Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 799 (2004) 245–254
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous determination of tyrosine, phenylalanine and deoxyguanosine oxidation products by liquid chromatography–tandem mass spectrometry as non-invasive biomarkers for oxidative damage

Hilmi Orhan[1], Nico P.E. Vermeulen, Cornelis Tump, Herman Zappey, John H.N. Meerman*

Department of Pharmacochemistry, Division of Molecular Toxicology, Leiden/Amsterdam Center for Drug Research (LACDR), Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

Received 5 May 2003; received in revised form 23 October 2003; accepted 23 October 2003

Abstract

We developed an isotope dilution HPLC–atmospheric pressure chemical ionization–tandem mass spectrometry (HPLC–APCI–MS/MS) method for the simultaneous determination of *p*-tyrosine, phenylalanine, *o*,*o*′-dityrosine, *m*-tyrosine, *o*-tyrosine, 3-chlorotyrosine and 3-nitrotyrosine and 8-hydroxy-2′-deoxyguanosine (8-OHdG) that requires no extensive sample pre-treatment. *p*-[$^2H_4$]Tyrosine and *o*,*o*′-[$^2H_6$]dityrosine were used as internal standards. Calibration curves of the method were linear ($r^2 = 0.990$–0.999) over a concentration range of 0.03–10 μM for *o*-tyrosine; 0.04–10 μM for 3-nitrotyrosine and 3-chlorotyrosine; 0.05–10 μM for *o*,*o*′-dityrosine; and for *m*-tyrosine; 1.0–100 μM for *p*-tyrosine and for phenylalanine; and 0.01–10 μM for 8-OHdG. The detection limits were from 0.025 to 0.05 μM for the tyrosine derivatives; 0.01 μM for 8-OHdG; and 0.5 μM for *p*-tyrosine and for phenylalanine, respectively. Within-day coefficients of variation (CV) for spiked human urine samples ranged from 2.7 to 7.0%, except for 8-OHdG (13.7%). Between-day variations ranged from 7.9 to 13.0%, except for *o*-tyrosine (CV = 18.2%), and for 8-OHdG (CV = 24.7%).

The background levels of *p*-tyrosine, phenylalanine, *o*,*o*′-dityrosine, and *o*-tyrosine in morning urine of eight healthy volunteers were 3890 ± 590, 3420 ± 730, 5.8 ± 0.3, and 9.2 ± 1.5 μmol/mol creatinine, respectively. Using the present HPLC–APCI–MS/MS method, the urinary background levels of *m*-tyrosine, 3-chlorotyrosine, 3-nitrotyrosine and 8-OHdG were below the limit of detection.

Keywords: Non-invasive biomarkers; Tyrosine; Phenylalanine; Deoxyguanosine

1. Introduction

Reactive oxygen species (ROS) and other free radicals may result from normal metabolic processes and from exposure to exogenous factors such as UV-light, radiation and various chemicals. Radical attack on cellular membrane lipids, proteins and DNA may cause oxidative injury and is thought to be involved in the aetiology of diseases like cancer [1,2], Parkinson's disease, Alzheimer's disease [3–5], rheumatoid arthritis [6,7], diabetes [8,9], Down's syndrome [10] and arteriosclerosis [2]. Experimental animal and controlled human intervention studies have demonstrated that oxidative damage by ROS can be reduced by dietary components [11–13] or by supplementation with antioxidants [14–16]. Determination of oxidative damage in humans is very useful, e.g. in order to study the effects of antioxidants administered to humans for the protecting of chemically/drug-induced oxidative damage or disease [16,17]. Since the half-lives of ROS are extremely short, they cannot be measured directly in humans. However, stable end products of oxidative damage to cellular macromolecules such as lipids, proteins and DNA that are excreted in exhalatory air or in urine may be used instead in a non-invasive manner [5].

Biomarkers are supposed to reflect changes in biological systems that are related to exposure to, or effects of toxic agents [18–20]. An ideal biomarker of effect should fulfil at least the following characteristics: (1) high specificity for the effect of interest, (2) reflection of an early effect, (3) easy and inexpensive analysis, (4) sample available by non-invasive sampling techniques, (5) low background

* Corresponding author. Tel.: +31-20-4447600; fax: +31-20-4447610.
E-mail address: meerman@few.vu.nl (J.H.N. Meerman).
[1] Present address: Toxicology Department, Faculty of Pharmacy, Hacettepe University, 06100 Ankara, Turkey.

1570-0232/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2003.10.056

level of the biomarker in the medium of interest, (6) a well-established relationship between the response of the biomarker and exposure, and (7) a well-established relationship between the response of the biomarker and the induced damage [5]. Recently, de Zwart et al. developed a set of biomarkers for oxidative damage to cellular membranes by measuring six aldehydes, i.e. formaldehyde, acetaldehyde, propanal, butanal, pentanal, and hexanal, and one keton (acetone) in urine by GC–ECD [21]. This multi-parameter biomarker set was successfully applied to determine radical damage in different organs of the rat, treated with various toxic chemicals [22–24].

Oxidized phenylalanine derivatives (*m*- and *o*-tyrosine) and oxidized tyrosine derivatives (*o*,*o*′-dityrosine, 3-nitrotyrosine, 3-chlorotyrosine, and 3,4-dihydroxyphenylalanine (dopa)) have been suggested as promising biomarkers for oxidative damage to proteins [25–29]. 8-OHdG has been shown to be a biomarker for oxidative damage to DNA [30,31]. Measurement of these biomarkers could in principle provide information about the radical source causing the oxidative damage: 3-nitrotyrosine is formed upon reaction of tyrosine with nitrogen radicals or peroxynitrite [32,33], 3-chlorotyrosine upon reaction of tyrosine with hypochlorous acid (HOCl) [34,35], *m*-, and *o*-tyrosine by reaction of phenylalanine with hydroxyl radicals [36,37], similarly *o*,*o*′-dityrosine is formed when hydroxyl radicals cross-link tyrosine residues [36,37], while 8-OHdG is formed as a result of attack by hydroxyl radicals to DNA [31,38]. The latter biomarker, 8-OHdG, has successfully been utilised by us to determine the oxidative DNA damage in rats treated with Fe–nitriloacetate [23].

Currently, there is no method available for the simultaneous determination of the six tyrosines and phenylalanine in biological matrices. The methods developed to date measure the tyrosines in separate runs by HPLC with electrochemical detection (HPLC–EC) [29,39,40] and by isotope dilution gas chromatography–mass spectrometry (GC–MS) [37]. Basically, these techniques require extensive sample pre-treatment such as (solid phase) extraction, derivatization and concentration. Very recently, a study was published on an atmospheric pressure chemical ionization (APCI)–MS/MS method for the analysis of *o*,*o*′-dityrosine, *o*-tyrosine, and 3-nitrotyrosine in cat urine. Although a similar sensitivity was reported for *o*,*o*′-dityrosine as in the present study, analysis of *o*-tyrosine, and 3-nitrotyrosine required rather extensive sample clean-up and derivatization [41]. In the present study, we aimed at developing an isotope dilution HPLC–APCI–MS/MS method for the simultaneous determination of *o*,*o*′-dityrosine, *m*-tyrosine, *o*-tyrosine, 3-chlorotyrosine and 3-nitrotyrosine in human urine, as non-invasive multi-parameter biomarker for oxidative protein damage. *p*-Tyrosine and phenylalanine were also measured as parent amino acids for the oxidized products. *o*,*o*′-[$^2H_6$]dityrosine was synthesized as internal standard for *o*,*o*′-dityrosine. For all other analytes, *p*-[$^2H_4$]tyrosine was used as internal standard, except for 8-OHdG. We compared the HPLC–APCI–MS/MS method to a GC–ECD method that was developed by us for the quantification of the tyrosines in human urine after derivatization with chloroformate and heptafluorobutanol.

2. Experimental

2.1. Chemicals

p-[ring-$^2H_4$]Tyrosine (*p*-[$^2H_4$]tyrosine) was obtained from Cambridge Isotope Laboratories (Andover, MA). 8-Hydroxy-2′-deoxyguanosine (8-OHdG), DL-*m*-tyrosine, DL-*o*-tyrosine, 3-nitro-L-tyrosine, *m*-fluoro-DL-tyrosine, DL-β-3,4-dihydroxyphenylalanine (dopa), picric acid, diethylenetriaminepentacetic acid (DTPA) and horseradish peroxidase (HRP) were obtained from Sigma (St. Louis, MO, USA). 3-Chloro-L-tyrosine hydrochloride was obtained from ICN Biomedicals (Zoetermeer, The Netherlands). *p*-Tyrosine and perchloric acid (PCA) were purchased from BDH (England). L-Phenylalanine was obtained from Janssen Chimica (Belgium). Methanol, *n*-heptane, acetic acid, hydrochloric acid and ammonium acetate were obtained from Riedel-de-Haën (Seelze, Germany). 2,2,3,3,4,4,4-Heptafluoro-1-butanol was obtained from Acros (Belgium). Isobutyl chloroformate and heptafluorobutyric anhydride (HFBA) were obtained from Fluka (Buchs, Switzerland). 9-Fluorenylmethyl chloroformate and *N*-(heptafluorobutyryl)imidazole (HFBI) were purchased from Pierce (Rockford, Illinois, USA). Pyridine, ammonium hydroxide, creatinine and hydrogen peroxide were obtained from J.T. Baker (Deventer, The Netherlands). Trifluoroacetic acid (TFA) was obtained from Merck (Germany). All chemicals were of the highest purity grade that was available. Nanopure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). DEAE cellulose was purchased from Whatman (Whatman International, Amsterdam, The Netherlands) and BioGel P2 gel beads from Bio-Rad (Bio-Rad Laboratories, Veenenedaal, The Netherlands). The analytical and the preparative HPLC columns were obtained from Phenomenex (California, USA) and from Alltech (Illinois, USA), respectively.

7-Methoxy-4-(aminomethyl)coumarin (MAMC), used as internal standard for the HPLC-fluorescence detection analysis of the various tyrosines, was synthesized as described [42].

2.2. Sample preparation for analysis by GC–ECD

After the addition of 1 μM 3-fluorotyrosine as internal standard (IS) to 2 ml urine, clean-up procedures were applied by using C-18 solid-phase extraction (SPE) columns (3 ml Bakerbond, J.T. Baker, Deventer, The Netherlands), or cation-exchange columns according to Heinecke et al. [27] and Crowley et al. [28], respectively.

2.3. Derivatization and analysis of tyrosines by GC–ECD

The oxidized protein products were converted into their *N*(*O*,*S*)-isobutoxycarbonyl heptafluorobutyl esters by the method of Wang et al. [43]. We optimized this derivatization method for oxidized urinary products and analysis by GC–ECD as described in the results section. A 1 μl aliquot was injected onto the GC column. Analysis by ECD was carried out on a Hewlett Packard 5890 series II gas chromatograph equipped with a 15 mCi ^{63}Ni electron-capture detector. GC separation was achieved on a CP Sil 8 CB (25 m × 0.15 mm i.d.) WCOT fused-silica capillary column with a 0.12 μm film coating from Chrompack (Middelburg, The Netherlands). Split injection of 40:1 was used. The temperature of the GC oven was programmed from 200 °C (0.5 min) to 250 °C at a rate of 25 °C/min and kept at this temperature for 9 min. Subsequently; the temperature was increased to 325 °C at a rate of 25 °C/min., and kept at 325 °C for 12 min. The temperatures for the injector and detector were 270 and 300 °C, respectively. The carrier gas (helium) flow rate was 0.7 ml/min.

2.4. Synthesis and characterization of *o,o′*-dityrosine and *o,o′*-[ring-$^2H_6$]dityrosine

Synthesis and purification of *o,o′*-dityrosine was accomplished by the method of Malencik et al. [44]. Briefly, *p*-tyrosine was oxidized by horseradish peroxidase in the presence of hydrogen peroxide. Subsequently, *o,o′*-dityrosine was purified by Whatman DEAE cellulose column chromatography, followed by BioGel P-2 column chromatography. Final purification was achieved by preparative HPLC; *o,o′*-dityrosine was recovered by gradient elution from the C-18 column (Econosil C18, 250 mm × 10 mm). The composition of eluent varied linearly from acetonitrile–water–TFA (1:99:0.02) to acetonitrile–water–TFA (20:80:0.02) over 25 min. The gradient was started 5 min after the injection. A flow rate of 4 ml/min was used. Purified *o,o′*-dityrosine was obtained as a pale yellow powder following freeze drying of the combined fractions. *o,o′*-[ring-$^2H_6$]Dityrosine (*o,o′*-[$^2H_6$]dityrosine) was synthesized and purified similarly by using *p*-[$^2H_4$]tyrosine instead of *p*-tyrosine. The yield for both *o,o′*-dityrosine and *o,o′*-[$^2H_6$]dityrosine synthesis was 25%. Typical side-products and/or impurities of the dityrosine synthesis by HRP/H_2O_2 oxidizing system are *p*-tyrosine (starting compound), isodityrosine, trityrosine, and pulcherosine [45]. Both *o,o′*-dityrosine and *o,o′*-[$^2H_6$]dityrosine batches were analyzed for these and other contaminants by reversed-phase HPLC with simultaneous UV-detection (280 nm) and fluorescence-detection (ex. 280 m, em. 410 nm). A phenomenex Inertsil ODS 2 (150 mm × 4.6 mm, 5 μm) HPLC column (Bester, Amsterdam, The Netherlands) equipped with a guard column was used for these analyses. A gradient was formed from 10 mM ammonium acetate, adjusted to pH 4.5 with acetic acid, and methanol, starting with 1% methanol and increasing to 10% over 30 min. The flow rate was 0.8 ml/min. These analyses of *o,o′*-dityrosine and *o,o′*-[$^2H_6$]dityrosine showed that the combined and lyophilized fractions contained only one 410 nm-emitting component and only one 280 nm-absorbing component. ^{1}H NMR spectra were consistent with the structures and confirmed the purity of both products. As expected, *o,o′*-[$^2H_6$]dityrosine did not contain aromatic protons at 7.1 ppm, compared to the spectrum of *o,o′*-dityrosine. HPLC–APCI–MS analysis at positive ion mode showed the expected protonated molecular ions, *m/z* 361 for *o,o′*-dityrosine, and *m/z* 367 for *o,o′*-[$^2H_6$]dityrosine.

2.5. Urine collection

From eight healthy volunteers, morning urines (the first urine after wake-up) and the first following urine fractions were collected in 500 ml containers. After arrival in the laboratory (∼1 h), 1 ml of the urines were mixed with 0.1 ml of antioxidant solution (containing 5% phenol as bactericidal agent, and 5 mM DTPA as metal chelator) and subsequently they were either analyzed freshly or stored (usually 2–3 days) at −35 °C until analysis. Urinary creatinine concentrations were determined in non-frozen aliquots by the Jaffée method [46]. *p*-Tyrosine, phenylalanine, *o,o′*-dityrosine, *m*-tyrosine, *o*-tyrosine, 3-chlorotyrosine, 3-nitrotyrosine and 8-OHdG levels were analyzed in the urine samples by HPLC–APCI–MS/MS as described below.

2.6. Sample preparation for analysis by HPLC–APCI–MS/MS

Weimann et al. [31] reported that 8-OHdG may precipitate in urine upon freezing. Therefore, any precipitate that was formed upon storage was re-dissolved by heating the urine samples to 37 °C for 10 min. Subsequently, *p*-[$^2H_4$]tyrosine and *o,o′*-[$^2H_6$]dityrosine (5 μM final concentrations) were added as internal standards either to fresh or thawed urine samples. After mixing, the samples were centrifuged at 14,000 × *g* for 15 min. The clear supernatant was used for the analyses.

2.7. Analysis of tyrosines by HPLC–APCI–MS/MS

Fifty microlitres of the clear supernatant was injected onto the Phenomenex Inertsil ODS 2 HPLC column using a HPLC system consisting of a Shimadzu SCL-10 AD*vp* system controller, two LC 10 AD*vp* pumps, a CTO 10 AS*vp* column oven and an SIL 10 AD*vp* injector (Shimadzu, Hertogenbosch, The Netherlands). A methanol gradient was applied as described in Section 2.4. The flow rate was 0.8 ml/min. The HPLC was coupled to an ion-trap mass spectrometer (Finnigan LCQ Deca, ThermoQuest, San Jose, CA, USA) equipped with an APCI source. To obtain maximum selectivity, the mass spectrometer was operated in the selected reaction-monitoring (SRM) mode with

Table 1
Time window settings, retention times and precursor/product ions for the HPLC–APCI–MS/MS analysis of the tyrosines, phenylalanine and 8-OHdG

Time Window	Time frame (min)	Compound	Retention time (min)	Precursor/product (*m*/*z*)
1	5–8	*p*-Tyrosine	6.8	182/165
		p-[$^2H_4$]tyrosine	6.7	186/169
2	8–11	*o*,*o*′-Dityrosine	8.9	361/315
		o,*o*′-[$^2H_6$]dityrosine	8.9	367/321
		m-Tyrosine	9.7	182/136
3	11–19	*o*-Tyrosine	12.8	182/136
		Phenylalanine	13.7	166/120
		3-Chlorotyrosine	17.7	216/199
4	19–30	3-Nitrotyrosine	22.4	227/181
		8-OHdG	27.8	284/168

positive ionization. Ionization temperature was 450 °C. The precursor/product reactions and the other time window settings are shown in Table 1. We divided the run time-window into four segments. After the last time-window, at 30 min, the column was equilibrated with 10 mM ammonium acetate containing 1% methanol for 20 min. The precursor and product ions were determined by MS/MS analyses of protonated analyte ions from standard solution. The activation amplitudes were 30% for the tyrosines and phenylalanine and 40% for 8-OHdG. The amounts of total microscans and maximum injection time were optimized to 3 and 200 ms, respectively.

To save the ion-source from contamination, the first five minutes and the last 20 min were diverted to waste. Since dopa elutes around 5 min, it was not analyzed for the sake of robustness of the method.

2.8. Linearity, reproducibility and sensitivity of the HPLC–APCI–MS/MS method

The sensitivity and linearity of the analytical method was tested for all six tyrosines and 8-OHdG both in water and in urine (concentration range: 0.01, 0.025, 0.05, 0.10, 0.50, 1.0, 5.0 and 10.0 μM). Information on reproducibility was obtained from the analysis of spiked urine samples that contained 2.5 μM of each analyte. For within-day variation, five individual samples were analyzed on the same day. For between-day variation, 20 samples were analyzed on four different days.

2.9. Stability of tyrosines

The stability of the tyrosines at three different pH values (4.0, 7.4 and 9.0) in ammonium acetate buffer (10 mM) was determined after 0, 1, 2, 5 and 7 days, 2 and 3 weeks of storage at room temperature and after 0, 1, 2, 3, 5 and 7 days, 2 and 3 weeks, 1, 2 and 3 months of storage at −35 °C. The six tyrosines were stored in buffered solutions in the presence or absence of two different antioxidants: 0.5% phenol + 0.5 mM DTPA or 100 μM Vitamin C. Samples were analyzed by HPLC with UV (280 nm), and fluorescence-detection (280/410 nm for *o*,*o*′-dityrosine; 275/305 nm for *o*- and *m*-tyrosine). Peak ratios of the compounds relative to the internal standards (3-fluorotyrosine for the UV-detection, MAMC for the fluorescence detection) were used for evaluation of the stability.

3. Results

3.1. GC–ECD

Our initial objective was to simultaneously measure *p*-tyrosine, *o*,*o*′-dityrosine, *m*-tyrosine, *o*-tyrosine, 3-chlorotyrosine, 3-nitrotyrosine and dopa in biological samples by GC–ECD using a derivatization method converts the analytes to heptafluorobutyryl derivatives by HFBA described for GC–MS by Heinecke et al. [27]. However, HFBA proved to be inappropriate as reagent for GC–ECD as it generated a relatively high and long lasting background trace in the GC–ECD chromatograms. This made detection of trace amounts of the different tyrosines impossible. Changing the HFBA reagent for HFBI did not solve this problem.

A second derivatization method, i.e. using chloroformate and halogenated alcohol combinations as described by Wang et al. [43], proved to be more suitable for GC–ECD application. Derivatization of tyrosines took less than 5 min, a stable baseline was obtained in the GC–ECD chromatogram, and the method is well applicable to aqueous samples. The highest responses and the best chromatography were obtained with combination of isobutyl chloroformate and heptafluorobutanol. However, *o*,*o*′-dityrosine showed two peaks. Identification by gas chromatography with negative chemical ionization (GC–NCI) showed that the extra peak corresponded to a *o*,*o*′-dityrosine derivative in which one of the ester moieties was an isobutyl ester. In some cases, the other compounds also showed two or more peaks instead of one in the chromatogram. We tried to eliminate these minor peaks by varying the amounts of chloroformate, pyridine and alcohol in the reaction mixture, and by varying reaction times and the inlet temperatures of the GC-apparatus. These changes, however, did not eliminate the minor peaks

completely. Nevertheless, *p*-tyrosine, 3-chlorotyrosine and *o*,*o*′-dityrosine were well detectable in spiked urine sample, while *m*-tyrosine, 3-nitrotyrosine and dopa appeared as shoulders on other peaks and *o*-tyrosine could not be detected at all in urine samples. Three different clean-up methods using C-18, anion-, or cation-exchange columns were applied as described in methods section. However, none of these methods were able to eliminate all disturbing peaks in the GC–ECD chromatograms.

3.2. HPLC–APCI–MS/MS analysis of six tyrosines and 8-OHdG

As an alternative for the GC–ECD method described above, we developed a HPLC–APCI–MS/MS method for the analysis of the various tyrosines. For all seven compounds, the positive ion mode proved to be more sensitive than the negative ion mode and the main ions that showed up in the mass spectra were the pseudo-molecular ions $[M + H]^+$.

Fragmentation patterns of the analyzed compounds by HPLC–APCI–MS/MS in positive ion mode were as follows: *p*-tyrosine and 3-chlorotyrosine lost *m*/*z* 17 (NH_3^+), and gave *m*/*z* 165 and 199 as most intense fragment ions respectively; phenylalanine, *o*-tyrosine, *m*-tyrosine and 3-nitrotyrosine lost *m*/*z* 46 (HCOOH) during secondary fragmentation, resulting in *m*/*z* 120 (phenylalanine), *m*/*z* 136 (*o*- and *m*-tyrosine), and *m*/*z* 181 (3-nitrotyrosine), as fragment ions, respectively. *o*,*o*′-Dityrosine and *o*,*o*′-[$^2H_6$]dityrosine also lost HCOOH fragment to yield *m*/*z* 315 and 321 as most intense fragment ions, respectively.

Fig. 1 represents typical SRM chromatograms of a control water sample spiked with 5 μM of the internal standards, a standard water sample spiked with 0.5 μM of all seven compounds to be measured, and a control urine sample spiked with 5 μM of the internal standards. The trace of *p*-tyrosine observed in control water spiked with the internal standards (Fig. 1A) was due to an impurity of commercial *p*-[$^2H_4$]tyrosine, the purity of which was 98%. The retention times for *p*-tyrosine, *o*,*o*′-dityrosine, *m*-tyrosine, *o*-tyrosine, phenylalanine, 3-chlorotyrosine, 3-nitrotyrosine and 8-OHdG were 6.8, 8.9, 9.7, 12.8, 13.7, 17.7, 22.4 and 27.8 min, respectively (Fig. 1B). The retention times of all the analytes were slightly longer (range 0.01–0.35 min) in urine than in water. This was not problem, however, since the retention times were very reproducible both in water and in urine for over ∼60 injections.

3.3. Linearity, reproducibility and sensitivity of the HPLC–APCI–LC–MS/MS assay

Calibration curves of the HPLC–APCI–MS/MS method were linear (r^2 = 0.990–0.999) over a concentration range of 0.05–10 μM for *o*,*o*′-dityrosine and *m*-tyrosine; 0.03–10 μM for *o*-tyrosine; 0.04 –10 μM for 3-chlorotyrosine and 3-nitrotyrosine; 0.01–10 μM for 8-OHdG; and 1.0–100 μM for *p*-tyrosine and for phenylalanine.

The detection limits (LODs; S/N = 3) were 0.5 μM for *p*-tyrosine and phenylalanine, 0.025 μM for *o*,*o*′-dityrosine and 3-nitrotyrosine, 0.05 μM for *m*-tyrosine, 0.03 μM for *o*-tyrosine and for 3-chlorotyrosine, and 0.01 μM for 8-OHdG.

The precision of the present HPLC–APCI–MS/MS assay was acceptable. Within-day variations (CV, %) were 7.0% for 3-chlorotyrosine and 13.7% for 8-OHdG and less than 6% for all the others. Between-day variations were somewhat higher than the within-day variations. The highest between-day variation was seen with 8-OHdG, which was 24.7% (Table 2).

3.4. Detection of six tyrosines and 8-OHdG in human urine by HPLC–APCI–MS/MS

In order to determine background levels of the six tyrosines, phenylalanine and 8-OHdG in humans, morning urine and the first following urine fractions of four male and four female healthy volunteers were analyzed.

The demographic data of the volunteers are presented at Table 3. Urinary levels of *p*-tyrosine, phenylalanine, *o*,*o*′-dityrosine and *o*-tyrosine were detectable, and the mean concentrations in μM were 65.6 ± 13 (mean ± S.E.M.) for *p*-tyrosine, 52.2 ± 8 for phenylalanine, 0.1 ± 0.01 for *o*,*o*′-dityrosine, and 0.15 ± 0.02 for *o*-tyrosine in morning urine fractions. In the following first urine fractions, the concentrations were 51.4 ± 11.7 for *p*-tyrosine, 43.1 ± 4.5 for phenylalanine, 0.11 ± 0.01 for *o*,*o*′-dityrosine, and 0.17 ± 0.07 for *o*-tyrosine. The urinary concentrations were then normalized to creatinine by division by the creatinine concentration, since this corrects for differences in glomerular filtration rate between subjects, and expressed as μmol/mol creatinine (Table 4). In morning urine fractions, creatinine concentrations varied from 11.9 to 26.4 mM, with an average of 17.0 ± 1.7 (mean ± S.E.M.). In the first following urine fractions, creatinine concentrations varied from 4.6 to 27.9 mM, with an average of 13.5 ± 3.2 (mean ± S.E.M.). These values are comparable to reference values [47].

p-Tyrosine and phenylalanine were excreted in relatively higher concentrations, so they were easily detectable in all urine samples. *o*,*o*′-Dityrosine was detected in six out of eight morning urines, and five out of eight first following urines. The mean values were 5.8 ± 0.3, and 12.3 ± 5.0 μmol/mol creatinine, respectively. *o*-Tyrosine was detectable in seven out of eight morning urines, and in three out of eight first following urines. The mean concentrations of *o*-tyrosine were 9.2 ± 1.5, and 9.8 ± 0.3 μmol/mol creatinine, respectively. When compared to the morning urine fractions, the concentrations of *p*-tyrosine and *o*,*o*′-dityrosine were higher in the first following urine fractions. The differences, however, were not statistically significant. Urinary concentrations of the other four compounds, *m*-tyrosine,

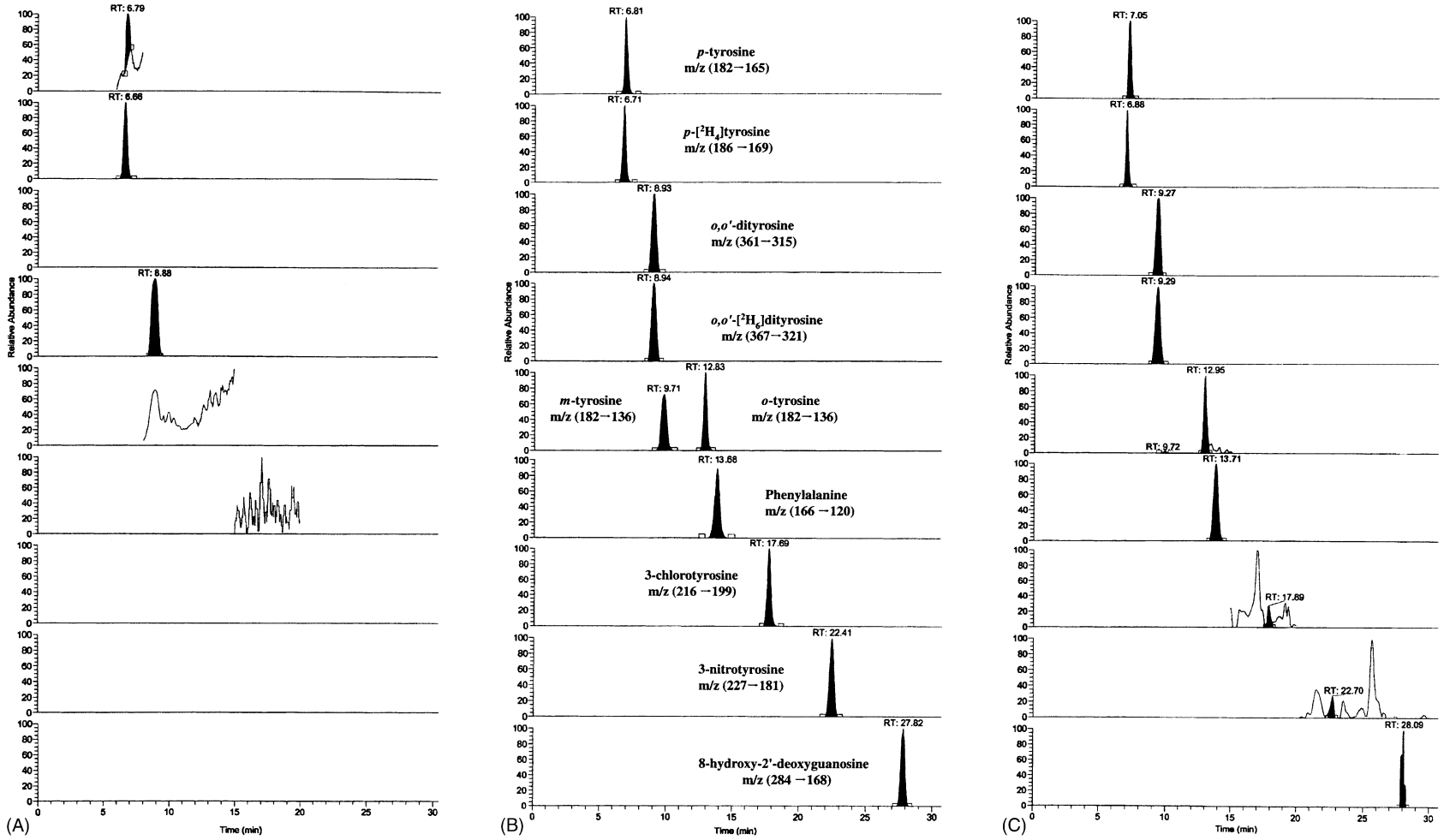

Fig. 1. HPLC–APCI–MS/MS selected reaction-monitoring (SRM) chromatograms of oxidized protein products and 8-hydroxy-2′-deoxyguanosine: (A) control (water) sample spiked with internal standards; (B) 0.5 μM standards in water; (C) a morning urine sample. The specific precursor product ion transitions that are monitored for each analyte are shown below the name of the compound.

Table 2
Coefficients of variation (CV) ($n = 5$ for within-day, $n = 20$ for between-day; 2.5 μM spiked urine), LODs, range of linearity, calibration formulae ($y = ax + b$) including S.D.s of slope and intercept in parentheses, and correlation coefficients (r^2) of the tyrosines and 8-OHdG in spiked human urine samples by HPLC–APCI–MS/MS method

Compound	CV (%)		LOD (μM)	Linearity range (μM)	Calibration curve ($y = ax + b$)	r^2
	Within-day	Between-day				
p-Tyrosine	3.5	11.5	0.50	1.00–100.0	0.165 (±0.007)x + 0.600 (±0.265)	0.990
Phenylalanine	4.3	10.5	0.50	1.00–100.0	0.135 (±0.002)x − 0.137 (±0.078)	0.998
o,*o*′-Dityrosine	5.2	11.0	0.025	0.05–10.0	0.306 (±0.006)x + 0.006 (±0.025)	0.998
m-Tyrosine	2.8	13.0	0.05	0.05–10.0	0.565 (±0.011)x − 0.099 (±0.044)	0.997
o-Tyrosine	2.7	18.2	0.03	0.03–10.0	0.800 (±0.004)x + 0.022 (±0.015)	0.999
3-Chlorotyrosine	7.0	7.9	0.03	0.04–10.0	1.161 (±0.008)x − 0.073 (±0.031)	0.999
3-Nitrotyrosine	4.0	9.2	0.025	0.04–10.0	0.365 (±0.010)x − 0.046 (±0.040)	0.995
8-OHdG	13.7	24.7	0.01	0.01–10.0	0.105 (±0.004)x + 0.022 (±0.016)	0.990

3-chlorotyrosine, 3-nitrotyrosine and 8-OHdG, were below the LODs.

Urinary concentrations of *o*,*o*′-dityrosine and *o*-tyrosine were normalized also to the parent amino acids and presented in Table 5. According to this normalization, *o*,*o*′-dityrosine concentration was 1.8 ± 0.2 mmol/mol *p*-tyrosine (mean ± S.E.M.) in morning urine and 3.3 ± 1.5 mmol/mol *p*-tyrosine in the first following urine. The values for *o*-tyrosine were 3.1 ± 0.6 and 4.4 ± 2.4 mmol/mol phenylalanine, respectively.

3.5. Stability of tyrosines upon storage

Stability of tyrosines was determined by analysis in buffer at pH values varying from 4.5 to 9.0, in the presence and absence of 100 μM of ascorbic acid or 0.5% phenol + 0.5 mM DTPA as antioxidants by HPLC with fluorescence detection. No significant changes in concentrations were observed after storage at −35 °C for at least 3 months. Analysis of samples stored at room temperature showed that tyrosines were stable until 3 weeks. No differences were observed for the two different antioxidants.

4. Discussion

Our attempts to measure six tyrosines as potential biomarkers for oxidative damage with GC–ECD were found to have significant drawbacks as the selectivity of the GC–ECD assay was not high enough to determine all six tyrosines in urine with sufficient sensitivity. Therefore, it was decided to evaluate the use of HPLC–MS as a possible technique.

Table 3
Demographic data of the volunteers

Subject no.	Age	Sex	Weight (kg)	Cigarettes per day	Alcoholic drinks per week
1	51	Female	62	7	<7
2	22	Female	59	–	<7
3	27	Female	58	4	<7
4	30	Female	58	–	<7
5	22	Male	78	–	–
6	31	Male	69	–	<7
7	26	Male	70	–	8–14
8	34	Male	74	–	<7

First, we tried to quantify of all six tyrosines by HPLC–APCI–MS/MS using 3-fluorotyrosine as internal standard. However, we observed a non-linear response for *o*,*o*′-dityrosine, probably due to ion suppression. Subsequently, we synthesized *o*,*o*′-[$^2H_6$]dityrosine for use as internal standard. The use of this deuterium labeled compound completely solved this problem, as can be seen by the value of 0.998 for the correlation coefficient of the *o*,*o*′-dityrosine standard curve (Table 2). Using stable isotope labeled internal standards increased the reliability of the method for *o*,*o*′-dityrosine and *p*-tyrosine significantly. For the other compounds, except 8-OHdG, commercially available *p*-[$^2H_4$]tyrosine was used as internal standard. The retention time of *p*-[$^2H_4$]tyrosine was slightly shorter than its [$^2H_0$]-isotope. This chromatographic behavior of deuterium labeled isotopes is well known [27]. However, this deuterium labeled internal standard could be used safely, since its retention time was very reproducible.

p-Tyrosine and phenylalanine are natural amino acid residues in cellular and extra-cellular proteins. Since *p*-tyrosine is the parent amino acid of *o*,*o*′-dityrosine, 3-chlorotyrosine and 3-nitrotyrosine, and phenylalanine is the parent for the others, the urinary levels of *p*-tyrosine and phenylalanine were also determined. They allow normalizing the urinary concentrations of oxidized derivatives, next to the urinary creatinine concentrations. However, validation of this approach would require further study.

A good chromatographic separation of all six tyrosines, phenylalanine and two labeled internal standards could be achieved with a C-18-based HPLC column and a gradient of methanol in ammonium acetate buffer at pH 4.5. This good separation of the analytes allowed selecting time windows in which a small number of transitions was monitored (Table 1), which increased the sensitivity of the assay because the dwell time of the detector for each transition can be long.

Table 4
Urinary excretions of tyrosine and phenylalanine oxidation products and 8-OHdG in human morning urine and in first following urine ($n = 8$), normalized to creatinine

Compound	Morning urine			First following urine		
	Number	Range (μmol/mol creatinine)	Mean ± S.E.M. (μmol/mol creatinine)	Number	Range (μmol/mol creatinine)	Mean ± S.E.M. (μmol/mol creatinine)
p-Tyrosine	8	2280–7020	3890 ± 590	8	2530–6430	4120 ± 600
Phenylalanine	8	1360–6000	3420 ± 730	8	1210–8090	4250 ± 800
o,*o*′-Dityrosine	6	5.1–6.4	5.8 ± 0.3	5	3.1–25.2	12.3 ± 5
m-Tyrosine	–	<LOD[a]	–	–	<LOD	–
o-Tyrosine	7	5.6–16.8	9.2 ± 1.5	3	9.2–10.2	9.8 ± 0.3
3-Chlorotyrosine	–	<LOD	–	–	<LOD	–
3-Nitrotyrosine	–	<LOD	–	–	<LOD	–
8-OHdG	–	<LOD	–	–	<LOD	–

Number of urines in which a compound is detected, the range of the concentration and the mean concentration ± standard error of the mean (S.E.M.) are shown.

[a] Below the LOD, which were 3.3 μmol/mol (*m*-tyrosine), 2.0 μmol/mol (3-chlorotyrosine), 1.6 μmol/mol (3-nitrotyrosine), and 0.70 μmol/mol (8-OHdG) creatinine.

All six tyrosines could be measured simultaneously by the HPLC–APCI–MS/MS assay described here. The calibration curves prepared in control urine yielded r^2 values between 0.980 and 0.999. The assay was well reproducible in day and between-days. The LODs varied between 0.01 and 0.05 μM for oxidation products, and they were 0.5 μM for *p*-tyrosine and for phenylalanine (parent amino acids).

We applied the assay to human urine in which *p*-tyrosine, phenylalanine, *o*,*o*′-dityrosine, and *o*-tyrosine were detected in the morning and in first following urine of eight healthy volunteers. Morning urine reflects the night time period in which basal metabolism of the body is low. First following urine, in contrast, reflects the daytime period when the basal metabolism is high. The excretion of *p*-tyrosine, phenylalanine and *o*,*o*′-dityrosine were higher in the first daytime urine fraction compared to morning urine fractions, although the differences were statistically not significant.

Using the present HPLC–APCI–MS/MS method, the healthy volunteers' urinary background levels of *m*-tyrosine, 3-chlorotyrosine, 3-nitrotyrosine and 8-OHdG were below the LOD. Under pathological disease conditions, however, their urinary levels may rise and this may make those compounds detectable. This was demonstrated by de Zwart [48] for urinary aldehydes after chemotherapy in cancer patients.

All six tyrosines were stable upon storage for at least 3 months at −35 °C, and for ∼3 weeks at room temperature in aqueous buffer (pH = 4.5–9.0). Presence or absence of ascorbic acid or phenol + DTPA as antioxidants did not affect the stability. Therefore, it might be possible to store urine samples for tyrosine analysis without adjustment of the pH and without addition of antioxidant. This is currently being investigated. We did not check the stability of urinary 8-OHdG. However, it is known that it remains stable for up to 1 year, when frozen at −80 °C [49].

As yet, no assays are described in literature in which six tyrosine derivatives in urine or in other biological matrices are determined in one single analysis. Recently, one method capable of measuring the six tyrosines separately in rat tissue extracts and in rat or human urine by negative chemical ionization-gas chromatography-mass spectrometry (NCI–GC–MS) was described by Heinecke et al. [27]. The same group reported also a liquid chromatography-electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) assay for the simultaneous analysis of 3-chlorotyrosine, 3-nitrotyrosine and 3-bromotyrosine in human plasma [50]. Very recently, another group described an isotope dilution LC–ESI–MS/MS assay for urinary *o*,*o*′-dityrosine, *o*-tyrosine, and 3-nitrotyrosine [41]. The main difference between that and our study is that the authors extracted the oxidized amino acids from urine by SPE, while we analyzed the urine samples directly. They also derivatized *o*-tyrosine and 3-nitrotyrosine by butylation prior to analysis. They applied the assay to cat urine and found the LOD for *o*,*o*′-dityrosine (underivatized), *o*-tyrosine (butylated), and *o*-nitrotyrosine (butylated) to be 37, 7.0 and 4.2 nM, respectively. Our LOD for *o*,*o*′-dityrosine is somewhat

Table 5
Urinary excretions of *o*,*o*′-dityrosine and *o*-tyrosine in human morning urine and in first following urine, normalized to the parent amino acids; *p*-tyrosine and phenylalanine, respectively

Compound	Morning urine		First following urine	
	Range (mmol/mol parent amino acids)	Mean ± S.E.M.	Range (mmol/mol parent amino acids)	Mean ± S.E.M.
o,*o*′-Dityrosine	0.9–2.0	1.8 ± 0.2	0.7–8.6	3.3 ± 1.5
o-Tyrosine	1.1–5.3	3.1 ± 0.6	2.2–8.4	4.4 ± 2.4

lower (25 nM) than the reported 37 nM, while our LOD value for *o*-tyrosine is 30 nM, which is ∼4-fold higher than the reported LOD of the butylated analyte. Our LOD for 3-nitrotyrosine is also 25 nM, which is ∼6-fold higher than the reported LOD of the butylated analyte [41]. Although butylation of *o*-tyrosine and 3-nitrotyrosine may enhance their MS response, butylation decreases the response for *o*,*o*′-dityrosine due to formation of mono- and doubly-butylated species which makes it necessary to analyze samples also without derivatization [41]. Therefore, we did not apply butylation of tyrosines since our aim was to develop a least laborious method for human urine and our LODs for *o*,*o*′-dityrosine and *o*-tyrosine seem already sufficiently low for the analysis of these compounds in human urine without further derivatization (Table 4).

Manary et al. [51] recently reported *o*,*o*′-dityrosine and *o*-tyrosine levels in urine of healthy and diseased children, based on analysis by NCI–GC–MS. The levels were 26.3 ± 10.0 (*o*,*o*′-dityrosine), and 29.4 ± 10 μmol/mol creatinine (*o*-tyrosine) in healthy children. These levels of *o*,*o*′-dityrosine and *o*-tyrosine are two to four times higher than the levels we found in healthy adults in the present study. The reasons of these differences are unknown. One possibility might be the higher basal metabolism in infants compared to adults.

8-OHdG signals were observed in several morning and first following urine samples in the present study. However, these levels were below the LOD. Several urinary 8-OHdG levels have been reported in the literature [30,31,52,53]. By using the reference values for creatinine (1.3 g/24 h), urine volume (1.2 l/24 h), and an average body weight (70 kg) [47], these values correspond to 0.56–3.8 μmol/mol creatinine. The LOD in this study is 0.70 μmol/mol creatinine (using the present mean creatinine value). Thus, the urinary 8-OHdG levels in healthy humans are lower than 0.70 μmol/mol creatinine by the present method.

The urinary data on the healthy volunteers are expressed as ratios over urinary creatinine concentrations, since it is considered that urinary creatinine excretion is constant under normal conditions. However, if urine of other populations has to be analyzed (e.g. diseased persons), creatinine concentrations may not be constant. The data can then be expressed as ratios over the parent amino acids for each oxidized product, as has been practiced before by others [52]. We found *o*,*o*′-dityrosine concentrations as 1.8 ± 0.2 in morning urine and 3.3 ± 1.5 mmol/mol *p*-tyrosine in first following urine. For *o*-tyrosine, these concentrations were 3.1 ± 0.6 and 4.4 ± 2.4 mmol/mol phenylalanine, respectively. These urinary concentrations are similar to published concentrations [52].

5. Conclusion

In summary, the present HPLC–APCI–MS/MS method for the analysis of six tyrosines and 8-OHdG, it is highly selective and sensitive enough for the determination of *p*-tyrosine, phenylalanine, *o*,*o*′-dityrosine, and *o*-tyrosine in healthy human urine. However, urinary background concentrations of *m*-tyrosine, 3-chlorotyrosine, 3-nitrotyrosine and 8-OHdG in healthy individuals were too low to be detectable. In principle, sensitivity may be increased by concentrating the urine samples, e.g. by freeze–drying. Combination with SPE clean up would be a further option. Furthermore, this method provides positive identification of the analytes and there is no need for derivatization and/or sample pre-treatment, which makes it possible to routinely measure large number of samples.

Acknowledgements

This study was supported by a grant from the Dutch Technology Foundation STW (LBI.22.2822).

References

[1] J.M. Gutteridge, Free Radic. Res. Commun. 19 (1993) 141.
[2] J.A. Knight, Ann. Clin. Lab. Sci. 25 (1995) 111.
[3] D.A. Butterfield, C.M. Lauderback, Free Radic. Biol. Med. 32 (2002) 1050.
[4] B.I. Giasson, H. Ischiropoulos, V.M. Lee, J.Q. Trojanowski, Free Radic. Biol. Med. 32 (2002) 1264.
[5] L.L. de Zwart, J.H.N. Meerman, J.N.M. Commandeur, N.P.E. Vermeulen, Free Radic. Biol. Med. 26 (1999) 202.
[6] S. Basu, M. Whiteman, D.L. Mattey, B. Halliwell, Ann. Rheum. Dis. 60 (2001) 627.
[7] T. Jikimoto, Y. Nishikubo, M. Koshiba, S. Kanagawa, S. Morinobu, A. Morinobu, R. Saura, K. Mizuno, S. Kondo, S. Toyokuni, H. Nakamura, J. Yodoi, S. Kumagai, Mol. Immunol. 38 (2002) 765.
[8] S.S. Kim, D.D. Gallagher, A.S. Csallany, Lipids 35 (2000) 1225.
[9] S. Pennathur, J.D. Wagner, C. Leeuwenburgh, K.N. Litwak, J.W. Heinecke, J. Clin. Invest. 107 (2001) 853.
[10] J. Buscigilo, B.A. Yankner, Nature 378 (1995) 776.
[11] H. Verhagen, H.E. Poulsen, S. Loft, G. van Poppel, M.I. Willems, P.J. van Bladeren, Carcinogenesis 16 (1995) 969.
[12] D.U. Song, D.Y. Jung, K.O. Chay, M.A. Chung, K.H. Lee, S.Y. Yang, B.A. Shin, B.W. Ahn, Arch. Biochem. Biophys. 397 (2002) 424.
[13] S.J. Duthie, A. Ma, M.A. Ross, A.R. Collins, Cancer Res. 56 (1996) 1291.
[14] D.J. Socci, B.M. Crandall, G.W. Arendash, Brain Res. 693 (1995) 88.
[15] L.J. Packer, Sports Sci. 15 (1997) 353.
[16] P. Jaruga, B. Jaruga, D. Gackowski, A. Olczak, W. Halota, M. Pawlowska, R. Olinski, Free Radic. Biol. Med. 32 (2002) 414.
[17] J.J. Hageman, A. Bast, N.P.E. Vermeulen, Chem. Biol. Interact. 82 (1992) 243.
[18] R.F. Henderson, W.E. Bechtold, J.A. Bond, J.D. Sun, Crit. Rev. Toxicol. 20 (1989) 65.
[19] R.T. van Welie, R.G. van Dijck, N.P.E. Vermeulen, N.J. van Sittert, Crit. Rev. Toxicol. 22 (1992) 271.
[20] H.R. Griffiths, L. Moller, G. Bartosz, A. Bast, C. Bertoni-Freddari, A. Collins, M. Cooke, S. Coolen, G. Haenen, A.M. Hoberg, S. Loft, J. Lunec, R. Olinski, J. Parry, A. Pompella, H. Poulsen, H. Verhagen, S.B. Astley, Mol. Aspects Med. 23 (2002) 101.
[21] L.L. de Zwart, J. Venhorst, M. Groot, J.N.M. Commandeur, R.C.A. Hermanns, J.H.N. Meerman, B.L.M. van Baar, N.P.E. Vermeulen, J. Chromatogr. B 694 (1997) 277.

[22] L.L. de Zwart, N.P.E. Vermeulen, R.C. Hermanns, J.N.M. Commandeur, P.J. Salemink, J.H.N. Meerman, Chem. Biol. Interact. 117 (1999) 151.
[23] R.C. Hermanns, L.L. de Zwart, P.J. Salemink, J.N. M Commandeur, N.P.E. Vermeulen, J.H.N. Meerman, Toxicol. Sci. 43 (1998) 241.
[24] L.L. de Zwart, R.C. Hermanns, J.H.N. Meerman, J.N.M. Commandeur, P.J. Salemink, N.P.E. Vermeulen, Toxicol. Appl. Pharmacol. 148 (1998) 71.
[25] G. Cohen, S. Yakushin, D. Dembiec-Cohen, Anal. Biochem. 263 (1998) 232.
[26] C. Leeuwenburgh, P.A. Hansen, J.O. Holloszy, J.W. Heinecke, Am. J. Physiol. 276 (1999) R128.
[27] J.W. Heinecke, F.F. Hsu, J.R. Crowley, S.L. Hazen, C. Leeuwenburgh, D.M. Mueller, J.E. Rasmussen, J. Turk, Methods Enzymol. 300 (1999) 124.
[28] J.R. Crowley, K. Yarasheski, C. Leeuwenburgh, J. Turk, J.W. Heinecke, Anal. Biochem. 259 (1998) 127.
[29] K. Hensley, K.S. Williamson, R.A. Floyd, Free Radic. Biol. Med. 28 (2000) 520.
[30] S. Loft, A. Fischer-Nielsen, I.B. Jeding, K. Vistisen, H.E. Poulsen, J. Toxicol. Environ. Health 40 (1993) 391.
[31] A. Weimann, D. Belling, H.E. Poulsen, Nucleic Acids Res. 30 (2002) E7.
[32] J.S. Beckman, T.W. Beckman, J. Chen, P.A. Marshall, B.A. Freeman, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 1620.
[33] C. Leeuwenburgh, M.M. Hardy, S.L. Hazen, P. Wagner, S. Oh-ishi, U.P. Steinbrecher, J.W. Heinecke, J. Biol. Chem. 272 (1997) 1433.
[34] E.L. Thomas, M.B. Grisham, M.M. Jefferson, Methods Enzymol. 132 (1986) 569.
[35] S.L. Hazen, F.F. Hsu, D.M. Mueller, J.R. Crowley, J.W. Heinecke, Clin. Invest. 98 (1996) 1283.
[36] T.G. Huggins, M.C. Wells-Knecht, N.A. Detorie, J.W. Baynes, S.R. Thorpe, J. Biol. Chem. 268 (1993) 12341.
[37] C. Leeuwenburgh, J.E. Rasmussen, F.F. Hsu, D.M. Mueller, S. Pennathur, J.W. Heinecke, J. Biol. Chem. 272 (1997) 3520.
[38] M. Dizdaroglu, D.S. Bertgold, Anal. Biochem. 156 (1986) 182.
[39] K. Hensley, M.L. Maidt, Z. Yu, H. Sang, W.R. Markesbery, R.A. Floyd, J. Neurosci. 18 (1998) 8126.
[40] H. Wiseman, B. Halliwell, Biochem. J. 313 (1996) 17.
[41] L.F. Marvin, T. Delatour, I. Tavazzi, L.B. Fay, C. Cupp, P.A. Guy, Anal. Chem. 75 (2003) 261.
[42] R.C. Onderwater, J. Venhorst, J.N.M. Commandeur, N.P.E. Vermeulen, Chem. Res. Toxicol. 12 (1999) 555.
[43] J. Wang, Z.H. Huang, D.A. Gage, J.T. Watson, J. Chromatogr. A. 663 (1994) 71.
[44] D.A. Malencik, J.F. Sprouse, C.A. Swanson, S.R. Anderson, Anal. Biochem. 242 (1996) 202.
[45] J.S. Jacob, D.P. Cistola, F.F. Hsu, S. Muzaffar, D.M. Mueller, S.L. Hazen, J.W. Heinecke, J. Biol. Chem. 271 (1996) 19950.
[46] R.C. Rock, S.W. Walker, D.C. Jennings, in: N.W. Tietz (Ed.), Textbook of Clinical Chemistry, Saunders, Philadelphia, 1986, p. 1270.
[47] J. Wallach (Ed.,) Interpretation of Diagnostic Tests: A Handbook Synopsis of Laboratory Medicine, third ed., Little, Brown and Company Inc., 1978, p. 18.
[48] L.L. de Zwart, Development of non-invasive biomarkers for free radical induced damage, Ph.D. thesis, Vrije Universiteit, Amsterdam, 1997.
[49] M.S. Cooke, J. Lunec, M.D. Evans, Free Radic. Biol. Med. 33 (2002) 1601.
[50] J.P. Gaut, J. Byun, H.D. Tran, J.W. Heinecke, Anal. Biochem. 300 (2002) 252.
[51] M.J. Manary, C. Leeuwenburgh, J.W. Heinecke, J. Pediatr. 137 (2000) 421.
[52] T. Renner, T. Fechner, G.J. Scherer, Chromatogr. B 738 (2000) 311.
[53] C. Tagesson, M. Kallberg, C. Klintenberg, H. Starkhammar, Eur. J. Cancer 31A (1995) 934.