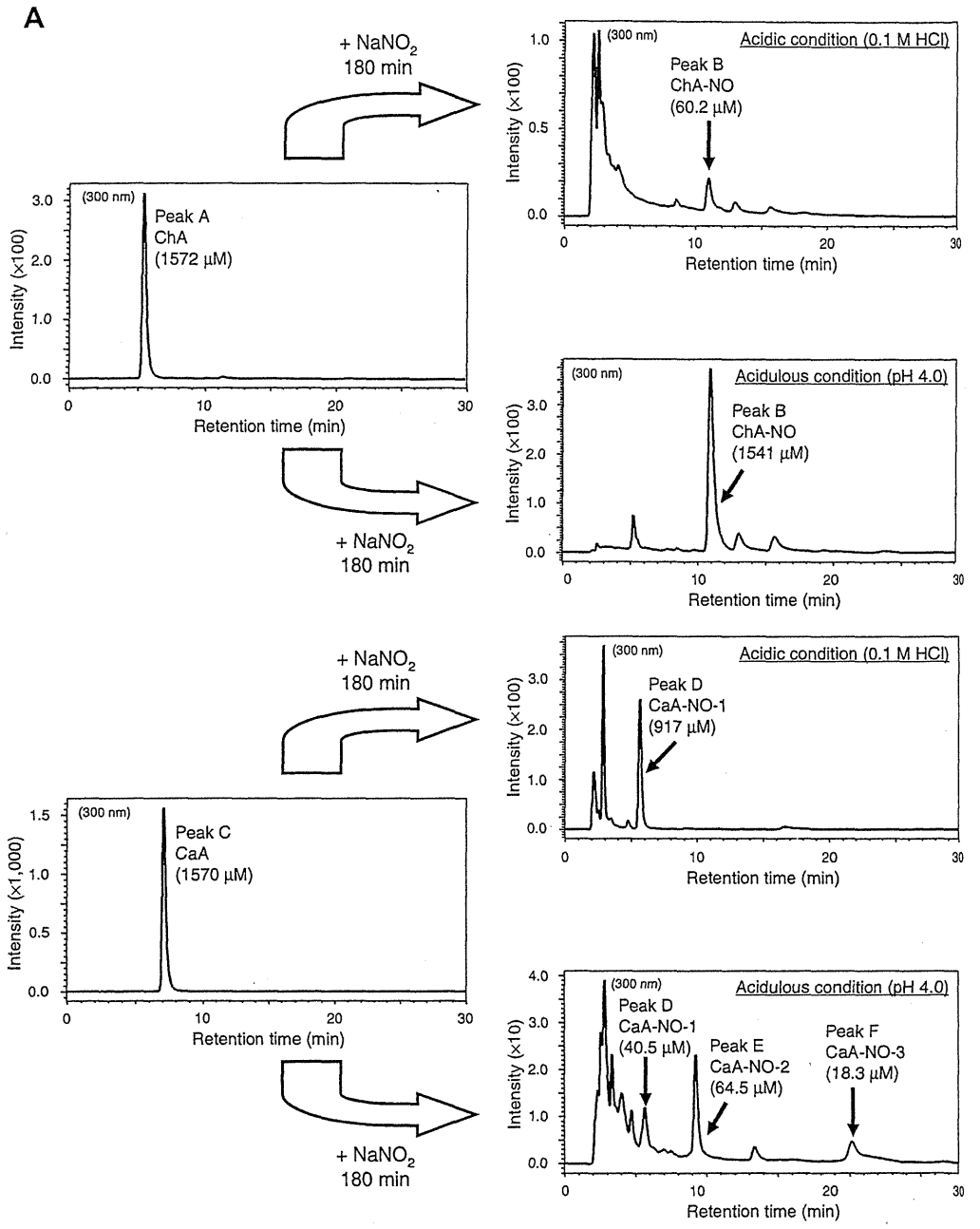

Fig. 3. Reaction between phenolic compound and sodium nitrite under the acidic condition. (A) UV chromatograms of the reaction between phenolic compound and sodium nitrate. (B) Time course of generated compounds under the acidic or acidulous condition.

Electron spin resonance measurement of hydroxyl radical

The ESR method was used for the determination of hydroxyl radical (·OH) [21]. Instead of directly trapping ·OH, this method traps and measures $\cdot CH_3$ by using POBN that is formed during the interaction of DMSO with ·OH. Moreover, this method can detect ·OH, which is not affected by Cu^{2+}. The analysis of $\cdot CH_3$ was carried out with an ESR spectrometer (JES-RE1X, JEOL Co., Tokyo, Japan). The ESR spectrum was measured at a microwave frequency of 9.43 GHz, a magnetic field of 335.5 ± 5 mT, a microwave power of 9 mW, a modulation of 100 kHz, a time constant of 0.03 s, and a sweep time of 30 s, using the ESR spectrometer. The spectra of the samples were scanned to record the signal intensities (peak-to-peak heights).

A typical reaction mixture for incubation consisted of phosphate buffer (50 mM, pH 7.4), POBN (10 mM), DMSO (10%), phenolic compound (2 mM), and copper(II) sulfate pentahydrate (1 mM) in a final volume of 0.3 mL. Samples were used in the reaction that was performed at 37 °C for 30 min.

DNA digestion and determination of dG and 8-OHdG

In order to prevent the formation of oxidative by-products during DNA isolation, DNA was digested by using the slightly modified

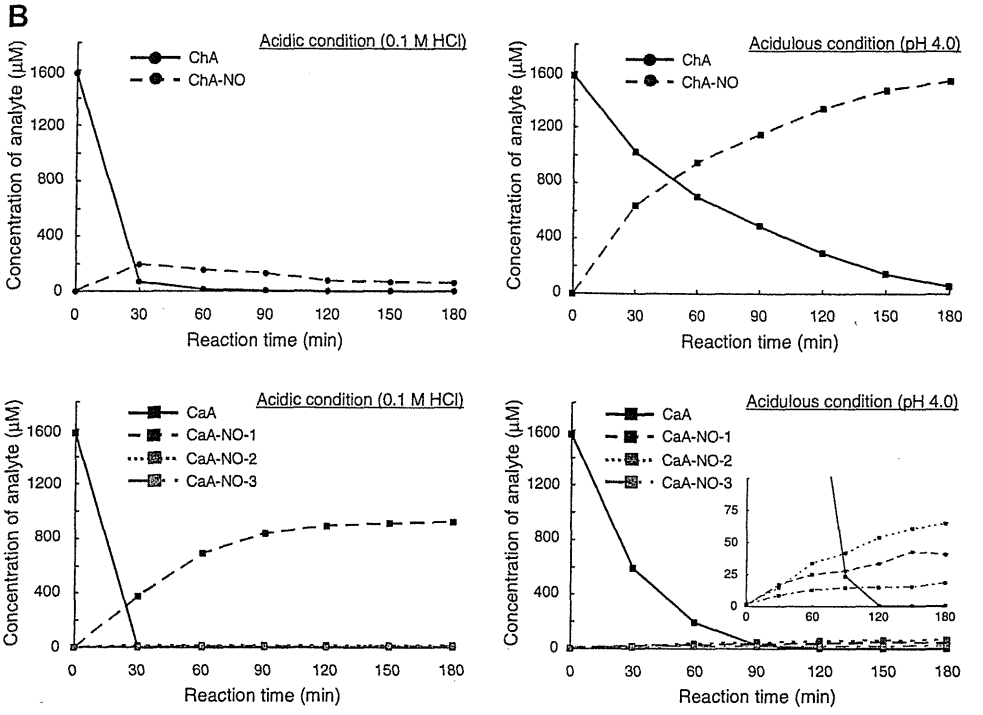

Fig. 3 (continued)

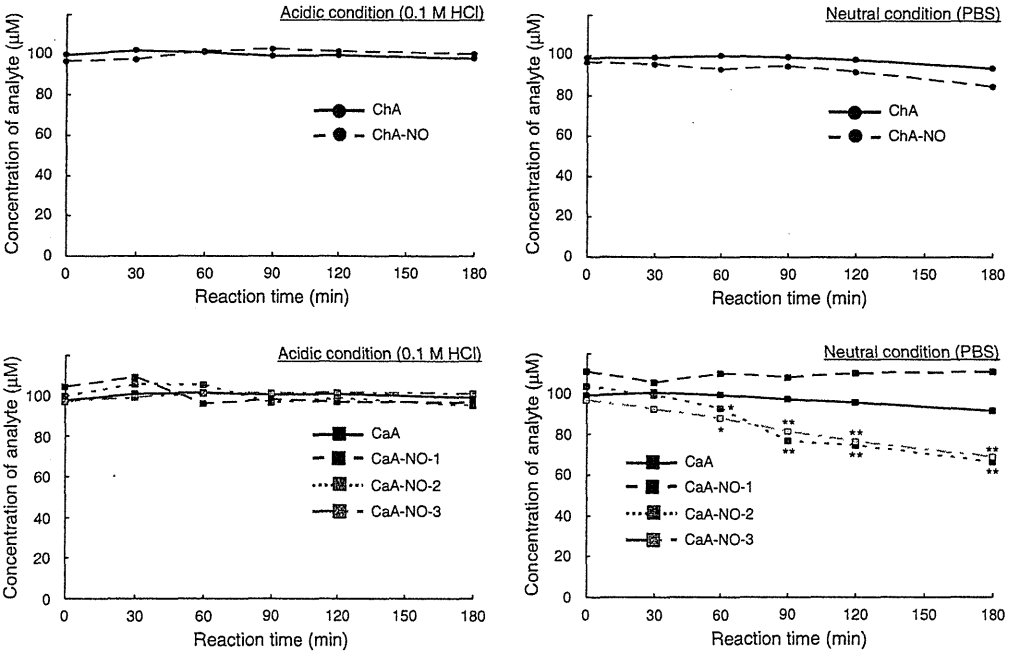

Fig. 4. Sample stability of phenolic and nitrated phenolic compounds under acidic and neutral conditions. *$P < 0.05$ and **$P < 0.01$ vs. 0 min).

method of Ishii et al. [22]. Calf thymus DNA (2 mg/mL) at 250 μL was incubated at 37 °C for 30 min after adding 50 μL of phenolic compound (2 mM) and 50 μL of copper(II) sulfate pentahydrate (1 mM) in 0.15 mL of phosphate buffer (50 mM, pH 7.4). Treated calf thymus DNA was immediately centrifuged at 10,000g for 5 min at 10 °C after the total volume was adjusted to 1.5 mL by adding NaI and 2-propanol. After washing with ethanol, the pellet was dissolved in 0.2 mL of 20 mM sodium acetate buffer, pH 4.8. DNA was enzymatically hydrolyzed by adding 5.0 μL of nuclease P1 to obtain a concentration of 500 U/mL. The mixture was incubated at 60 °C for 15 min. After the addition of 20 μL of 1.0 M Tris–HCl buffer (pH 8.0), 5.0 μL of alkaline phosphatase was added to give a final concentration of 20 U/mL. The mixture was passed through a 3000 NMWL filter (Millipore, Tokyo, Japan) after incubating at 37 °C for 60 min. Then, the digested solution was injected into the HPLC–UV–ECD instrument for 8-OHdG and dG analysis.

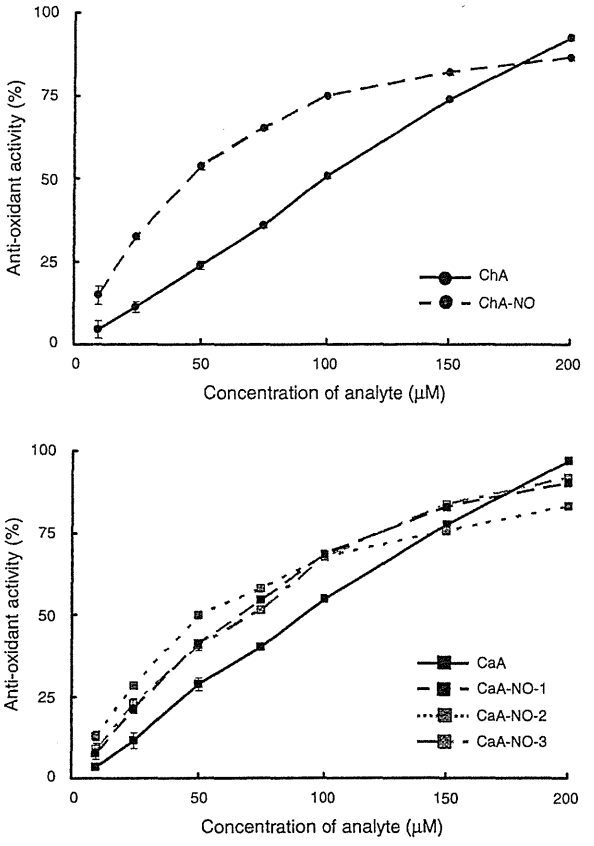

Fig. 5. Anti-oxidant activities of phenolic compounds as assessed by DPPH assay. DPPH concentration was 1 mM. Data points represent means ± SD ($n = 6$).

HPLC–UV–ECD conditions for dG and 8-OHdG analysis

In the assay for dG and 8-OHdG, the UV detector and the ECD instrument used were a Shimadzu SPD10A (Tokyo, Japan) and an ESA Coulochem III (Tokyo, Japan), respectively. A Shimadzu 10Avp pump (Tokyo, Japan) was used to induce flow through the analytical column. An Inertsil ODS3 (4.6 mm × 150 mm, 3.0 μm, GL Sciences, Tokyo, Japan) was used for separation. An aliquot (20 μL) of the sample was injected into the ODS column whose temperature was maintained at 40 °C. The mobile phase was a mixture of 10 mM sodium dihydrogen phosphate/methanol (97/3, v/v). The compounds were eluted isocratically at a flow rate of 1.0 mL/min. The wavelength of the UV detector was set at 290 nm for the detection of dG. The Coulochem III ECD instrument was used with a guard cell (Model 5020; −350 mV) and an analytical cell (Model 5011; electrode 1, 150 mV; electrode 2, 300 mV).

Analysis of nitrate by the Griess assay

Solutions (1 mM) of all compounds were prepared at 37 °C using sodium phosphate buffer (pH 7.4) or 0.1 M HCl. After incubation, an aqueous solution of NO_2^- was mixed with Griess reagent (25 mM sulfanilamide and 2.5 mM *N*-(1-naphthyl)ethylenediamine in 2.5% phosphoric acid) and the whole was incubated at room temperature for 10 min. Absorbance was monitored at 540 nm with a BIO-RAD Model 550 microplate reader. Acidification of the buffered solution resulted in an absorbance band at 540 nm, which was indicative of the corresponding azo dye [23,24].

Statistical analysis

All results are expressed as means ± SD. Statistics were analyzed using one-way analysis of variance (ANOVA) and if statistically significant, post hoc analysis using the Dunnett method was conducted for multiple comparisons among groups. Values of $P < 0.05$ and 0.01 were considered statistically significant.

Results

Characterization of products formed in the reaction of phenolic compounds with $NaNO_2$

In order to identify compounds produced by the reaction between ChA or CaA and $NaNO_2$ in artificial gastric juice, both standards and synthesized compounds were prepared and analyzed (Fig. 2). In the first series of experiments, ChA or CaA (1 mmol) was reacted with $NaNO_2$ (2 mmol) in the acidic condition (0.1 M HCl) or the acidulous condition (0.05 M acetate buffer; pH 4.0). To identify the substrates and the products, HPLC was performed after incubation of ChA or CaA in the presence of $NaNO_2$. HPLC of the reaction mixture after 3 h incubation indicated complete substrate consumption and the presence of a complex pattern of products (Fig. 3A). Peaks B, D, E, and F corresponded to the major components after incubation. Peaks A and C, which are ChA and CaA, respectively, showed a marked decrease in intensity in the presence of $NaNO_2$. Peaks B, D, E, and F showed an increase in intensity and corresponded to the major components after incubation (Fig. 3B). The complexity of the reaction mixture was confirmed by MS and NMR measurements. Peaks B, D, E, and F each gave a deprotonated ion peak $[M-H]^-$ in their ESI/MS spectra, which appeared at *m/z* 398, 180, 178, and 193, respectively, suggesting the formation of nitrated derivatives of ChA-NO, CaA-NO-1, CaA-NO-2, and CaA-NO-3.

The chemical stability of the analytes was assessed under several conditions and the results are shown in Fig. 4. All compounds were stable under the acidic condition. On the other hand, compounds CaA-NO-2 and CaA-NO-3, which are nitrated CaA, decomposed under the neutral condition. These data suggest that nitrated phenolic compounds were formed by reacting each phenolic compound (ChA or CaA) with $NaNO_2$ and could exist stably under the gastric acid condition.

DPPH radical scavenging activity of phenolic and nitrated phenolic compounds

The anti-oxidant activities of the phenolic compounds were measured by the DPPH method, which is one of the oldest and the most frequently used methods for evaluating anti-oxidant activity. The scavenging effects of phenolic compounds on DPPH radicals are shown in Fig. 5. All the phenolic and nitrated phenolic compounds exhibited anti-oxidant activity. The DPPH radical scavenging activities of nitrated phenolic compounds, such as ChA-NO, CaA-NO-1, CaA-NO-2, and CaA-NO-3, were stronger than those of the non-nitrated phenolic compounds.

Measurement of hydroxyl radical and oxidative damage in calf thymus DNA

To investigate ROS generated by the reaction of phenolic compounds with Cu^{2+} we performed electron spin resonance (ESR) measurements and determined whether these compounds have the ability to generate ROS. The pro-oxidant activities induced by the reaction of phenolic compounds with copper are shown in Fig. 6A. The results indicated that ChA and CaA significantly gener-

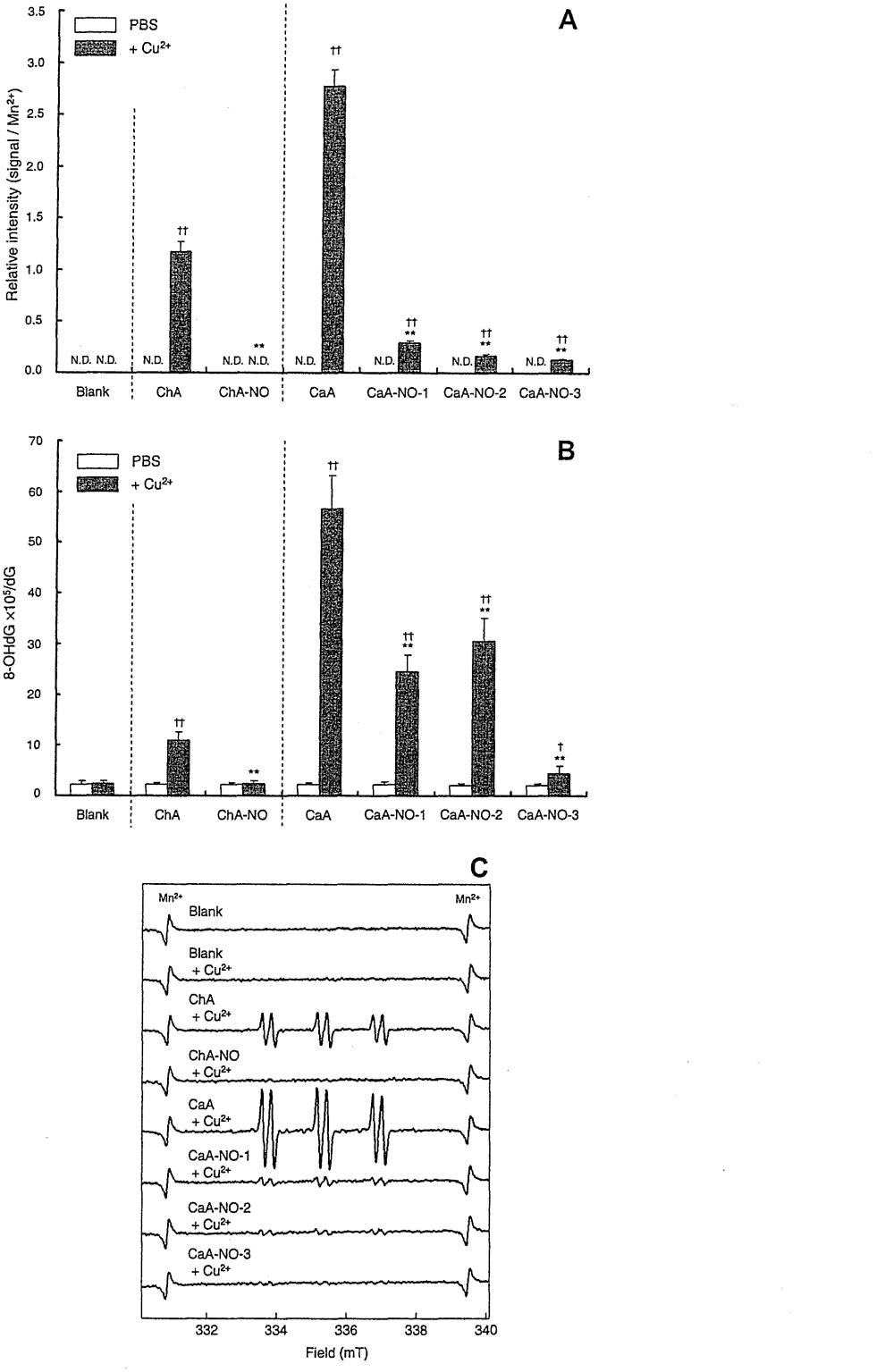

Fig. 6. Pro-oxidant activities produced by the reaction between phenolic compounds and Cu^{2+}. Data represent means ± S.D. ($n = 6$). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. phenolic compound + copper). $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ vs. phenolic compound alone). (A) ESR. (B) HPLC–UV–ECD. (C) Representative ESR spectra of the interaction between ChA, CaA, or nitrated phenolic compounds and copper.

ated ROS in the presence of Cu^{2+}. Meanwhile, nitrated phenolic compounds generated much less ROS in the same conditions.

In the next set of experiments, we examined the effects of the reaction between phenolic or nitrated phenolic compounds and Cu^{2+} on 8-OHdG formation using the same ESR conditions. When calf thymus DNA was treated with phenolic compounds in the presence of $CuSO_4$, 8-OHdG was formed. ChA and CaA induced DNA oxidation in the presence of Cu^{2+} (Fig. 6B). Meanwhile,

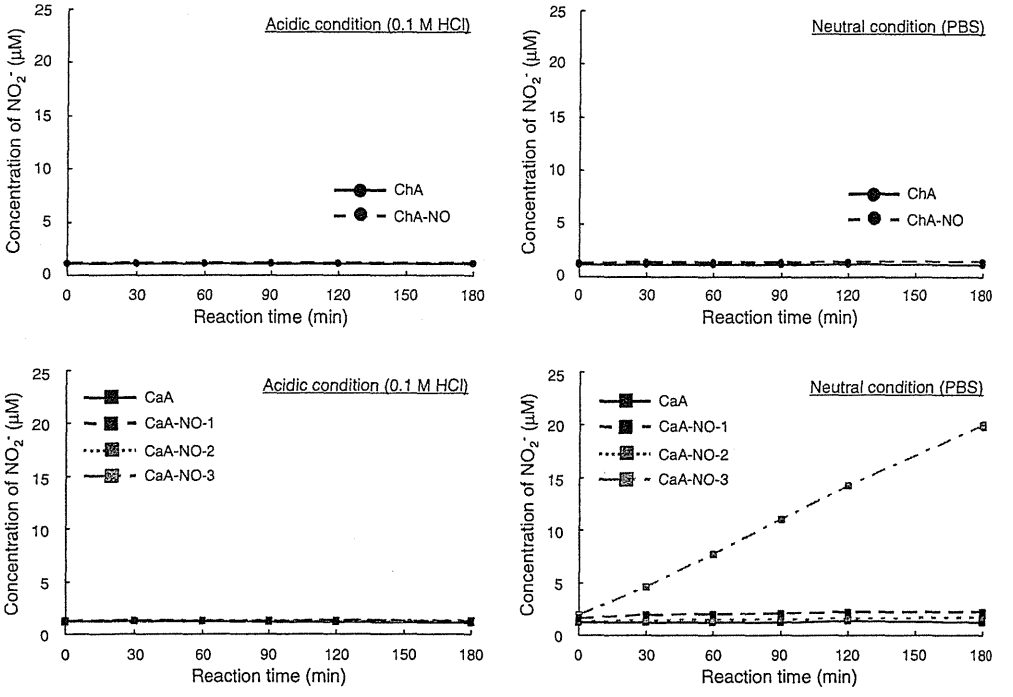

Fig. 7. Concentration of NO_2^- as assessed by Griess assay. Data points represent means ± SD ($n = 6$).

nitrated phenolic compounds obviously showed reduced pro-oxidant activity in the presence of Cu^{2+}. Thus, nitrated phenolic compounds do not have pro-oxidant activity.

Measurement of nitric oxide by Griess assay

The most famous and frequently used method of analysis of nitrite and nitrate is the Griess assay. To examine whether or not NO_2^- is generated from phenolic or nitrated phenolic compounds, we performed the Griess assay. As shown in Fig. 7, all the compounds did not generate NO_2^- under the acidic condition and only CaA-NO-3 was found to generate a significant amount of NO_2^- in the neutral condition. These data suggest that CaA-NO-3 decomposed while generating NO_2^- under the neutral condition.

Discussion

We have identified nitrated phenolic compounds generated by the reaction between ChA or CaA and $NaNO_2$ in artificial gastric juice and assessed their anti-oxidant, pro-oxidant, and nitration activities by an *in vitro* assay.

The mean pH of gastric juice is 1.2. However, it is significantly increased by an H2 receptor antagonist [25]. In our first series of experiments, ChA or CaA was reacted with $NaNO_2$ in the acidic condition (0.1 M HCl) or the acidulous condition (0.05 M acetate buffer; pH 4.0). The reaction after 3 h indicated complete substrate consumption and the presence of a complex pattern of products (Fig. 3A). In particular, several compounds, such as ChA-NO and CaA-NO, were generated at high concentrations under the acidulous condition.

The chemical stability of the analytes was assessed under several conditions and the results are shown in Fig. 4. All compounds existed stably under the acidic condition. On the other hand, compounds CaA-NO-2 and CaA-NO-3, which are nitrated CaA compounds, decomposed under the neutral condition. This result indicated that these compounds were stable in gastric juice but decomposed after being absorbed by the body (i.e., blood and liver).

Fig. 5 shows the results of determination of the anti-oxidant activity of the phenolic compounds by measuring DPPH radical scavenging activity. Our results indicated that nitrated phenolic compounds have stronger anti-oxidant activity than non-nitrated phenolic compounds. Sroka and Cisowski [26] reported the anti-oxidant activity of phenolic acid. The free radical scavenging activity of phenolic acids is correlated with the number of hydroxyl groups bound to the aromatic ring and/or the position of the hydroxyl groups (*ortho* or *para*) in the chemical structure. On the other hand, Kawabata et al. [27] investigated iron complexes of catechol and nitrocatechol derivatives. In the Fenton-like reaction, iron-catechol generated more ·OH than iron–nitrocatechols. Nitrocatechol derivatives having a conjugated structure can sequester chelated iron more effectively than catechol. These results indicated that nitrated phenolic compounds can bind to metal ion and prevent the generation of a large amount of ·OH.

In general, phenolic compounds can switch from being anti-oxidants to pro-oxidants in the presence of Cu^{2+} to induce ROS production and subsequently DNA damage. It is said that *ortho*-dihydroxyl groups that can chelate with Cu^{2+} induce the greatest pro-oxidant activity. The initial electron-transfer oxidation of phenolic compounds by Cu^{2+} generates the corresponding semiquinone radical and the radical undergoes a second electron-transfer reaction with O_2 to form *ortho*-quinone and superoxide anion ($O_2^{\cdot-}$). $O_2^{\cdot-}$ reacts with Cu^+ to give hydrogen peroxide (H_2O_2), which is readily converted via a Fenton-like reaction into ·OH [11]. Pro-oxidant experiments (Fig. 6) have shown that ChA and CaA generate ·OH when mixed with Cu^{2+}. On the other hand, nitrated phenolic compounds showed decreased pro-oxidant activity. These results corresponded to the results of anti-oxidant activity measurements.

A number of NO donors, namely, molecules that are able to release NO in the physiological condition, such as furoxans and organic nitrates, were proven to display cytotoxic and cytostatic effects on viruses and microbial agents [28–30]. Furoxan (1,2,5-oxa-

diazole-2-oxide) is a heterocyclic system known to chemists for its intriguing chemistry and structure. It is known that furoxans are able to release NO at physiological pH, in the presence of thiol cofactors. The mechanism of this release appears to be complex and may involve more than one redox form of NO [30]. Hybrid NO donor furoxan-based drugs are novel drugs that retain the pharmacological activity of the parent compound yet also has the biological activities of NO. To examine whether NO is generated from phenolic or nitrated phenolic compounds, we performed the Griess assay. CaA-NO-3 was found to significantly increase NO_2^- concentration in the neutral condition (Fig. 7), because CaA-NO-3 has a furoxan ring.

Conclusions

We identified the characteristics of compounds generated by reacting ChA or CaA contained in coffee with $NaNO_2$ in artificial gastric juice. All the compounds existed stably under the acidic condition. In addition, ChA, CaA, and the nitrated phenolic compounds were assessed for their anti-oxidant, pro-oxidant, and nitration activities by an *in vitro* assay. The nitrated phenolic compounds seemed to show an increase in anti-oxidant activity and a decrease in pro-oxidant activity. However, CaA-NO-3, which has a furoxan ring, significantly increased NO_2^- concentration in the neutral condition. Further studies are required to reveal the chemical mechanisms underlying the anti-oxidant, pro-oxidant, and nitration activities of phenolic compounds.

Acknowledgment

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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 ($^{\cdot}OH$), which can be generated by the interaction of superoxide anion ($^{\cdot}O_2^-$) 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.

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.

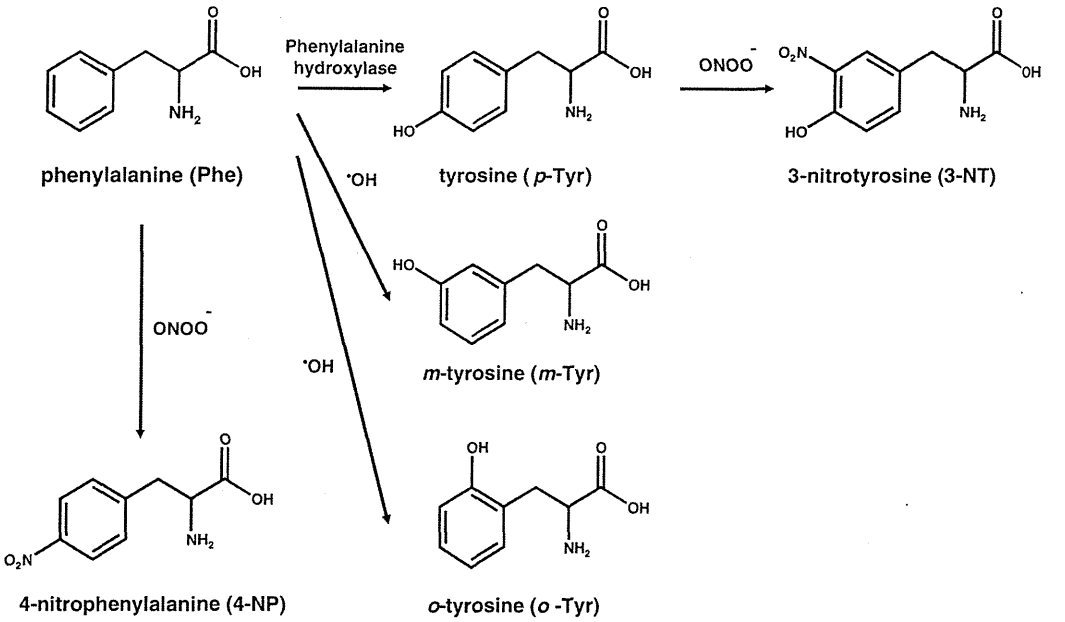

Figure 1. Chemical structures of phenylalanine, tyrosine, and their hydroxylated and nitrated compounds.

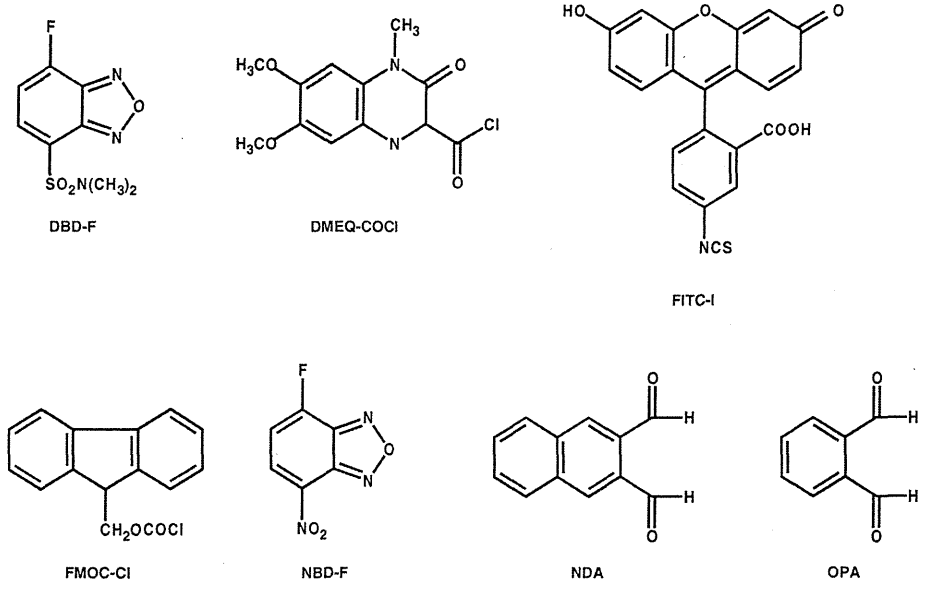

Figure 2. Chemical structures of FL reagents.

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.

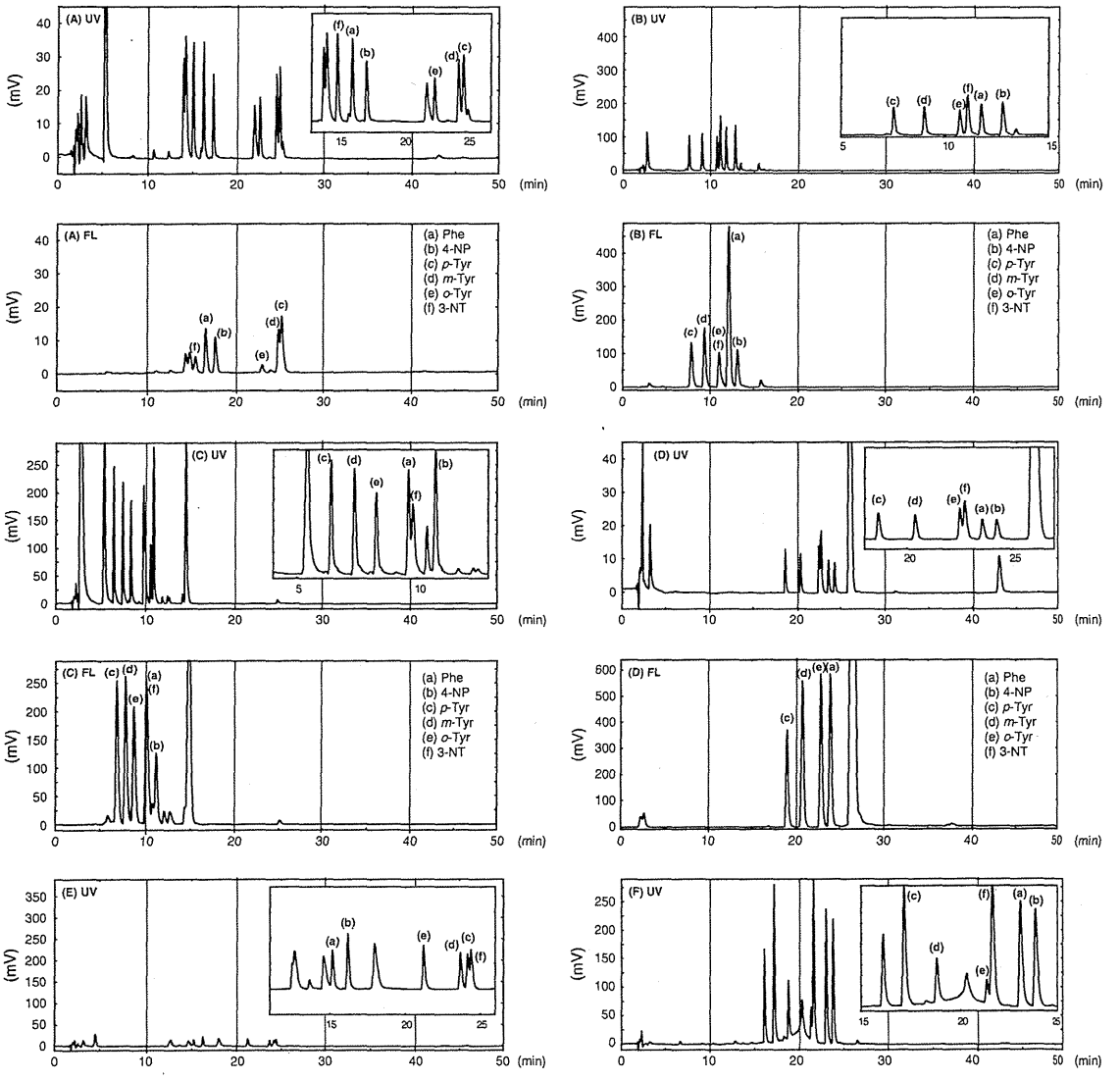

Figure 3. UV and FL chromatograms for the determination of hydroxylated phenylalanine and nitrated tyrosine (50 μM) products that were derivatized by several reagents. The derivatized standards were separated with a Capcell Pak C_{18} MGII (150 × 2.0 mm, 3 μm) and a gradient mobile phase consisting of 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.

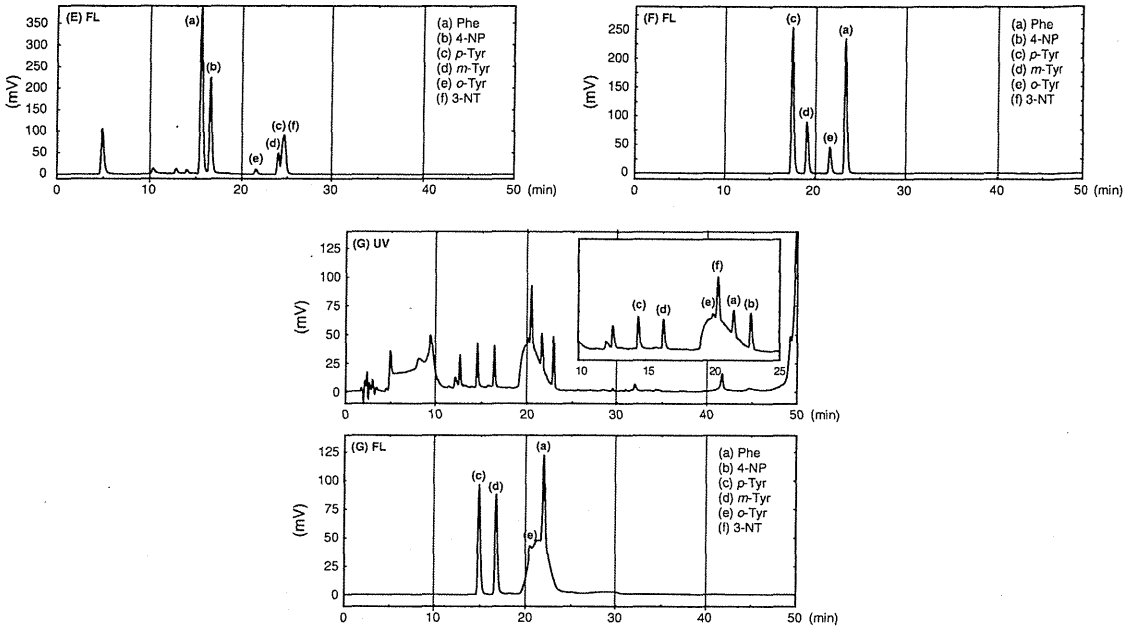

Figure 3: *Continued*

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

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.

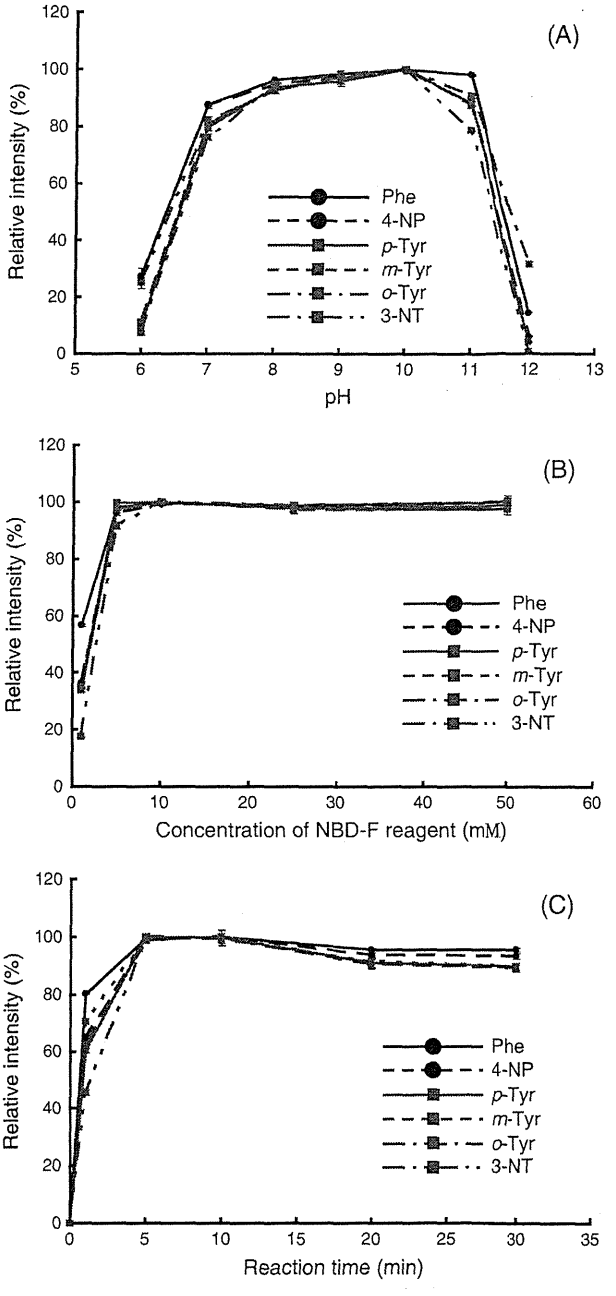

Figure 4. Optimization of derivatization conditions: (A) pH of reaction buffer; (B) concentration of NBD-F; and (C) reaction time.

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.

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).

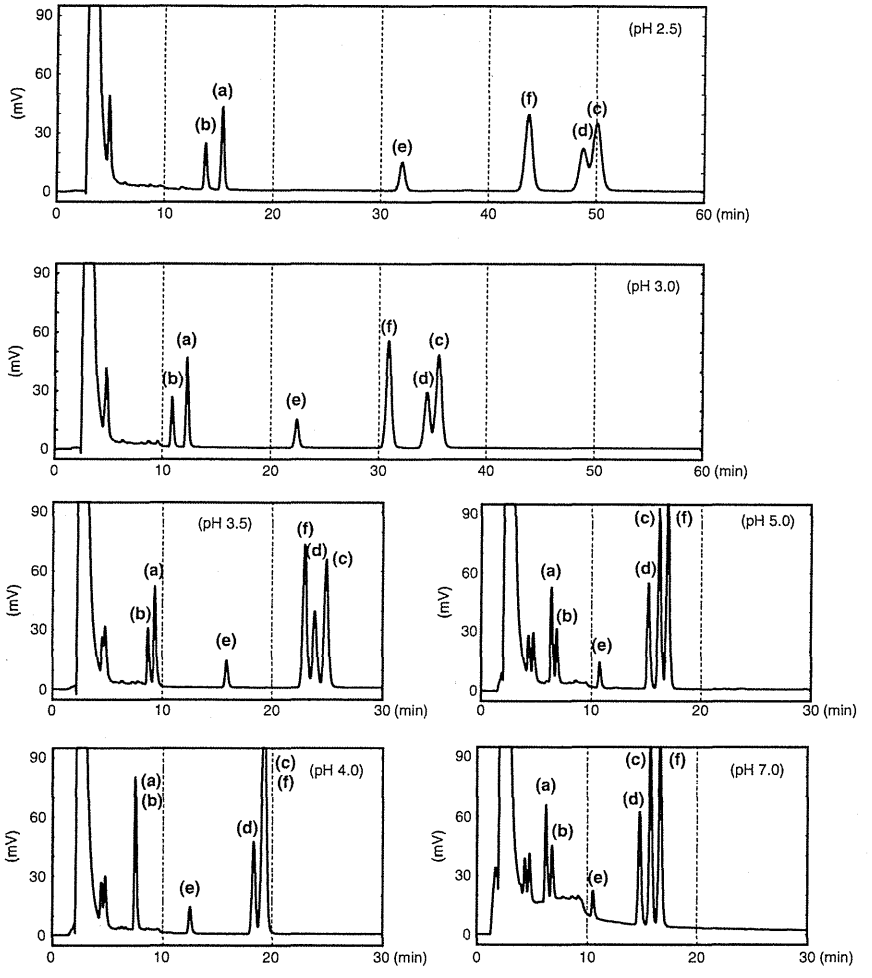

Figure 5. Effect of mobile phase pH on the separation of analytes. Chromatographic separation was achieved with a Capcell Pak C_{18} MGII (150 × 2.0 mm, 3 μm) and a gradient mobile phase consisting of 20 mM sodium phosphate buffer (solvent A) and acetonitrile (solvent B). A gradient program was used 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

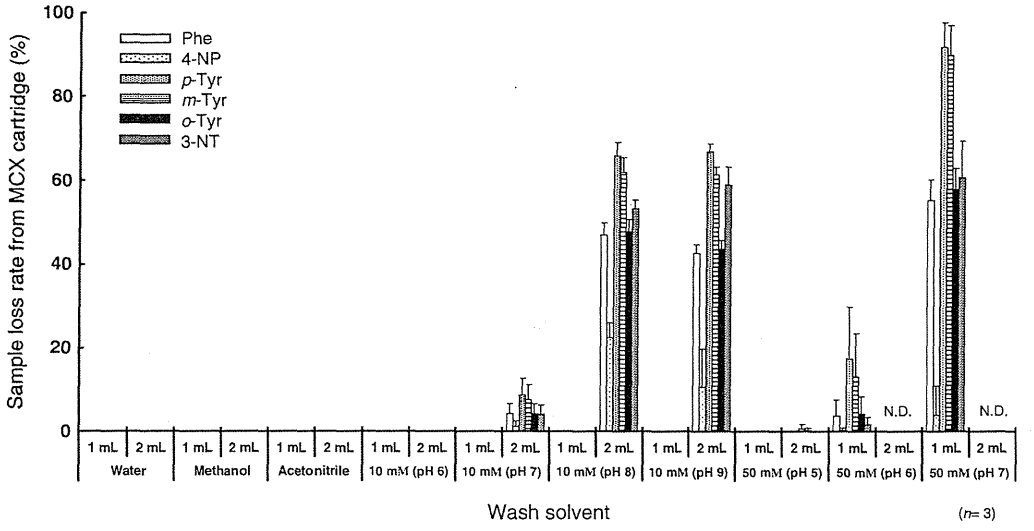

Figure 6. Optimization of wash conditions for solid-phase extraction. Washing solvents were prepared with sodium phosphate buffer. N.D., not determined.

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.

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 $^{\cdot}OH$ 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 $^{\cdot}OH$. 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 $^{\cdot}O_2^-$ 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

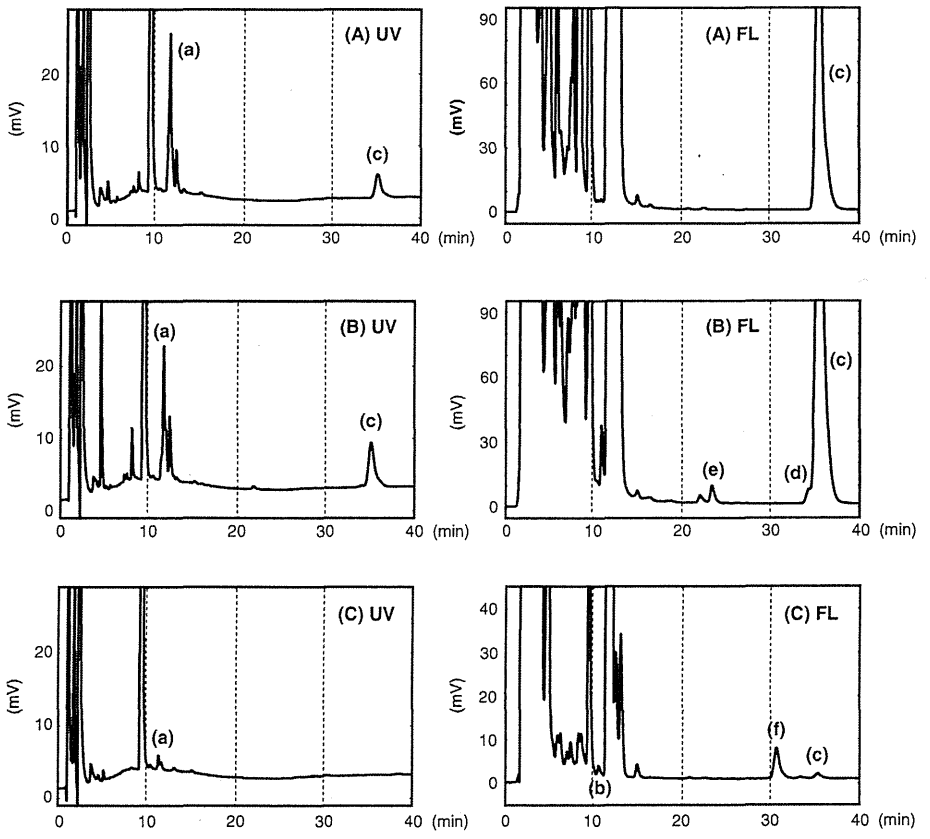

Figure 7. Chromatograms of (A) blank serum sample, (B) serum hydroxylated with $^{\cdot}OH$ by the Fenton reaction, and (C) serum nitrated with peroxynitrite (ONOO-). Chromatographic separation was achieved using a Capcell Pak C_{18} MGII (150 × 2.0 mm, 3 µm) and gradient mobile phase consisting of 20 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.

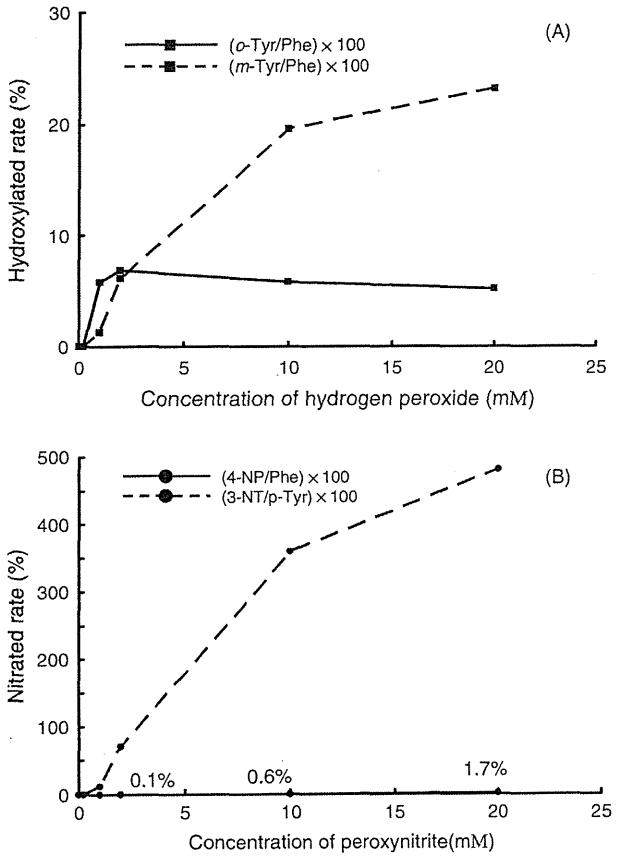

Figure 8. Hydroxylated phenylalanine and nitrated tyrosine formation in human serum treated with (A) hydrogen peroxide or (B) peroxynitrite (ONOO-).

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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