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Comparison of fluorescence reagents for simultaneous determination of hydroxylated phenylalanine and nitrated tyrosine by high-performance liquid chromatography with fluorescence detection

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ABSTRACT: Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are well-known and important contributors to oxidative and nitrosative stress in several diseases. Hydroxylated phenylalanine and nitrated tyrosine products appear to be particularly susceptible targets of oxidative and nitrosative stress. We compared fluorescence reagents for their potential use in the analysis of hydroxylated phenylalanine and nitrated tyrosine products with a high-sensitivity and high-specificity HPLC-UV-FL technique. The analytes were extracted from serum via solid-phase extraction on Waters Oasis MCX cartridges. Chromatographic separation was achieved on an ODS column (Capcell Pak MG II; 150 × 2.0 mm) using a gradient mobile phase consisting of 20 mM sodium phosphate buffer (adjusted to pH 3.0) and acetonitrile. The method quantification limit for 4-nitrophenylalanine, *m*-tyrosine, and 3-nitrotyrosine was 0.1 μM. The relative standard deviation of the precision and accuracy was acceptable at the spiked concentration of 0.1 μM for 4-nitrophenylalanine, *m*-tyrosine and 3-nitrotyrosine. The method could be used for the *in vitro* analysis of serum samples. **Copyright © 2011 John Wiley & Sons, Ltd.**

Keywords: phenylalanine; 4-nitrophenylalanine; *o*-tyrosine; 3-nitrotyrosine; *m*-tyrosine; *p*-tyrosine; derivatization

Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are well-known and important contributors to oxidative and nitrosative stress in several diseases, such as inflammation (Kielland *et al.*, 2009), Parkinson's disease (Tsang and Chung, 2009) and Alzheimer's disease (Aslan and Ozben, 2004). Oxidative stress, as well as nitrosative stress, is a result of an imbalance between oxidants and antioxidants. Oxidants can damage all biological molecules, including DNA, RNA, lipids, proteins, carbohydrates and antioxidants. Protein oxidation causes the loss of protein function, cellular dysfunction and ultimately cell death (Watson and Loweth, 2009; Grishko *et al.*, 2009). Oxidative damage can be measured by determining the levels of protein carbonyls (Zitnanová *et al.*, 2007) and protein adducts of alkenals, such as acrolein and 4-hydroxynonenal (Spickett *et al.*, 2010), which are themselves reactive products of lipid peroxidation.

One major difficulty in the evaluation of the roles of oxidants in human disease is the lack of precise measures of oxidative and nitrosative stress *in vivo*. Many of the currently available methods are nonspecific and prone to artifact formation. One powerful approach to studying oxidative and nitrosative damage *in vivo* is the analysis of normal and diseased tissues for specific markers (Wijnhoven *et al.*, 2006; Tian *et al.*, 2010). Such markers have been identified as stable products of protein oxidation through *in vitro* studies. For example, phenylalanine (Phe) is converted into tyrosine (*p*-Tyr) by Phe hydroxylase. However, an unnatural isomer, *o*-tyrosine (*o*-Tyr), is formed when a hydroxyl radical ($^{\bullet}OH$), which can be generated by the interaction of superoxide anion ($^{\bullet}O_2^-$) and hydrogen peroxide (H_2O_2), especially in the presence of catalytic metal ions, oxidizes protein-bound Phe residues (Leeuwenburgh *et al.*, 1997, Blount and Duncan, 1997). Peroxynitrite ($ONOO^-$) is a strong RNS that causes the nitration of the aromatic ring. Such amino acids as Phe and/or *p*-Tyr appear to be particularly susceptible targets of nitration, and the formation of free or protein-bound 4-nitrophenylalanine (4-NP) and 3-nitrotyrosine (3-NT)

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Abbreviations used: 3-NT, 3-nitrotyrosine; 4-NP, 4-nitrophenylalanine; DBD-F, 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; Fmoc-Cl, 9-fluorenylmethyl chloroformate; MDL, method detection limit; MQL, method quantification limit; NDA, 2,3-naphthalenedialdehyde; OPA, *o*-phthalaldehyde; Phe, phenylalanine; RNS, reactive nitrogen species; ROS, reactive oxygen species; Tyr, tyrosine.

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has received much interest recently as a potential biomarker for the generation of RNS, such as $ONOO^-$, *in vivo* (Radi, 2004; Fig. 1).

The detection and quantification methods reported for 3-NT are high-performance liquid chromatography (HPLC)-based methods that utilize electrochemistry (Hitomi *et al.*, 2007), mass spectrometry (MS; Yi *et al.*, 2000), and tandem mass spectrometry (MS/MS; Ishii *et al.*, 2006; Rabbani and Thornalley, 2008; Radabaugh *et al.*, 2008; Kato *et al.*, 2009). Immunological methods have been applied extensively but are generally regarded as semiquantitative (Ischiropoulos, 1998). Each method has its own strengths and limitations. Importantly, these alternative 3-NT detection methods differ widely with regard to sensitivity, specificity, throughput and accessibility because of the different requirements for specialized instrumentation. In particular, LC-MS/MS and GC-MS/MS have very high sensitivity, selectivity and specificity (Tsikas and Caidahl, 2005). However, these instruments have not gained widespread use because MS is expensive and a stable isotope is needed for accurate determination.

In the present study, we compared several fluorescence (FL) reagents (Fig. 2) for their potential use in the analysis of hydroxylated Phe and nitrated Tyr products with a high-sensitivity and high-specificity HPLC-UV-FL technique. Moreover, the developed method was applied to an *in vitro* study to assess oxidative and nitrosative stress.

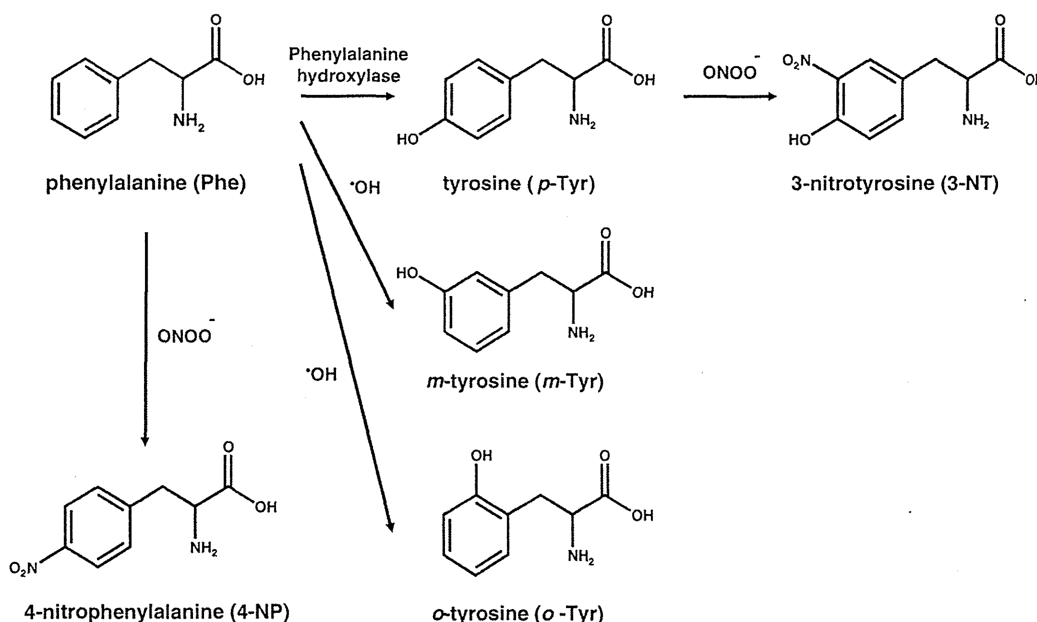

Figure 1. Chemical structures of phenylalanine, tyrosine, and their hydroxylated and nitrated compounds.

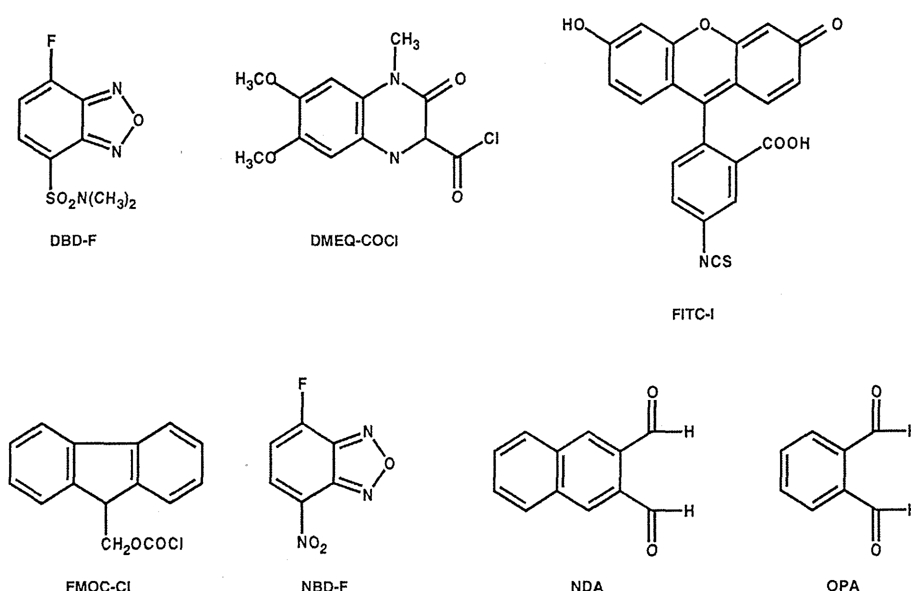

Figure 2. Chemical structures of FL reagents.

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Table 1. Optimum reaction conditions and comparison of LOD of hydroxylated and nitrated compounds with FL reagents

FL reagent	Reaction condition	Wavelength (nm)		LOD (nM) (S/N = 3)					
		Ex	Em	Phe	NO-Phe	*p*-Tyr	*o*-Tyr	*m*-Tyr	3-NT
DBD-F	50 mM PBS (pH 10) 60°C, 3 h	468	535	690	531	422	1298	355	1166
DMEQ-COCl	50 mM PBS (pH 10) 37°C, 10 min	408	478	1.0	9.9	10.3	50.6	10.1	60.3
FITC-I	50 mM PBS (pH 10) 60°C, 2 h	442	520	89.7	150	88.9	90.8	89.4	523
Fmoc-Cl	50 mM PBS (pH 12) 37°C, 1 h	265	313	5.0	6952	2.3	1.6	1.7	927
NBD-F	50 mM PBS (pH 9) 60°C, 5 min	470	540	1.2	2.2	15.6	26.1	68.1	13.7
NDA	50 mM PBS (pH 10) 37°C, 30 min	401	464	5.0	N.D.	5.0	28.5	24.8	N.D.
OPA	50 mM PBS (pH 10) 37°C, 1 h	340	450	101	N.D.	80.9	3544	87.4	N.D.

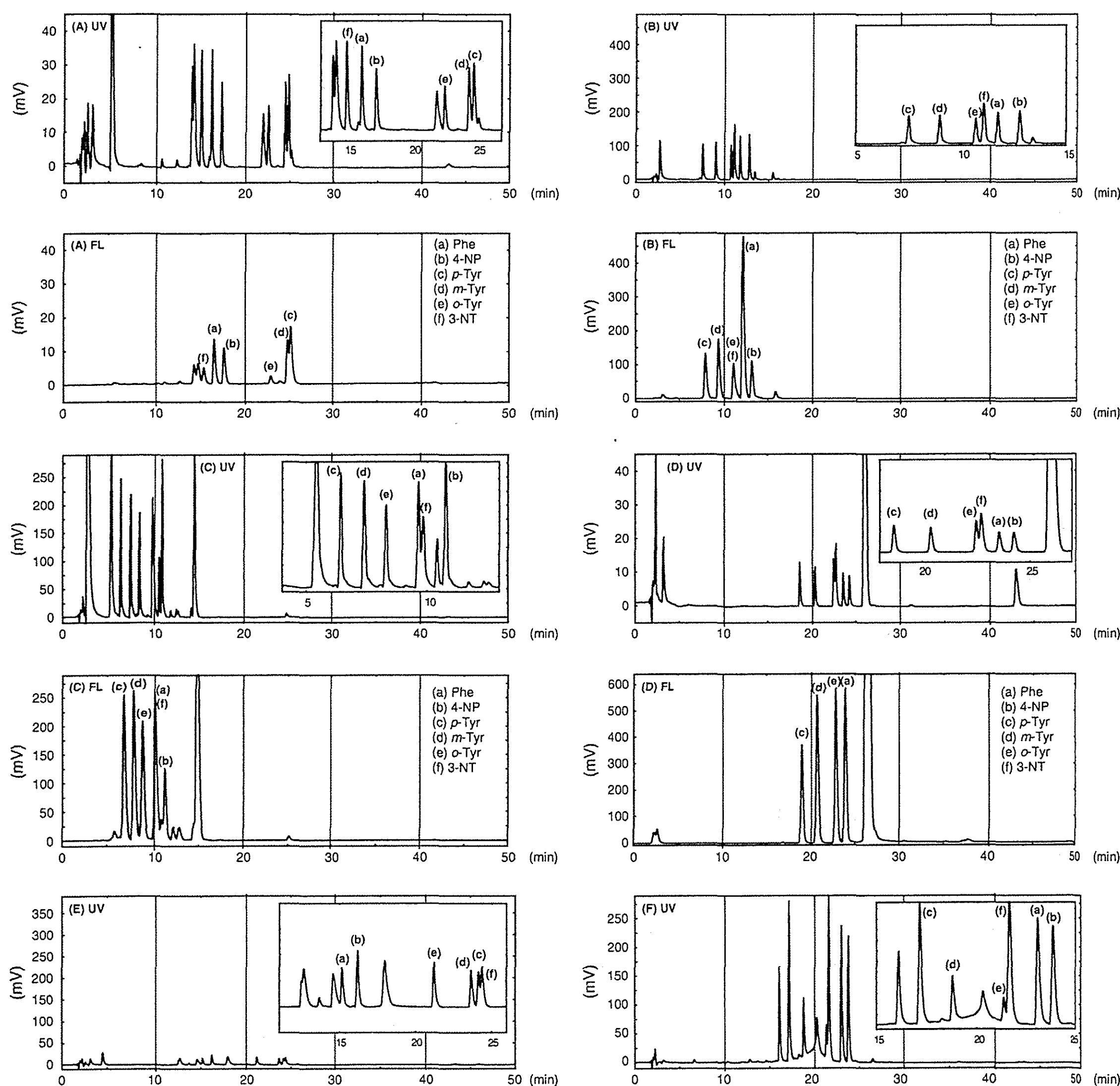

Figure 3. UV and FL chromatograms for the determination of hydroxylated phenylalanine and nitrated tyrosine (50 µM) products that were derivatized by several reagents. The derivatized standards were separated with a Capcell Pak C_{18} MGII (150 × 2.0 mm, 3 µm) and a gradient mobile phase consisting of 20 mM sodium phosphate buffer (pH 7.0) (solvent A) and acetonitrile (solvent B). A gradient program was used according to the following profile: 0–50 min, 10–60% B. (A) DBD-F, (B) DMEQ-COCl, (C) FITC-I, (D) Fmoc-Cl, (E) NBD-F, (F) NDA and (G) OPA. (a) Phenylalanine, (b) 4-nitrophenylalanine, (c) *p*-tyrosine, (d) *m*-tyrosine, (e) *o*-tyrosine and (f) 3-nitrotyrosine.

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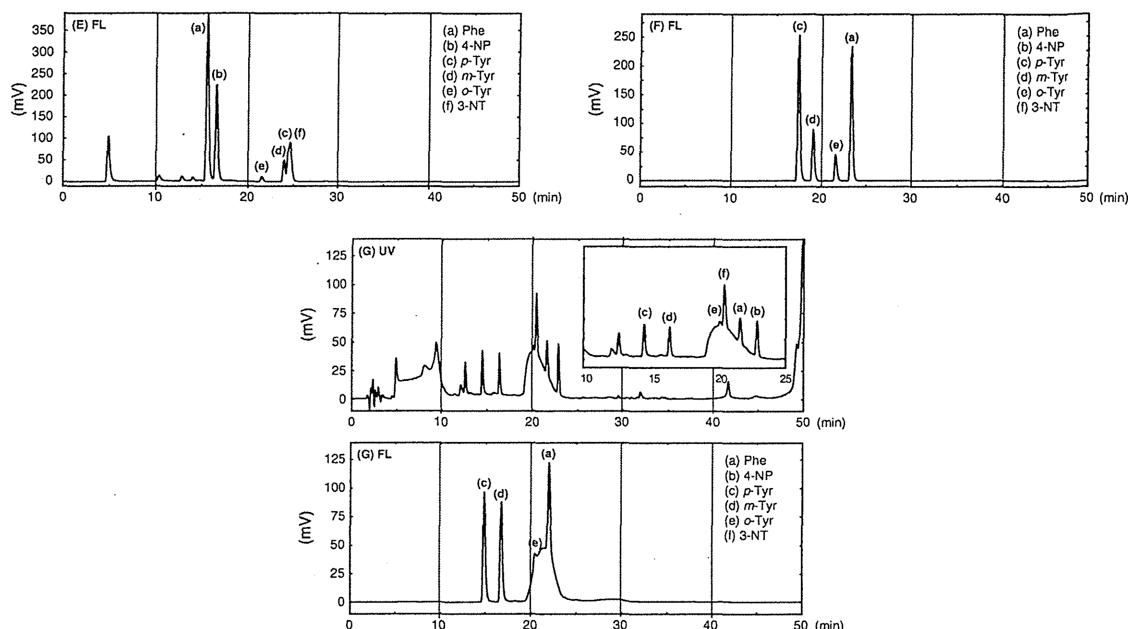

Figure 3: *Continued*

Experimental

Materials

DL-phenylalanine and 4-nitro-DL-phenylalanine were purchased from Kanto Chemical (Tokyo, Japan). 3-Nitro-L-tyrosine was purchased from Sigma (Tokyo, Japan). DL-tyrosine (*p*-Tyr), DL-*m*-tyrosine (*m*-Tyr), DL-*o*-tyrosine (*o*-Tyr), 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F), 9-fluorenylmethyl chloroformate (Fmoc-Cl), 2,3-naphthalenedialdehyde (NDA) and *o*-phthalaldehyde (OPA) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Peroxynitrite in NaOH solution ($ONOO^-$), 3-chlorocarbonyl-6,7-dimethoxy-1-methyl-2 (*1H*)-quinoxalinone (DMEQ-COCl), fluorescein-4-isothiocyanate (FITC-I) and 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) were purchased from Dojindo Laboratories (Kumamoto, Japan). All other reagents of analytical grade were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Solid-phase extraction (SPE) Oasis MCX (1 mL, 30 mg) cartridges were from Waters (Tokyo, Japan). Water was purified with a Milli-Q gradient A10 system (Millipore, MA, USA).

Liquid chromatography–fluorescence conditions

HPLC-UV-FL analyses were performed with a Shiseido system (Shiseido, Tokyo, Japan). Chromatographic separation was achieved with a Capcell Pak MGII column (150 × 2.0 mm i.d., Shiseido, Japan). Column temperature was maintained at 40°C and flow rate was set to 0.3 mL/min. The gradient mobile phase consisted of 20 mM sodium phosphate buffer (pH 3.0) and acetonitrile. Autosampler temperature was set to 4°C and 5 μL was injected. The wavelength of the ultraviolet detector was set at 210 nm. The excitation and emission wavelengths of the fluorescence detector were set at 470 and 540 nm, respectively.

Preparation of standards

Stock solutions of analytical compounds (10 mM) were prepared in 10 mM NaOH. Working solutions of analytical compounds were prepared by serial dilution of the stock solutions with water. Quality control (QC) samples for the determination of accuracy and precision at three concentrations for each calibration range were prepared by adding 50 μL of working solution into 500 μL of blank serum. All solutions were stored at −80°C and were stable under those conditions for at least one week.

Sample preparation by solid-phase extraction and derivatization

The serum sample (500 μL) was added to 1500 μL of acetonitrile to remove proteins. The solution was centrifuged at 10,000 *g* for 5 min after vortexing. The supernatant was evaporated to dryness under nitrogen gas stream at 50°C. The residue was dissolved 1 mL of 50 mM sodium phosphate buffer (pH 4.0). The reconstituted samples (1.0 mL) was applied to an SPE cartridge.

An Oasis MCX cartridge (Waters) was conditioned with 1 mL methanol and 1 mL water. Deproteinized serum sample was loaded and allowed to flow by aspiration. The cartridge was washed with 1 mL × 2 of acetonitrile and 10 mM sodium phosphate buffer (pH 6.0, 1 mL × 2), and dried for 5 min. The analytes were eluted with 0.5 mL of freshly prepared 50 mM sodium phosphate buffer (pH 11.0). The eluate was subjected to the derivatization step. To 250 μL of the eluate, 50 μL of 50 mM sodium phosphate buffer (pH 5.0) and 50 μL of 10 mM NBD-F in acetonitrile were added. This mixture was adjusted to pH 9.0. After 5 min at 60°C, the derivatized sample was dissolved in 100 μL of acetonitrile and 50 μL of 1 M HCl. The solution was centrifuged at 10,000 *g* for 5 min after vortexing. The supernatant was subjected to HPLC analysis.

Validation procedures

Prior to the application to real samples, the method was tested with a validation protocol following the accepted criteria for bioanalytical method validation. Linearity, accuracy, precision, method detection limit (MDL) and method quantification limit (MQL) were determined.

Calibration standards were prepared and analyzed in duplicate for three consecutive days. To establish linearity, the correlation coefficient (*r*) should be more than 0.99 and the deviation of the calculated concentrations should be within ±15% of the nominal concentrations.

Accuracy and precision of the method were evaluated over the linear range at two concentration levels (low, and high QC samples) in serum for three consecutive days. Six replicates of each concentration were

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analyzed per day. Accuracy was measured as the percentage deviation from the nominal concentrations and should not exceed 15%. The intra- and inter-day assay precision was calculated and should not exceed 15% expressed as relative standard deviation (RSD).

MQL, which is defined as the lowest concentration at which accuracy and precision should not exceed 20%, was evaluated by analyzing samples prepared in six replicates. The MDL was estimated at the concentration with a signal-to-noise ratio of at least 3.

Results and discussion

Comparison of fluorescence reagents for determination of hydroxylated Phe and nitrated Tyr

HPLC-FL is the most widely used method for amino acid analysis because of its high sensitivity and specificity. Because amino acids do not have fluorescence, several derivatization methods are used for the determination of amino-acid-containing compounds. Seven FL reagents, such as DBD-F, DMEQ-COCl, FITC-I, Fmoc-Cl, NBD-F, NDA and OPA, were examined. The optimum reaction conditions, FL wavelengths and limits of detection (LODs) are listed in Table 1. All derivatization reagents reacted with hydroxylated Phe and nitrated Tyr. The UV and FL chromatograms are shown in Fig. 3. Phe, *p*-Tyr, *m*-Tyr and *o*-Tyr were detected at high sensitivity by FL regardless of the FL reagent used. Meanwhile, 4-NP and 3-NT peaks were either very small or not detected at all in the FL chromatograms when DBD-F, Fmoc-Cl and NDA were used as the FL reagents. It seems that the FL intensity of the nitrated compounds was affected by the nitro group, which is a strong electron-attracting group. The peaks of DMEQ-COCl and FITC-I interfered with the detection of the analyte peaks. It is for these reasons that we decided to use NBD-F for the derivatization of hydroxylated Phe and nitrated Tyr.

Optimization of NBD-F derivatization conditions

NBD-F, an FL reagent, has excellent features with regard to sensitivity, FL characteristics, fluorophor stability and solubility in water (Toyo'oka *et al.*, 1989; Miyoshi *et al.*, 2009). However, NBD-F is affected by pH and its own concentration in the derivatization reaction (Zhang *et al.*, 2007). The pH of the reaction buffer, the concentration of NBD-F, and the reaction time were optimized (Fig. 4) and the optimum FL derivatization conditions were found to be 50 mM phosphate buffer (pH 9.0) and 10 mM NBD-F added to a sample. Then, the reaction was carried out at 60°C for 5 min and stopped by adding 50 μL of 1 M HCl.

Effect of mobile phase conditions on the separation of analytes

To determine the appropriate conditions for the chromatographic separation of the NBD-F derivatives of Phe, 4-NP, *o*-Tyr, *m*-Tyr, *p*-Tyr and 3-NT, we tried separation with a mobile phase whose pH was varied. When the mobile phase had a low pH value, all compounds were retained by the ODS column. In contrast, 3-NT and *p*-Tyr were detected at the same retention time when the mobile phase had a pH of 4.0 (Fig. 5). Consequently, the mobile phase that consisted of 20 mM phosphate buffer (pH 3.0) and acetonitrile was chosen as the optimum condition. No significant interference from other endogenous serum substances was apparent.

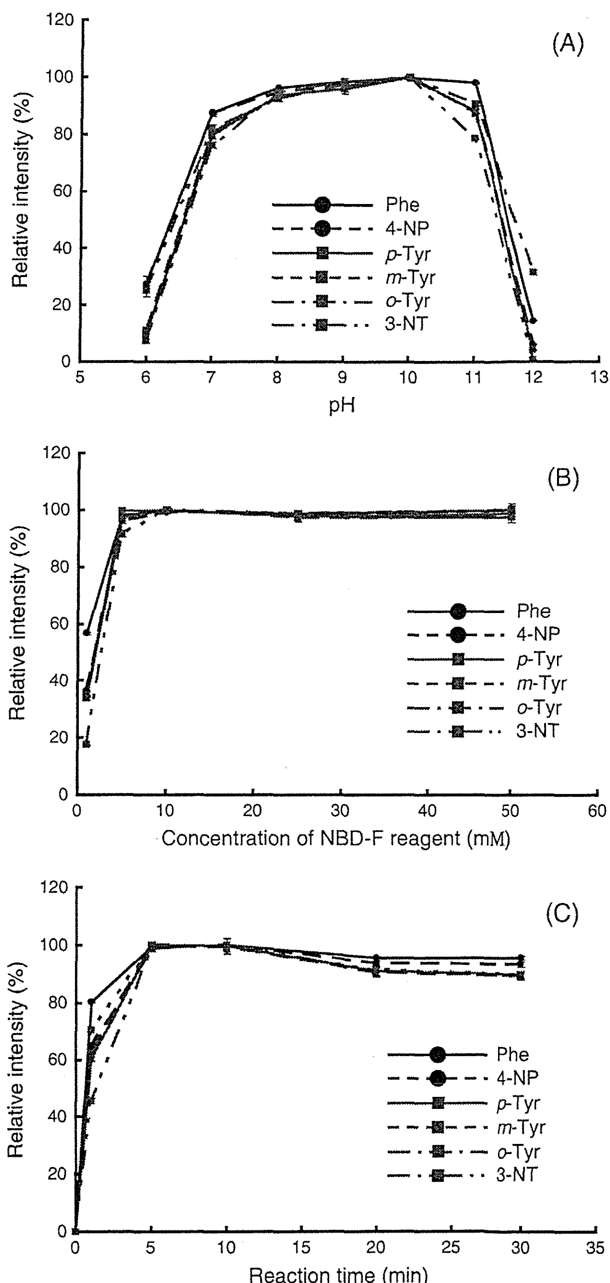

Figure 4. Optimization of derivatization conditions: (A) pH of reaction buffer; (B) concentration of NBD-F; and (C) reaction time.

Optimization of solid-phase extraction method

Solid-phase extraction (SPE) is a convenient and rapid method for sample preparation. The SPE method was optimized to meet three objectives. First, the overall conditions selected should provide the highest and most consistent absolute recovery of the target analyte. Second, the conditions for washing SPE cartridges containing bound analyte should allow for the removal of unwanted matrix components to the greatest extent possible without eluting the target analyte. Third, the elution conditions should enable the subsequent recovery of the analyte efficiently while minimizing the elution of less polar matrix components (Yu *et al.*, 2008).

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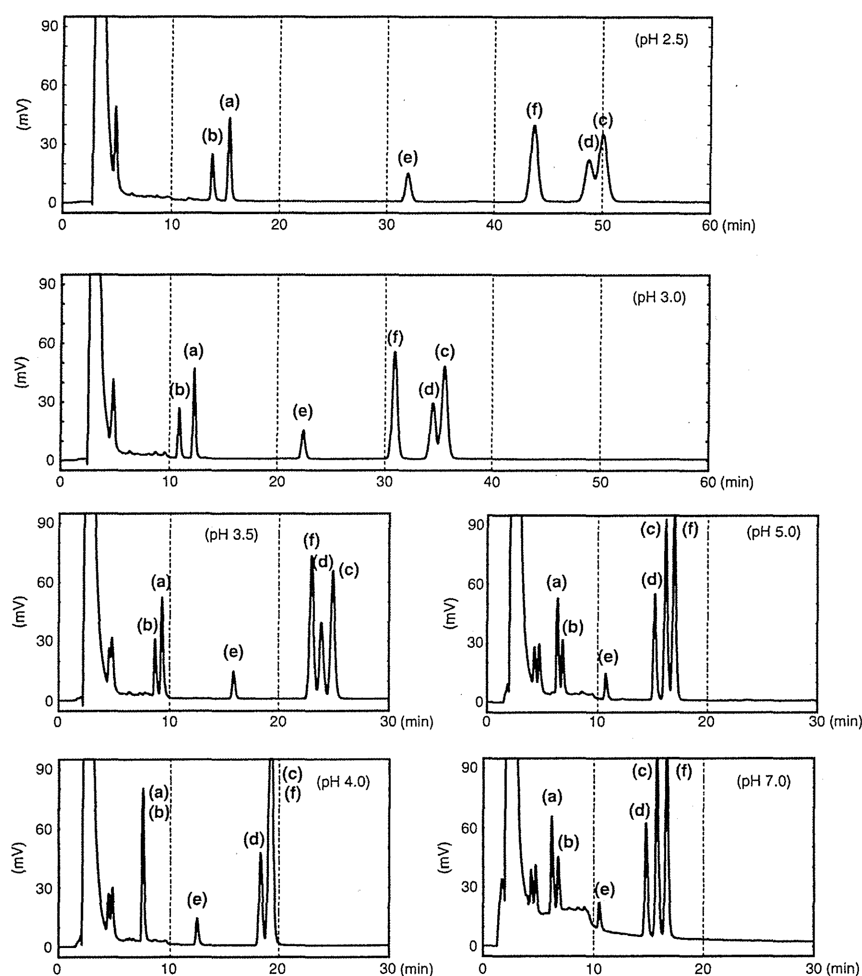

Figure 5. Effect of mobile phase pH on the separation of analytes. Chromatographic separation was achieved with a Capcell Pak C_{18} MGII (150 × 2.0 mm, 3 μm) and a gradient mobile phase consisting of 20 mM sodium phosphate buffer (solvent A) and acetonitrile (solvent B). A gradient program was used according to the following profile: 0–5 min, 20–30% B; and 5–40 min, 30% B. (a) Phenylalanine (0.1 μM), (b) 4-nitrophenylalanine (0.1 μM), (c) *p*-tyrosine (1 μM), (d) *m*-tyrosine (1 μM), (e) *o*-tyrosine (1 μM) and (f) 3-nitrotyrosine (1 μM).

The difficulty lies in not only the very low concentrations of hydroxylated Phe and nitrated Tyr products in the sample matrix but also their hydrophilicity. Several papers have given incorrect descriptions of the chemistry and biochemistry of the nitric oxide (NO) pathway and ignored almost completely previous reports on the pitfalls of 3-NT measurement (Oeckl and Ferger, 2009; Saravanabhavan *et al.*, 2010). One well-known example is the reaction of Tyr with nitrate and nitrite ions in acidic conditions, which can produce new 3-NT (Oldreive *et al.*, 1998). It is necessary to avoid the formation of 3-NT artifact from Tyr and ubiquitous nitrate and nitrite.

We compared the Oasis HLB cartridge with the Oasis MCX cartridge that has a mixed-phase mode (RP and cation exchange) in terms of sample extraction. *p*-Tyr could not be recovered with the Oasis HLB cartridge. Saravanabhavan *et al.* (2010) reported that urine sample was prepared by using the Oasis HLB cartridge and ion pairing reagents (trifluoroacetic acid and octanesulphonic acid). Urine samples were treated with trifluoroacetic acid before loading on the HLB cartridge. However, this condition led to artifact formation.

In contrast, hydroxylated Phe and nitrated Tyr products were well extracted from the Oasis MCX cartridge. The Oasis MCX cartridge is dependent on the pH and the concentration of the salt to be retained and eluted. When pH was increased, all analytes were well extracted from the Oasis MCX cartridge. We examined the recovery of the target analytes in relation to the wash conditions (water, methanol, acetonitrile, 10 and 50 mM sodium phosphate buffer). Loss rate from the Oasis MCX cartridge was calculated from the peak area ratio (as a percentage) of the extraction sample to the control sample at each concentration and the results are summarized in Fig. 6. The wash solvent of high pH and concentration (pH 6 and 50 mM) did not furnish good results. Moreover, our results indicated that the highest recovery of the analytes was accomplished with 10 mM sodium phosphate buffer (pH 6.0; range 95.1–97.8%). We decided that the loading and washing pH should be less than 5.0, and the eluting pH should be more than 11.0.

Validation

The MDL of the HPLC-FL method was determined by analyzing the response of the standard compounds. The MQL of 4-NP, 3-NT and *m*-Tyr was 0.1 μM. Excellent linearity was observed for all analytes over their respective concentration ranges with

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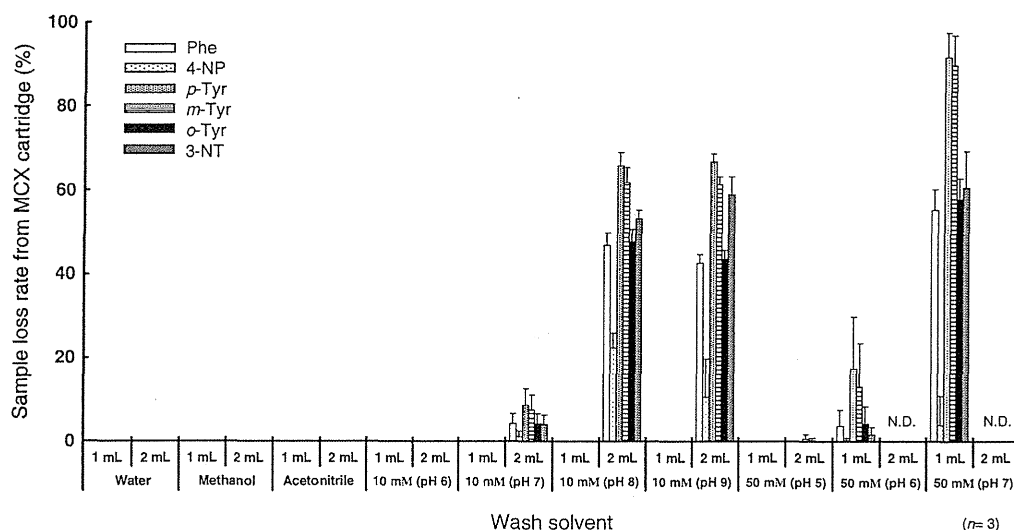

Figure 6. Optimization of wash conditions for solid-phase extraction. Washing solvents were prepared with sodium phosphate buffer. N.D., not determined.

Table 2. Validation results of phenylalanine, tyrosine, and their hydroxylated and nitrated compounds

Analyte	Detector	Calibration range (μM)	MDL[a] (μM)	MQL[b] (μM)	r
Phe	UV	10–1000	3	10	0.9999
4-NP	FL	0.1–10	0.03	0.1	0.9999
p-Tyr	UV	10–1000	3	10	0.9999
m-Tyr	FL	0.1–10	0.03	0.1	0.9999
o-Tyr	FL	1–100	0.3	1	0.9999
3-NT	FL	0.1–10	0.03	0.1	0.9999

[a] MDL: S/N = 3.
[b] MQL: S/N > 10, precision and accuracy <20%.

Table 3. Recovery rates of QC samples during routine analysis ($n = 6$)

	LQC	HQC		LQC	HQC
Phe	10 μM	100 μM	*m*-Tyr	0.1 μM	1 μM
Recovery (%)	99.9	95.7	Recovery (%)	93.9	96.2
Intra-day (%RSD)	3.1	1.0	Intra-day (%RSD)	6.0	2.3
Inter-day (%RSD)	2.1	4.4	Inter-day (%RSD)	2.1	3.0
4-NP	0.1 μM	1 μM	*o*-Tyr	1 μM	10 μM
Recovery (%)	110.5	107.9	Recovery (%)	101.9	98.5
Intra-day (%RSD)	8.8	9.3	Intra-day (%RSD)	7.3	2.4
Inter-day (%RSD)	8.1	10.3	Inter-day (%RSD)	3.9	4.3
p-Tyr	10 μM	100 μM	3-NT	0.1 μM	1 μM
Recovery (%)	101.1	101.4	Recovery (%)	99.7	95.2
Intra-day (%RSD)	8.7	2.5	Intra-day (%RSD)	10.2	2.8
Inter-day (%RSD)	3.9	6.6	Inter-day (%RSD)	6.0	3.0

LQC: low quality control.
HQC: high quality control.

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correlation coefficients (r) > 0.99 (Table 2). The results indicated a good linear relationship between peak ratio and concentration.

Precision and accuracy were assessed for low, and high QC samples. Table 3 contains intra- and inter-day assay precision and accuracy data for each concentration. Precision was determined by multiple analyses of the same sample at different days, and is expressed as RSD. In this method, the intra-day precision was less than 15% for all the analytes ($n = 6$). The inter-day precision ($n = 6$) was also less than 15% for each compound. The %CV values for the intra- and the inter-day assay were in the range of ±15% and were considered to be acceptable according to FDA guidelines for bioanalytical method validation (FDA, 2001).

Pooled serum samples were spiked with the analyte at low, and high concentrations and six samples were analyzed immediately following sample preparation. The remaining pooled serum sample was divided into two subsets (stable samples and control samples). The stable samples were stored at 25, 4 and −80°C, respectively. Loss of less than 15% was observed on storage for 3 h at 25°C (room temperature storage) and for 24 h at 4°C (autosampler storage; data not shown). The degradation was not significant and stability was maintained under routine laboratory conditions.

Application

Measurements of Phe, *o*-Tyr, 3-NT, *m*-Tyr and *p*-Tyr were also carried out in each serum sample nitrated with $ONOO^-$ or hydroxylated with ˙OH formed by the Fenton reaction (Fe^{2+}/H_2O_2; Fig. 7). When the concentration of $ONOO^-$ was increased, the concentrations of 4-NP and 3-NT were increased as well (Fig. 8). On the other hand, a high concentration of H_2O_2 yielded *o*-Tyr and *m*-Tyr, which were synthesized from Phe by ˙OH. The formation of ONOO- is particularly important because it can cause both oxidative and nitrosative stress under physiological conditions (Gunaydin and Houk, 2009). During inflammatory processes, cells produce high levels of ˙O_2^- and NO, and consequently ONOO- formation is expected to be facile during inflammatory processes. Peroxynitrous acid can nitrate free or protein-bound *p*-Tyr. *p*-Tyr nitration by ONOO- may also be dependent on the local protein environment. These results indicate that the developed method could be used for the assessment of oxidative and nitrosative stress in serum.

Conclusion

We have developed and validated a high-sensitivity, high-accuracy and specific HPLC-UV-FL method for the determination of hydroxylated Phe and nitrated Tyr products, to assess ROS and RNS. To determine hydroxylated Phe and nitrated Tyr, we compared several fluorescence reagents. We decided to use NBD-F for the derivatization of hydroxylated Phe and nitrated Tyr, because it has higher sensitivity and specificity than other fluorescence reagents. The simple mixed-mode SPE method provides highly efficient sample clean-up with excellent

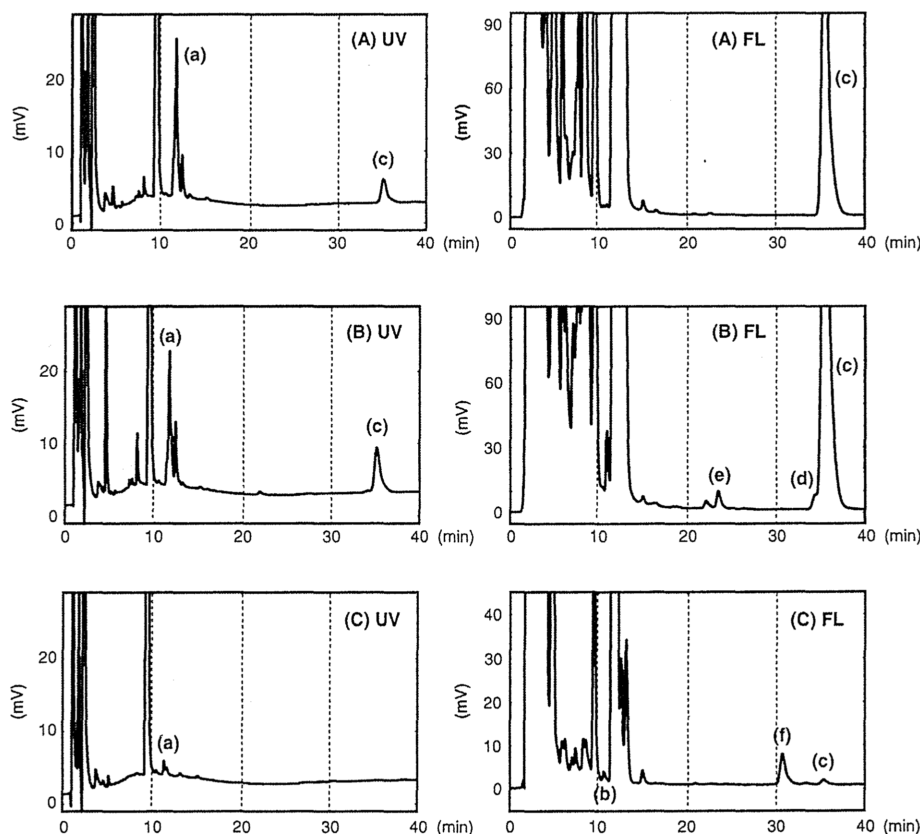

Figure 7. Chromatograms of (A) blank serum sample, (B) serum hydroxylated with ˙OH by the Fenton reaction, and (C) serum nitrated with peroxynitrite (ONOO-). Chromatographic separation was achieved using a Capcell Pak C_{18} MGII (150 × 2.0 mm, 3 μm) and gradient mobile phase consisting of 20 mM sodium phosphate buffer (pH 3.0; solvent A) and acetonitrile (solvent B). A gradient program was used according to the following profile: 0–5 min, 20–30% B; and 5–40 min, 30% B. (a) Phenylalanine, (b) 4-nitrophenylalanine, (c) *p*-tyrosine, (d) *m*-tyrosine, (e) *o*-tyrosine, and (f) 3-nitrotyrosine.

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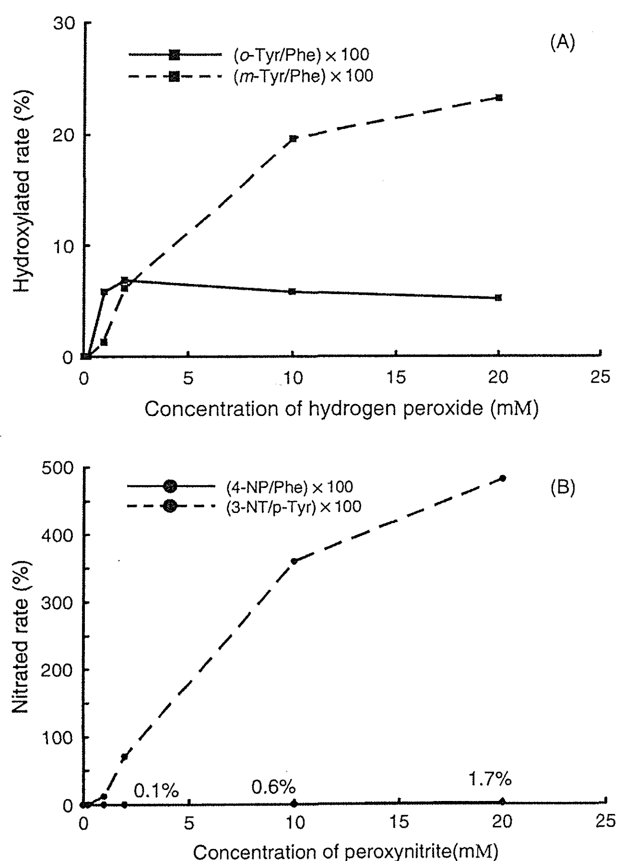

Figure 8. Hydroxylated phenylalanine and nitrated tyrosine formation in human serum treated with (A) hydrogen peroxide or (B) peroxynitrite (ONOO-).

recovery. MQL, accuracy and precision were acceptable according to FDA guidelines for bioanalytical method validation. The MQLs of Phe and *p*-Tyr were 0.1 μM. Our method is useful for the *in vitro* study of oxidative and nitrosative stress.

Acknowledgments

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