b** were prepared (Scheme 5) and the corresponding MS data are given in Table II and shown in Figs. 2b and 2c. It is of interest to observe that this derivatisation technique offers the advantage of a sufficient abundance of high molecular mass fragments that should facilitate the identification of the compound.



8 a. $R_1=R_2=R_3=CH_3$
b. $R_1=R_2=CH_3$, $R_3=t$Bu

Scheme 5. Structures of the silyl derivatives of NY previously reacted with TFA/TFEOH mixture.

TABLE II. Characteristic ion peaks in the mass spectra of NY silyl derivatives of N-trifluorethyl esters

Derivative	*m/z*	Main peak, *m/z*	Characteristic peaks, *m/z*
TMS derivate of NY *N*-trifluoracetyl trifluorethyl ester (**8a**)	473	461	179, 224, 348
TBDMS derivate of NY *N*-trifluoracetyl trifluorethyl ester (**8b**)	518[a]	461	179, 210, 445,503

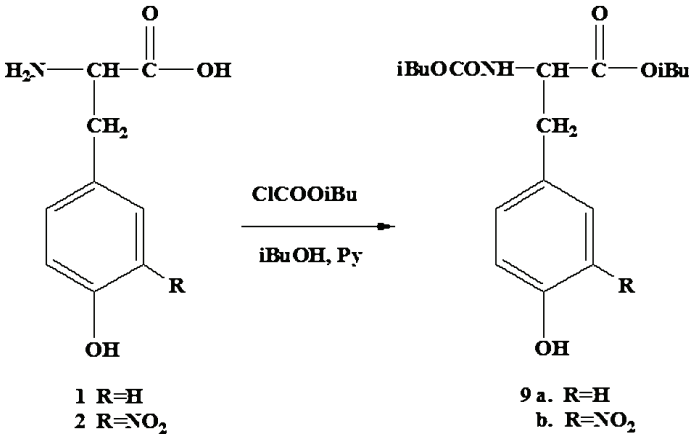
[a]No molecular ion identified

GC analysis of the silyl derivatives **8a** and **8b** showed that, as opposed to derivatives **3** and **7**, the peaks remained constantly sharp after many injections onto the same column; thus confirming that a tris-derivative of NY would be highly desirable.

In any event, the impossibility of obtaining the same derivatives for both NY and Y by an anhydride/alcohol mixture, led to the exploration for alternative methods. The Husek method[16,22] is a validated procedure for proteinogenic amino acids that offers distinct advantages due to its simplicity and rapidity. According

to this procedure, aqueous solutions of practically all natural amino acids react with alkyl chloroformates and alcohols in pyridine to form *N*(*O*)-alkoxycarbonyl alkyl esters suitable for GC analysis. A combination of alkyl chloroformates and fluoroalkanols has already been used for amino acids analysis,[17,15,23–26] but, to the best of our knowledge, has not been exploited for the purpose of NY determination. In accordance with previous studies,[17] the reaction of the carboxylic group with a chloroformate and an alcohol leads to the formation of an ester, the alkyl moiety of which is provided by the alcohol. The same reaction with an amine group leads to an *N*-alkoxycarbonyl derivative bearing the alkyl group of the chloroformate. In the case of an amino acid, such as Y or NY, tris-substituted *N*,*O*-alkoxycarbonyl derivatives are possible, due to the presence of the phenolic OH. The main experiments in this work were addressed to the study of the different behaviour of NY with respect to Y, of which some Husek derivatives have already been reported. The purpose was the careful monitoring of the influence of the nitro group on the outcome of the derivatisation procedures performed with different commercial chloroformate reagents and perfluoroalcohols.

Among *N*(*O*,*S*) alkoxycarbonyl alkyl esters of amino acids, isobutoxycarbonyl isobutyl esters can be considered as an optimal solution to start with, from the viewpoint of both sensitivity and stability.[25] Therefore, the classical combination *i*BuCF/*i*BuOH and bis-substituted derivatives of Y and NY was first investigated and *N*-*iso*-butoxycarbonyl *iso*-butyl esters **9a** and **9b** were exclusively obtained (Scheme 6). This result is in line with previously reported data for Y.[25,26]

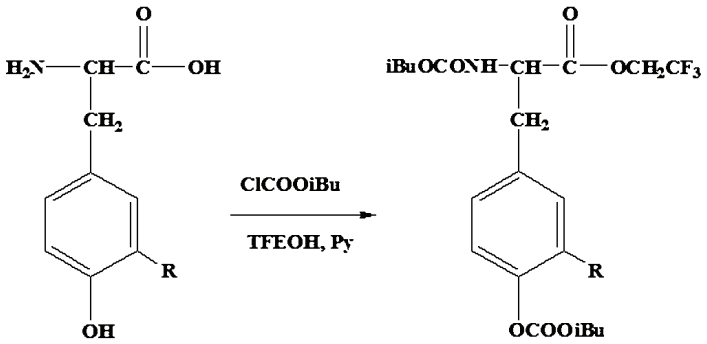


Scheme 6. Derivatisation of tyrosine and 3-nitrotyrosine by isobutyl chloroformate/isobutanol combinations.

In this case, the reaction did not occur at the phenolic OH of either Y or NY, which could be due to the presence of the apolar alcohol *i*BuOH in the reaction mixture. This may affect some chemico–physical parameters of the derivatisation

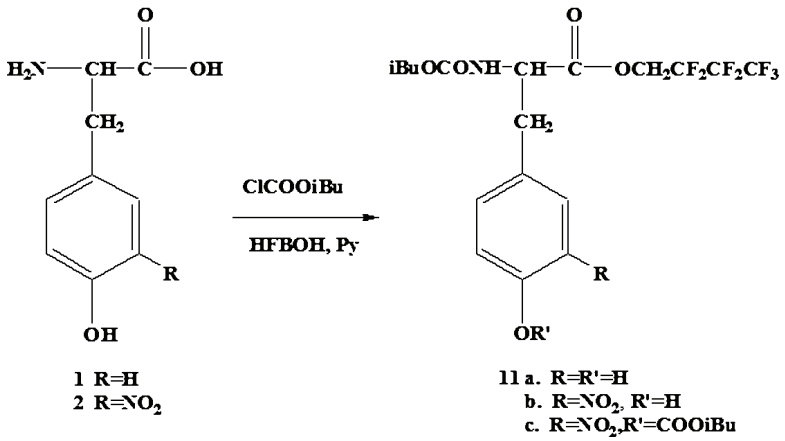
medium, with the effect of decreasing the reactivity of the phenolic OH toward chloroformate.

Being as the aim was to prepare volatile fluorinated derivatives, TFEOH was initially selected as the perfluoroalcohol in the Huseck procedure. The combination *i*BuCF/TFEOH afforded the tris-substituted derivative as the single product for both Y and NY (Scheme 7, compounds **10a** and **10b**).



Scheme 7. Derivatisation of tyrosine and 3-nitrotyrosine by isobutyl chloroformate/TFEOH combinations.

In a subsequent experiment, HFBOH was tested (Scheme 8). The encouraging result obtained with TFEOH was not confirmed by the combination of *i*BuCF with the less hydrophilic HFBOH. In fact, Y afforded the tris-substituted derivative **11a** as a single product, whereas NY afforded two products (bis- and tris-substituted **11b** and **11c**), as shown in Fig. 3. As the efficiency of the deri-



Scheme 8. Derivatisation of tyrosine and 3-nitrotyrosine by isobutyl chloroformate/HFBOH combinations.

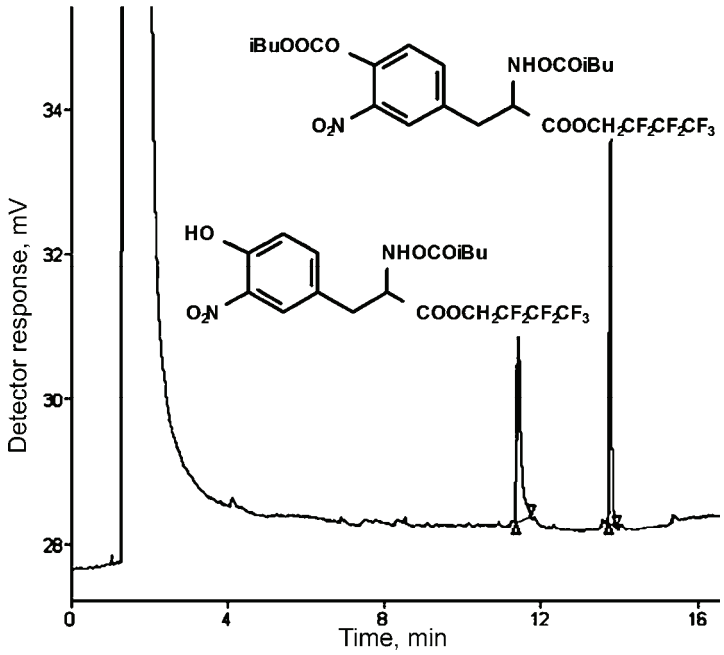


Fig. 3. GC–FID Chromatogram of NY derivatives obtained by treatment with iBuCF and HFBOH.

vatisation procedure may be attributed to different experimental conditions, an attempt was made to eliminate the double products obtained with the iBuCF/ /HFBOH combination by varying the reaction time and the amounts of chloroformate, pyridine and alcohol in the reaction mixture. These changes, however, did not completely lead to a single product. One possible explanation can be related to the low solubility of *i*BuCF in the mixture H_2O/HFBOH/pyridine that might cause incomplete derivatisation of NY. In this context, the high polarity of NY,[27] which renders this amino acid more hydrophilic than tyrosine itself, may also have contributed to the incomplete derivatisation of NY when the *i*BuCF/HFBOH combination was applied.

MS analysis of the derivatives obtained with *i*BuCF was used for the identification of the products. In Table III, the most significant peaks in the mass spectra of derivatives are listed. The EI spectra could be interpreted with reference to that reported by Wang *et al.*[17] and Sobolevsky *et al.*[25] for tyrosine.

As a general comment, using *i*BuCF only in combination with TFEOH afforded a single tris-substituted derivative product for NY and Y (compounds **10a** and **10b**). Although, the fluorine content was low, this result evidences that it is possible to obtain tris-substituted products for both Y and NY by a suitable combination of alkyl choroformates and alcohols.

TABLE III. Characteristic ion peaks in the mass spectra of *N*-isobutoxycarbonyl(perfluoro)alkyl esters of tyrosine and 3-nitrotyrosine

Amino acid	Chloroformate/alcohol	Derivative	*m*/*z*	Main peak, *m*/*z*	Characteristic peaks, *m*/*z*
Tyrosine	*i*BuCF/*i*BuOH	*N*-isobutoxycarbonyl isobutyl ester **9a**	337	107	164, 220, 320
	*i*BuCF/TFEOH	*N*,*O*-di-isobutoxycarbonyl trifluoroethyl ester **10a**	463[a]	107	207, 235, 246, 390
	*i*BuCF/HFBOH	*N*,*O*-di-isobutoxycarbonyl heptafluorbutyl ester **11a**	563	107	57, 207, 256, 346
3-Nitrotyrosine	*i*BuCF/*i*BuOH	*N*-isobutoxycarbonyl isobutyl ester **9b**	382[a]	57	137, 152, 209,280
	*i*BuCF/TFEOH	*N*,*O*-di-isobutoxycarbonyl trifluoroethyl ester **10b**	508	57	135, 152, 156, 291, 334, 435
	*i*BuCF/HFBOH	*N*-isobutoxycarbonyl heptafluorbutyl ester **11b**	508	152	57, 234, 256, 281, 391, 434
		N,*O*-isobutoxycarbonyl heptafluorbutyl ester **11c**	608[a]	152	57, 106, 256, 391, 462, 490, 508, 535

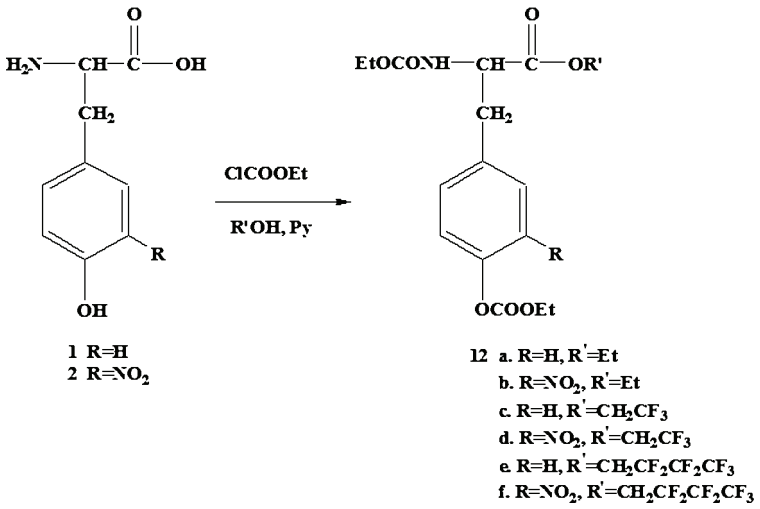
[a]No molecular ion identified

This prompted the study of other combinations of reagents, and ethyl chloroformate (EtCF) was selected, which, although less stable than iBuCF, has already been used in combination with different alcohols for Y derivatisation.[17,19,23] The combination EtCF/EtOH that gave the tris-substituted derivative **12a** with Y[28] was tested with NY. The non-proteinogenic amino acid NY also afforded the *N*,*O*-diethoxycarbonyl ethyl ester **12b** (Scheme 9)**.**

It was gratifying to observe that both NY and Y reacted also with TFEOH or HFBOH in the presence of EtCF and pyridine to give always the tris-derivatives (compounds **12c**–**f**).

The MS spectra of the derivatives obtained with EtCF are collected in Table IV, in which the molecular ions and the most characteristic fragments of the mass spectra of derivatives **12a**–**f** are presented. The mass fragmentation pattern of the NY tris-substituted derivative (**12f**) is shown in Fig. 4.

The results obtained with EtCF and fluorinated alcohols open several analytical perspectives, since it has been demonstrated that by a suitable selection of alkyl chloroformates and alcohols, NY and Y can be determined in the same run, affording the corresponding tris-substituted derivative. This opens a new opportunity for the determination of their ratio as nitro-oxidative marker.



Scheme 9. Derivatisation of tyrosine and 3-nitrotyrosine by ethyl chloroformate/ethanol or perfluoro alcohols combinations.

TABLE IV. Characteristic ion peaks in the mass spectra of *N*,*O*-diethoxycarbonyl (perfluoro)alkyl esters of tyrosine and 3-nitrotyrosine

Amino acid	Chloroformate/alcohol	Derivative	*m/z*	Main peak, *m/z*	Characteristic peaks, *m/z*
Tyrosine	EtCF/EtOH	*N*,*O*-diethoxycarbonyl ethyl ester **12a**	353	107	135, 192, 264, 280
	EtCF/TFEOH	*N*,*O*-diethoxycarbonyl trifluoroethyl ester **12c**	407	107	135, 179, 246, 280, 318
	EtCF/HFBOH	*N*,*O*-diethoxycarbonyl heptafluorbutyl ester **12e**	507	107	135, 179, 280, 346, 434
3-Nitrotyrosine	EtCF/ EtOH	*N*,*O*-diethoxycarbonyl ethyl ester **12b**	398	102	106, 135, 152, 174, 237, 265, 235
	EtCF/TFEOH	*N*,*O*-diethoxycarbonyl trifluoroethyl ester **12d**	452	152	106, 135, 228, 291, 325, 362
	EtCF/HFBOH	*N*,*O*-diethoxycarbonyl heptafluorbutyl ester **12f**	552	152	135, 256, 391, 462

CONCLUSIONS

Several one-step preparations of fluorinated derivatives of Y and NY, which would be suitable for GC analysis, were investigated. The simultaneous determination of Y and NY could constitute a new, useful method for the evaluation of the nitro-oxidative stress of proteins; in this regard, the optimal derivatisation method should afford only a single product in a one-step procedure

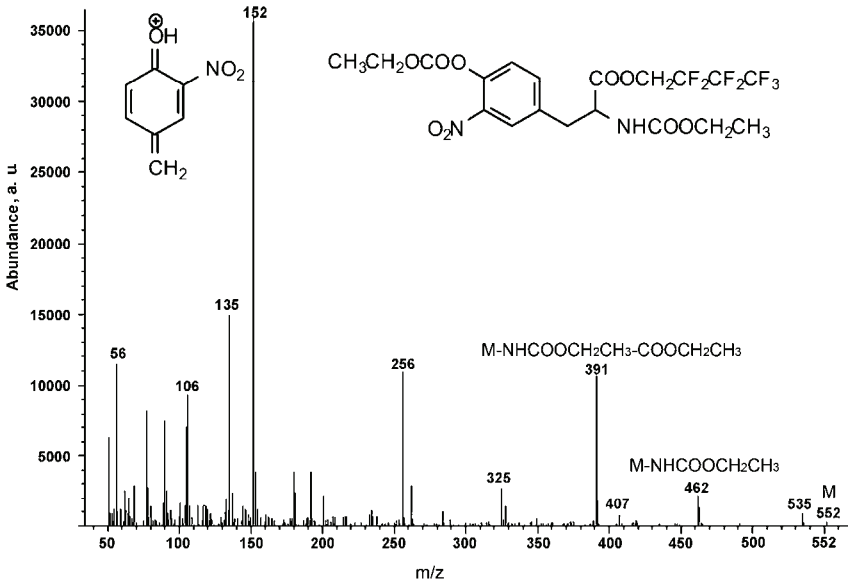


Fig. 4. Mass spectrum of the *N*,*O*-diethoxycarbonyl heptafluorbutyl ester derivative of NY (compound **12f**).

with both amino acids. Actually, in addition to inductive effect reasons, the presence of an *ortho*-nitro group causes different availability of the phenolic OH when bulky reagents are used, while small reagents are not influenced to the same extent by groups located near the phenolic OH. In addition, the structure of the alcohol appears to represent a critical parameter that could influence different reaction courses with NY and Y. The chloroformate/perfluoroalcohol procedure was evaluated by GC–FID and all the derivatives were identified by MS analysis. The best results were obtained with iBuCF/TFEOH and EtCF/HFBOH combinations. The *N*,*O*-diethoxycarbonyl heptafluorobutyl ester derivative **12f** is a tris--substituted product endowed with good GC characteristics. This derivative can be obtained in a single derivatisation step and could, therefore, be proposed as a suitable derivative for the simultaneous determination of Y and NY by GC analysis coupled to detectors sensitive to the presence of atoms with a high electron affinity, such as MS used in the negative ion chemical ionisation (NICI) mode.

Finally, the information presented herein could be useful from the viewpoint of exploiting the potential availability of perfluoroalkylchloroformates recently reported by Husek *et al.*[29] The use of this reagent could allow the introduction of a consistent number of fluorine atoms into the *N*,*O*-dialkoxycarbonyl part of the final derivative.

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И З В О Д

РАЗЛИЧИТО ПОНАШАЊЕ 3-НИТРОТИРОЗИНА И ТИРОЗИНА У ОДНОСУ НА ПЕРФЛУОРНЕ РЕАГЕНСЕ ПОГОДНЕ ЗА ЈЕДНОСТЕПЕНУ ПРИПРЕМУ ИСПАРЉИВИХ ДЕРИВАТА

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У циљу развијања гасно-хроматографске (GC) методе којом би се одредио однос 3-нитротирозина (NY) и тирозина (Y) као маркера нитро-оксидативног стреса, тестирани су различити перфлуорни реагенси. Сврха овог рада је била добијање јединственог испарљивог производа за сваку аминокиселину, и то у једностепеном дериватизационом поступаку. Процедура са хептафлуоробутирил-анхидридом (HFBA)/хептафлуоробутанолом (HFBOH) показала се неуспешном за симултано одређивање NY и Y. Детаљно су проучаване реакције с различитим хлорформијатима (изобутил-хлорформијат (iBuCF) и етил-хлорформијат (EtCF)) у присуству различитих перфлуорних алкохола (трифлуороетанол (TFEOH) и HFBOH). Комбинација EtCF/перфлуорни алкохол даје деривате обе аминокиселине као јединствене пикове који су погодни за одређивање NY/Y односа гасном хроматографском техником. Детаљно је разматрано различито понашање две аминокиселине у испитиваним дериватизационим поступцима, као што су разматрани и параметри који утичу на резултате одређивања.

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