Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 860 (2007) 78–85
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous determination of 3-nitrotyrosine, tyrosine, hydroxyproline and proline in exhaled breath condensate by hydrophilic interaction liquid chromatography/electrospray ionization tandem mass spectrometry

A. Conventz, A. Musiol, C. Brodowsky, A. Müller-Lux, P. Dewes, T. Kraus, T. Schettgen *

Institute and Outpatient Clinic of Occupational and Social Medicine, University Hospital, Aachen University of Technology, Pauwelsstrasse 30, D-52074 Aachen, Germany

Received 14 August 2007; accepted 10 October 2007

Abstract

The analysis of biomarkers from exhaled breath condensate (EBC) is a non-invasive but challenging method for the detection of pulmonary diseases. The amino acids L-proline (Pro) and L-tyrosine (Tyr) are precursors for two important metabolites, *trans*-L-4-hydroxyproline (*trans*-L-4-hydroxypyrrolidin-2-carboxylic acid, *t*-Hyp) and nitrotyrosine (NT). Whereas *t*-Hyp is supposed to be a biomarker for lung fibrosis, NT is a promising biomarker for inflammation in airway diseases. Analysis of EBC requires extremely sensitive methods, because the epithelial lining fluid of the lung and upper airway is highly diluted in EBC. The high intra- and interindividual variation of this dilution implicates additional problems for sample collection and the interpretation of EBC results. Hence, our aim was to work out a method that would compensate for these possible dilution effects. We have developed a new, reliable and very sensitive method for the simultaneous determination of Pro, *t*-Hyp, Tyr and NT from EBC. Except for *t*-Hyp, we used labelled internal standards (IS) L-proline $^{13}C_5$, ^{15}N (Pro $^{13}C_5$), L-tyrosine-$^{13}C_9$ (Tyr $^{13}C_9$), $^{13}C_9$-3-nitrotyrosine (NT$^{13}C_9$), IS for *t*-Hyp was *cis*-4-hydroxy-L-proline, which were added to the samples before they were lyophilised for concentration. For the separation of the analytes we used hydrophilic interaction liquid chromatography (HILIC), coupled to tandem-mass-spectrometry (MS/MS). The limit of detection (LOD) was 0.5 μg/l for Pro and Tyr and 5 ng/l for *t*-Hyp and NT. The relative standard deviation (RSD) of the precision from day to day was between 2.6 and 8.0% at spiked concentrations between 4 and 25 μg/l for Pro and between 4.2 and 7.3% for Tyr. The RSD of the precision from day to day was between 7.5 and 13.2% at spiked concentrations between 40 and 250 ng/l for *t*-Hyp and between 3.5 and 8.2% for NT. The method was established using 27 healthy subjects with a median age of 46 years. Concentrations ranged from 2.8 to 51.9 μg/l for Pro, from <5 to 516.5 ng/l for *t*-Hyp, from 2.4 to 99.1 for Tyr and for NT concentration ranged between <5 and 1686.5 ng/l.

Keywords: Proline; Hydroxyproline; Tyrosine; Nitrotyrosine; HILIC; LC/MS; ZIC®-HILIC column; EBC; Biomarkers; Pulmonary diseases; Standardisation parameter

1. Introduction

Biological effect monitoring plays an important role in the early diagnosis of diseases of the pulmonary system [1,2]. In the last decade, analysis of exhaled breath condensate (EBC) has gained great interest because sampling is easy, non-invasive and less stressful for the patient than other more invasive techniques [3]. Collection takes place by using an appliance which cools the exhaled breath to −20 °C, which results in condensate. The condensated droplets do not only consist of water and low-volatile organic compounds (VOCs) [4,5], they also contain non-volatile substances originating from the epithelial lining fluid of the lung and upper airways, such as cysteinyl-leukotrienes (cys-LTs), prostaglandine E_2 (PGE_2) and leucotriene B_4 (LTB_4) [6]. Consequently, analysis of biomarkers in EBC offers the unique possibility of exploring the effect of diseases or different inhalative pollutants directly at the lung as the target organ. EBC has been successfully used for the detection of different biomarkers of oxidative stress in the lower airways such as H_2O_2 (e.g. in the context of chromium exposure [7] or tuberculosis [8]), or aldehydes such as malondialdehyde or 4-hydroxy-2-nonenal [9].

A promising biomarker for the determination of nitrosative stress in the lung is the detection of 3-nitrotyrosine in EBC. In vivo, nitric oxide (NO) and superoxide ions can react

* Corresponding author.
E-mail address: tschettgen@ukaachen.de (T. Schettgen).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.10.031

to peroxynitrite. Peroxynitrite is a potent oxidant, maybe the major reactive form of NO in vivo, especially during inflammation processes where the production of free oxygen radicals is prominent [10]. Peroxynitrite can react with free or protein-bound tyrosine creating 3-nitrotyrosine (NT) [11]. NT has been applied as a marker of inflammation in airway diseases and high levels were found in serum, bronchoalveolar lavage fluid (BALF) and lung tissue of subjects with lung diseases [11,12]. In comparison to control persons, high levels of nitrotyrosine were found especially in asthmatic, allergic patients [13–15]. In particular, the ratio of nitrotyrosine levels to those of its precursor tyrosine found in EBC of asthmatic children [15] turned out to be very effective in differentiating between patients and controls, while the measurement of nitrotyrosine alone failed to be successful in distinguishing between asthmatic patients or patients with cystic fibrosis and controls [16]. Methods for the determination of nitrotyrosine in EBC used so far include: enzyme immunoassays [14], GC/MS/MS [13], GC/NCI–MS [16] and LC/MS/MS [4,15]. In particular the simultaneous determination of nitrotyrosine and tyrosine is a special challenge due to the high polarity of both compounds.

trans-Hydroxyproline (*t*-Hyp) is a major component of the protein collagen, the main protein of connective tissue and bone, which is responsible for the mechanical properties of these tissues by forming hydrogen bonds. Biochemically, *t*-Hyp is synthesized post-translationally by hydroxylation of its precursor proline (Pro) after the synthesis of pro-collagen using ascorbic acid as a cofactor. Because *t*-hydroxyproline is predominantly found in collagen, release of *t*-Hyp is normally due to an injury of this protein by inflammation processes. Therefore, *t*-Hyp is supposed to be an appropriate marker for the decomposition of bone and connective tissue and thus useful for the early diagnosis of associated bone diseases [17–20] and tumors [21].

Asbestosis is a fibrotic reaction of the lung parenchyma due to asbestos fibers and is connected with an increased deposition of collagen in lung tissue [22]. In this context the levels of *t*-Hyp found in blood of previously asbestos-exposed workers, suffering from asbestosis, were elevated in comparison to the levels of *t*-Hyp found in blood of non-exposed controls and even dose–response relations were reported [23]. As the lung is the target organ for asbestos and asbestos-related diseases, it may be hypothesized that inflammation processes involved in asbestosis might lead to decomposition of collagen in lung tissue. Consequently, the determination of *t*-Hyp in exhaled breath condensate might serve as a biomarker of asbestosis that is more closely related to the effect on the lung as the target organ.

As the ratio of nitrotyrosine levels to the levels of its precursor tyrosine determined in EBC turned out to be a more promising biomarker than nitrotyrosine levels alone [15], it should be expected that the same could be true for hydroxyproline and its biochemical precursor proline. This implicates a general problem of EBC collection and analysis: dilution effects occuring during sample collection. During exhalation, particles and droplets of the bronchoalveolar lining fluid are carried away with the airflow. The dilution of these droplets generated in the respiratory tract by water vapour varies considerably, as already observed by other authors [24]. Consequently, despite standardised sampling conditions, it is necessary to accurately compensate for this effect when analysing the results of EBC. [25]. Non-volatile substances like amino acids or peptides that are present within the airway-lining fluid of the lung would be ideally suited to correct for the degree of dilution of the lining-fluid in the collected EBC. Our own previous investigations concerning the analysis of *N*-ε-carboxymethyllysine (CML) and lysine in EBC [26] as well as the results of Baraldi et al. [15] give strong support to this theory.

Therefore, it was the aim of this work to develop and validate an analytical method for the accurate and specific simultaneous determination of 3-nitrotyrosine and its precursor tyrosine as well as *trans*-hydroxyproline and its precursor proline in exhaled breath condensate as markers of nitrosative/inflammative stress as well as fibrotic alterations in the lung. The simultaneous determination of compounds with such a high polarity at the ng/l-level was achieved using hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry (HILIC/MS/MS). As a first application, the method was used for the analysis of EBC of 27 healthy persons of the general population.

2. Experimental

2.1. Chemicals and solvents

All chemicals were of the highest purity available unless stated otherwise.

L-Proline, L-proline $^{13}C_5$, ^{15}N and *cis*-4-hydroxy-L-proline were purchased from Sigma–Aldrich (Germany). *trans*-L-4-Hydroxyproline (*trans*-L-4-hydroxypyrrolidin-2-carboxylic acid) was purchased from Applichem (Darmstadt, Germany). L-Tyrosine, ammonium acetate and formic acid were purchased from Merck (Darmstadt, Germany). L-Tyrosine$^{13}C_9$ was purchased from Cambridge Isotope Laboratories (Minnesota, USA) and 3-nitro-L-tyrosine from Cayman chemical company (Michigan, USA). $^{13}C_9$-3-nitrotyrosine was self-synthesised in our laboratory [4]. Acetonitrile (HPLC-grade) was purchased from Baker (Germany) and deionised water was produced by Millipore®-technique.

2.2. Solvent preparation

An aqueous solution was prepared containing 10% formic acid. For mobile phase, eluent A and eluent B were prepared as follows:

Eluent A: an aqueous 5 mM ammonium acetate solution was prepared and the pH was brought to pH = 4.0 using 10% aqueous formic acid. Eluent B consists of 0.025% formic acid in acetonitrile. All processed samples were reconstituted in a mixture of eluent A and B 20:80 (v/v) as starting eluent for HILIC/MS/MS. The preparation of the calibration curve requires 0.1 mM ammonium acetate buffer brought to pH 4.0 with 10% formic acid. For the preparation of all the solutions, deionised water was used.

2.3. Synthesis of $^{13}C_9$-nitrotyrosine as internal standard (IS)

The internal standard, $^{13}C_9$-3-nitrotyrosine, was synthesized according to the preparation of Schwedhelm et al. [27]. $^{13}C_9$-L-tyrosine (30 μmol) and sodium nitrate (90 μmol) were suspended in 600 μl of ice-cold water. Upon slow addition of 300 μl of sulphuric acid (97%) under stirring the colour of the cold solution changed to yellow. After 1 h incubation in the ice-bath, the pH of the solution was adjusted to pH = 3 by the addition of 1700 μl aqueous solution of sodium hydroxide (5 M). The target substance was removed from the reaction solution by adsorption onto a C18 POLAR RP cartridge (500 mg/3 ml) and elution by 1.5 ml of a solution of 2 mM NH_4Ac buffer and methanol (60/40, v/v) adjusted to pH 4.35. The purity of the reaction product in the eluent was proven by LC–MS/MS and was found to be 99.7%.

2.4. EBC sample collection

EBC samples were collected using a commercially available ECOSCREEN condenser from Viasys Healthcare (Hoechberg, Germany). EBC was collected from 27 healthy adults aged between 31 and 64, with a median age of 46. These subjects included 14 female and 13 male subjects, of which 8 were smokers and 8 were former smokers. The subjects wore noseclips and were instructed to perform tidal breath to a breath volume of 200 l through a mouthpiece connected to the condenser. Volumes of resulting EBC ranged from 1 to 6 ml. The samples were aliquoted and stored at −80 °C until analysis.

2.5. Standard and sample preparation

Two stock solutions were prepared, L-proline$^{13}C_5$ (IS) and *cis*-4-hydroxy-L-proline (IS), each containing 1 g/l in 0.01 mM ammonium acetate buffer (pH = 4). Afterwards 20 μl of stock solution *cis*-4-hydroxy-L-proline were placed in a 20 ml glass volumetric flask and diluted to the mark with 0.01 mM ammonium acetate resulting in a 1 mg/l intermediate solution.

A stock solution of the IS L-tyrosine$^{13}c_9$ was prepared by adding 5 mg of L-tyrosine $^{13}c_9$ in a 50 ml volumetric flask and filled up to the mark with 5 mM NH_4Ac, pH 4 (see also solution for eluent A). The resulting concentration is 0.1 g/l.

The stock solution containing 10 g/l $^{13}C_9$-nitrotyrosine in 2 mM NH_4Ac was prepared with the self-synthesised internal standard. This stock solution was diluted 1:1000 in 2 mM NH_4Ac obtaining a 10 mg/l solution. Further, 300 μl of this solution was filled up to the mark in a 10 ml volumetric flask using 2 mM NH_4Ac-buffer resulting in a 300 μg/l dilution (intermediate solution).

The working solution for the internal standards L-proline$^{13}C_5$, *cis*-4-hydroxy-L-proline, L-tyrosine$^{13}C_9$ and $^{13}C_9$-3-nitrotyrosine was prepared as described:

Twenty microlitres of L-proline$^{13}C_5$ stock solution were placed in a 20 ml volumetric flask (dilution 1:1000). Two hundred microlitres of intermediate solution of *cis*-4-hydroxy-L-proline (dilution 1:100), 200 μl of L-tyrosine$^{13}C_9$ stock solution (dilution 1:100) and 667 μl of $^{13}C_9$-nitrotyrosine intermediate solution (dilution 1:30) were added. Afterwards the volumetric flask was filled to the mark with 0.01 mM NH_4Ac. The concentrations of the internal standard working solution were 1 mg/l for L-proline$^{13}C_5$ and L-tyrosine$^{13}C_9$ and 0.01 mg/l for *cis*-4-hydroxy-L-proline and $^{13}C_9$-3-nitrotyrosine, respectively. All solutions were stored at −20 °C and are stable under these conditions for at least six months.

2.6. Preparation of standards for the calibration curves

Stock solutions of L-proline, *trans*-L-hydroxyproline, L-tyrosine and 3-nitrotyrosine were prepared, each containing 1 g/l in 0.01 mM NH_4Ac. The expected higher concentrations of L-proline and L-tyrosine compared to 3-nitrotyrosine and *trans*-L-hydroxyproline in EBC were taken into consideration during the following calibration standards:

Working solution A: 10 μl of stock solution *trans*-L-hydroxyproline and nitrotyrosine were placed in a 10 ml volumetric flask and diluted to the mark with 0.01 mM NH_4Ac (dilution factor 1:1000). The resulting concentration was 1 mg/l for both standards.

Working solution B: 1000 μl of L-proline stock solution were brought into a 10 ml volumetric flask and filled up to the mark with 0.01 mM NH_4Ac (dilution factor 1: 10), resulting in a concentration of 0.1 g/l.

Working solution C: 100 μl of working solution A and 100 μl of working solution B and 100 μl of the stock solution of L-tyrosine were placed in a 100 ml volumetric flask and diluted to the mark with 0.01 mM NH_4Ac. The dilution factor was 1:1000 for all standards. The final concentrations of L-proline and L-tyrosine were 100 μg/l and 1 μg/l for *trans*-L-hydroxyproline and nitrotyrosine. All stock and working solutions were stored at −20 °C and are stable under these conditions for at least six months.

Working solution C was used to prepare calibration standards. Concentrations were in the range from 0.01 to 1 μg/l for *trans*-L-hydroxyproline and 3-nitrotyrosine and in the range from 1 to 100 μg/l for L-proline and L-tyrosine. Calibration standards were prepared using 0.01 mM NH_4Ac for dilution, aliquoted into 1.1-ml-portions and stored at −80 °C until analysis. A blank value consisting of 0.01 mM NH_4Ac was included in every analytical series.

2.7. Sample preparation

Samples and standards had to be thawed at room temperature and intensively shaken before analysis. A volume of 1000 μl of each sample was pipetted into 1.8 ml glass vials. A volume of 50 μl of the working solution of the internal standard was added afterwards. The vials were kept at −80 °C for 30 min, resulting in complete freezing, before they were lyophilised overnight using a Piatkowski P-3-freeze-drying-system (Piatkowski, Munich, Germany) at −15 °C and a pressure of app. 10^{-2}–10^{-3} mbar. The dry residue was reconstituted in

50 μl, a mixture of eluent A and B 20:80 (v/v), and finally brought into a micro insert for the injection into the LC/MS/MS system for quantitative analysis. Calibration standards were treated in the same way. Linear calibration curves were obtained by plotting the quotients of the peak areas to the peak area of the according IS. The calibration curves were used to ascertain the unknown concentrations of the analytes in the EBC samples.

2.8. Liquid chromatography and mass spectrometry

Liquid chromatographic separation was performed on a Hewlett-Packard HP 1100 series HPLC system equipped with a binary gradient pump, degasser and autosampler. Chromatography was performed on a ZIC®-HILIC (3.5 μm, 4.6 mm × 100 mm) from SeQuant (Haltern am See, Germany) using 5 mM ammonium acetate (pH 4.0)/0.0025% formic acid in acetonitrile mixture (20:80, v/v) as mobile phase and a flow rate of 0.5 ml/min for the initial condition. The temperature of the column was kept at 30 °C using a Thermasphere TS-130 column oven from Phenomenex.

A gradient elution was started after injection of the sample by varying the proportion of ammonium acetate (solvent A) from 20 to 80% within 15 min. Next the gradient was held for 4 min before it was brought back to the initial conditions within 1 min and then held for another 10 min. In total, the whole chromatographic run required 30 min before a new sample could be injected. All steps were controlled by Analyst 1.3.2 software from Applied Biosystems.

The detection was accomplished with a Sciex API 3000 tandem mass spectrometry (MS/MS) system with an electrospray ionization (ESI) source in the positive ion mode. The applied ESI needle voltage was +5000 V. Nitrogen was used as the nebuliser gas (at a pressure of 8 psi) and turbo heater gas (500 °C) as well as for the collision gas settings at 10 instrument units. The curtain gas was set to 8 psi. The multiple reaction monitoring (MRM) mode was chosen to perform the MS/MS detection and the dwell time for each transition was 300 ms.

The retention times and the substance-specific mass spectrometric conditions for each compound under the given conditions are listed in Table 1. Mass spectra were obtained with continuous flow injections of standard solutions of each of the analytes (1 mg/l MeOH, 10 μl/min) with a syringe pump system coupled to the Sciex API 3000 LC/MS/MS system. The most specific and intense precursor-product ion transitions for Pro, *t*-Hyp, Tyr and NT could be determined in this manner. Resolution of the quadrupoles Q 1 and Q 3 was set to "unit".

3. Results

The method developed allows for the simultaneous determination of NT, Tyr, Pro and *t*-Hyp. The retention times (Table 1) for NT, Tyr and Pro were almost identical with their respective labelled internal standards because here, standards and internal standards exhibited the same chemical behaviour, but mass transitions shifted (see Table 1). For the determination of *t*-Hyp, a labelled internal standard was not commercially available. Consequently, we chose to use the stereoisomer *cis*-hydroxyproline. *cis*-Hydroxyproline is usually not present in collagen or other proteins, so that its determination is not disturbed by natural backgrounds. In fact, the investigation of pooled exhaled breath condensate without addition of *cis*-Hyp showed no detectable levels of *cis*-Hyp. From a chemical point of view, both stereoisomers should show almost identical properties, so that it was considered to be the best internal standard available, as already reported by other authors [28]. Under the given conditions for HILIC, *cis*-Hyp eluted approximately 0.3 min later than its isomer *t*-Hyp. Although quenching effects during mass spectrometric determination cannot be compensated for in this manner, *cis*-Hyp turned out to be a reliable internal standard for the accurate determination of *t*-Hyp. A chromatogram of the processed EBC-sample of a healthy person is shown in Fig. 1.

3.1. Reliability of the method

The volume of collected EBC is usually comparitively lower (between 1 and 6 ml) than the volume needed in our method (1 ml), thus making it difficult to collect adequate volumes for quality control materials for a full validation of the method. Therefore, we decided to use self-prepared control material for the validation of the method using 0.01 mM NH_4Ac. All standards for the calibration curves and quality control material were prepared in the laboratory by spiking 0.01 mM NH_4Ac with different amounts of Pro, Tyr, *t*-Hyp and NT. For quality control purposes, two different concentration levels were prepared. Q1, the quality control for low-concentrations, was spiked with 40 ng/l Hyp and NT and 4 μg/l Pro and Tyr. Q2 the quality control for high concentrations, 250 ng/l *t*-Hyp and NT and 25 μg/l Pro and Tyr was added to 0.01 mM NH_4Ac. The quality controls were aliquoted into 1.1 ml-portions and stored at −80 °C.

Table 1
Retention times and compound specific mass spectrometric conditions

	NT	NT $^{13}C_9$	Tyr	Tyr$^{13}C_9$	Pro	Pro$^{13}C_5$	*t*-Hyp	*c*-Hyp
Retention time (min)	4.95	4.95	6.59	6.60	7.43	7.42	7.99	8.34
Precusor ion (*m*/*z*)	227	236	182	191	116	121	132	132
Product ion (*m*/*z*)	181	189	165	144	70	75	86	68
Declustering potential (V)	41	41	41	41	31	31	41	41
Focusing potential (V)	170	170	210	200	170	200	60	60
Entrance potential (V)	9	9	10	10	10	10	10	10
Collision energy (V)	18	18	15	17	19	21	21	33
Collision exit potential (V)	11	11	10	10	6	6	4	12

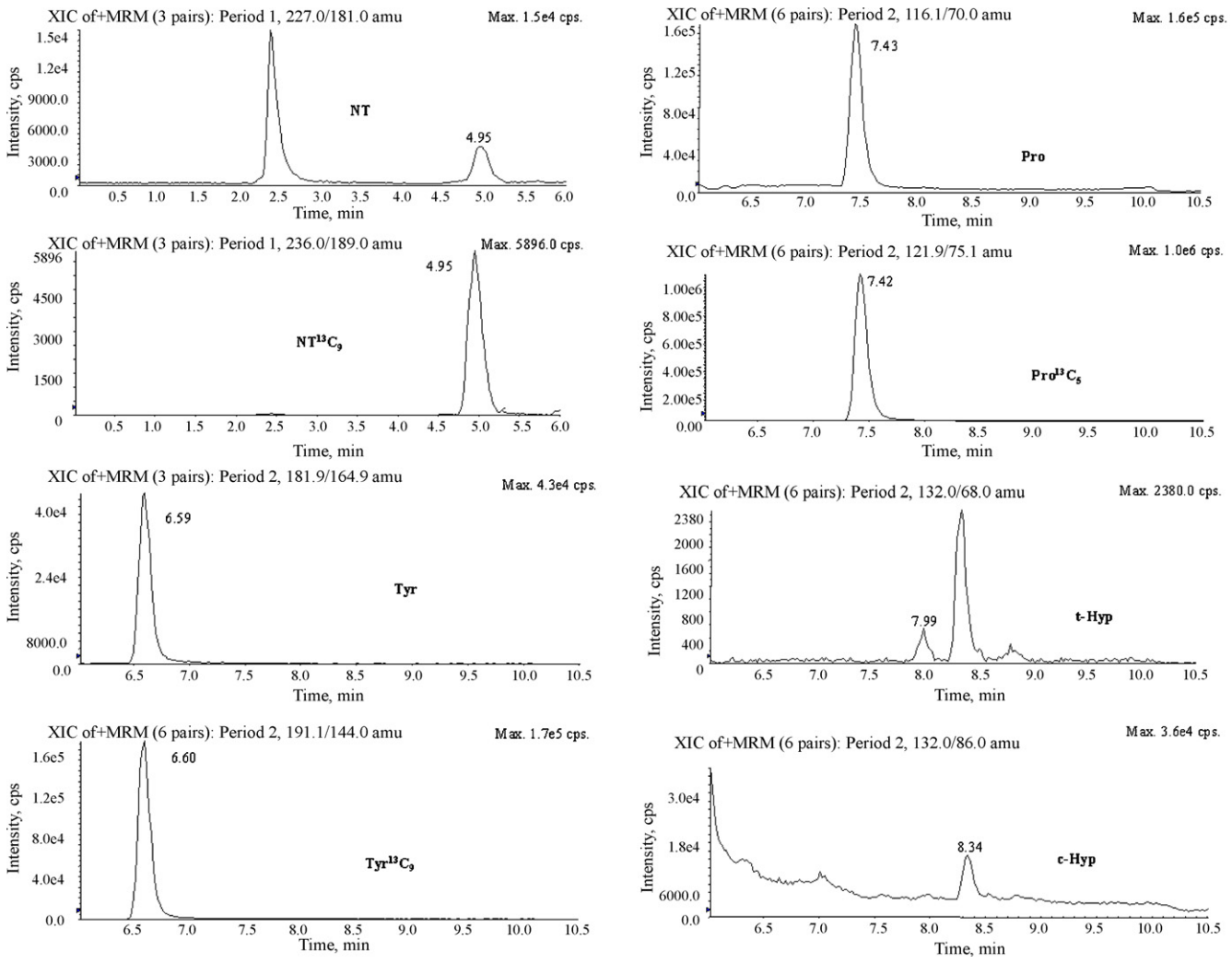

Fig. 1. Chromatogram of the processed EBC sample of a healthy person. Concentrations determined were 17.9 μg/l for Pro, 43 ng/l for *t*-Hyp, 21.4 μg/l for Tyr and 106 ng/l for NT.

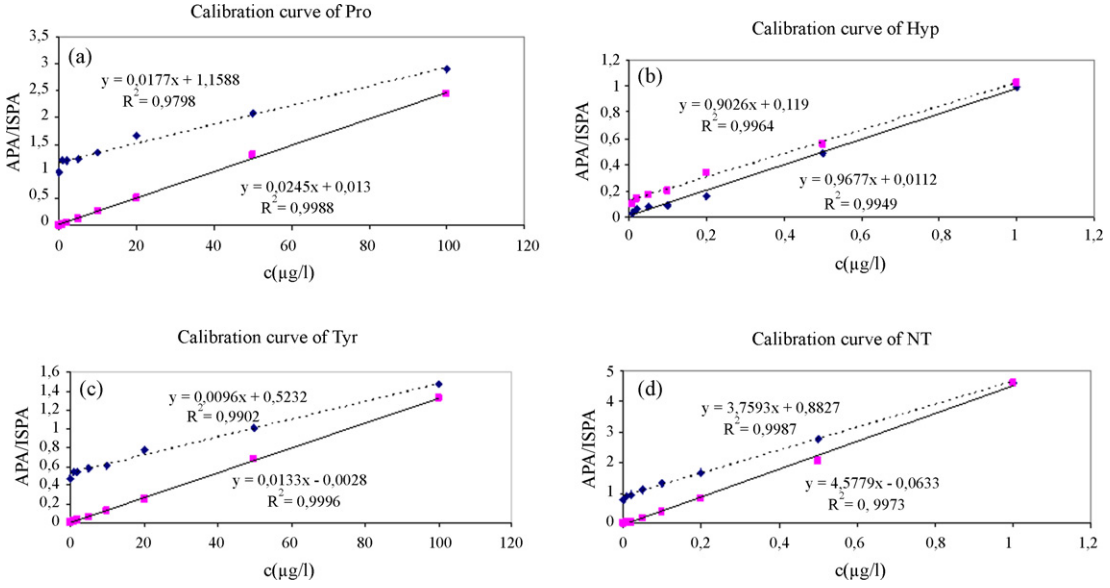

Fig. 2. Calibration curves of Pro, Hyp, Tyr and NT; APA = analyte peak area, ISPA = internal standard peak area; (- - -) calibration curve in EBC, (—) calibration curve in 0.01 mM NH_4Ac.

Table 2
Within-day and between-day precision for the determination of Pro, Hyp, Tyr and NT ($n = 8$)

	Mean ± SD (μg/l), RSD (%)[a]			
	Pro	Hyp	Tyr	NT
Within-day[b]				
QK1[d]	4.45 ± 0.21	0.05 ± 0.003	5.12 ± 0.26	0.04 ± 0.002
	4.6	7.0	5.1	5.8
QK2[e]	26.21 ± 0.60	0.27 ± 0.007	25.98 ± 0.92	0.25 ± 0.012
	2.3	2.7	3.5	4.9
Between-day[c]				
QK1[d]	4.6 ± 0.37	0.082 ± 0.01	5.21 ± 0.41	0.04 ± 0.03
	8.0	13.2	7.3	8.2
QK2[e]	26.4 ± 0.69	0.31 ± 0.02	25.43 ± 1.06	0.25 ± 0.01
	2.6	7.5	4.2	3.5

[a] RSD: relative standard deviation.
[b] Within-day precision was obtained from eight replicate assays on one day.
[c] Between-day precision was obtained from eight different days.
[d] QK1 spiked with 4 μg/l Pro and Tyr and 40 ng/l Hyp and NT.
[e] QK2 spiked with 25 μg/l Pro and Tyr and 250 ng/l Hyp and NT.

In order to investigate the influence of matrix on the calibration of the analytes, we prepared calibration standards by spiking pooled EBC in the same concentration range and processed these standards together with the "normal" calibration standards in 0.01 mM NH_4Ac. The resulting slopes and linearity of the calibration curves in 0.01 mM NH_4Ac and EBC were compared. Fig. 2 shows the results of this comparison graphically. As shown, both calibrations showed excellent linearity with slightly lower slopes in EBC calibration, especially regarding Pro and Tyr. This may be explained by the comparatively high background levels found in the pooled EBC used that ranged from 52 μg/l for Tyr to 65 μg/l for Pro. Consequently, spiking high levels of both amino acids might overstrain the linear range of our method that was determined to be 1–100 μg/l for both compounds and result in a flattening of the calibration at the higher concentration range.

The precision within-day and between day was evaluated using quality control Q1 and Q2 (cf. Table 2). Within-day precision was examined with eight replicates each in one day and the between-day precision were performed with replicates of Q1 and Q2 from eight different days.

In order to further investigate the effect of different matrices on the result obtained, accuracy tests were performed using five individual EBC samples from healthy subjects. An aliquot of each sample was analysed unspiked and then spiked with 10 μg/l Pro and Tyr and 100 ng/l of *t*-Hyp and NT. The mean accuracy, determined from the difference of the levels found in unspiked and spiked EBC was 104.2% for Pro, 115.8% for *t*-Hyp, 88.9% for Tyr and 101.9% for NT.

Table 3
Median level and range of Pro, Hyp, Tyr and NT in EBC of 27 healthy subjects

	Pro (μg/l)	Hyp (ng/l)	Tyr (μg/l)	NT (ng/l)
$n >$ LOD	27	26	27	25
Median	13.4	45.7	15.5	168.0
Range	2.8–51.9	<5–516.5	2.4–99.1	<5–1686.5

The limit of detection (LOD), defined as a signal-to-noise (S/N) ratio of 3 for the registered fragment ions, was estimated to be 5 ng/l for *t*-Hyp and NT. The LOD for Pro and Tyr in aqueous samples was slightly higher than for *t*-Hyp and NT (app. 0.05 μg/l), but regarding the high levels found in EBC samples, this LOD has never been reached in practice (see Table 3).

In Table 3, the results of our investigation of EBC samples of 27 healthy subjects are summarised. All analytes could be determined in more than 90% of the samples investigated. The concentration of Pro varies from 2.8 to 51.9 μg/l. *t*-Hyp differs in a range within <5 and 516.5 ng/l. Values for Tyr varied from 2.4 to 99.1 μg/l and those of NT from <5 to 1686.5 ng/l. No significant differences could be found for each of the analytes between smokers, ex-smokers and non-smokers as well as between women and men.

The concentrations of Pro and Tyr in the EBC samples showed a strong correlation ($r = 0.97$), indicating that both amino acids were present in the alveolar lining fluid and exhaled in a similar manner. The correlation between *t*-Hyp and Pro was strong ($r = 0.87$), but weaker than the correlation between Pro and Tyr, indicating that even in the lungs of healthy subjects, a certain portion of lung collagen (or its precursors) had decomposed and been exhaled. Fig. 3 shows the correlations for Pro and Tyr as well as *t*-Hyp and Pro.

4. Discussion

Analysis of EBC samples is complicated by the small sample volume available for analysis coupled with the need to detect very low concentrations. While the determination of *t*-hydroxyproline and proline is often described in the literature for matrices like serum, urine or tissue or collagen samples

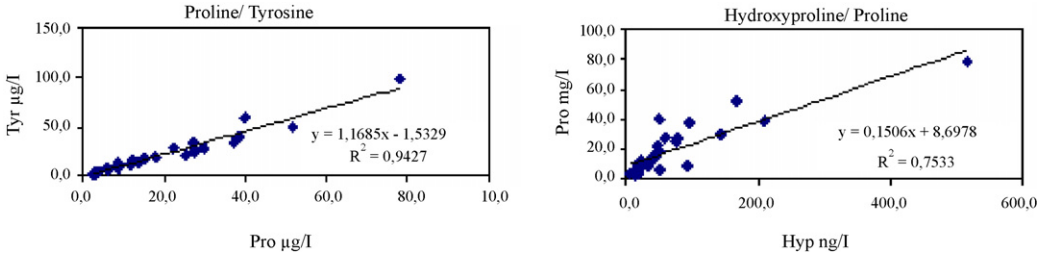

Fig. 3. Correlation between proline and tyrosine and *t*-hydroxyproline and proline in EBC samples of healthy subjects. Pro and Tyr are given in μg/l whereas *t*-Hyp is given in ng/l.

[28–33], we present here for the first time quantitative results for both compounds in EBC. Previous methods analysing both compounds used HPLC with fluorescence or mass spectrometric detection after elaborate derivatisation of the compounds to fluorescent derivatives [28–30] or UV detection after derivatisation with dinitrophenylhydrazine [31]. Other methods included GC/MS after derivatisation to *tert*-butyldimethylsilyl derivatives [32]. All these methods required extensive sample preparation and elaborate derivatisation steps that are expensive and might affect column lifetime and efficiency in HPLC. The same is true for the determination of 3-nitrotyrosine and tyrosine in EBC. While NT and Tyr levels have already been reported in EBC [4,13–16], the methods either failed to simultaneously determine Tyr [4,13,14,16] and/or needed extensive sample preparation for GC/MS [13,16] or LC/MS/MS [4] or lacked specifity due to the use of enzyme immunoassays [16].

In order to avoid derivatisation for the determination of both *t*-Hyp, Pro, NT and Tyr and still utilize tandem mass spectrometric detection as the most specific and sensitive detector, we decided to use hydrophilic ion liquid chromatography (HILIC) as an alternative. Normal reversed phase technique is not suitable for such highly polar compounds, because with the exception of NT [4] they usually show almost no retention on RP-columns. The use of "classic" normal phase chromatography would require organic phases which affect HPLC pump seals and make this technique in general hard to handle.

An alternative for the detection of highly polar compounds is the use of hydrophilic interaction liquid chromatography (HILIC). HILIC is similar to normal phase technique as the stationary phase is polar (silica gel modified with strongly polar groups). The HILIC-technique has initially been successfully applied for the separation of carbohydrates or peptides [34–37]. Further development of different stationary phases has led to an increase in the application of this technique in analytical chemistry, e.g. in the analysis of polar food compounds [38] or in the screening for polar metabolites in bodyfluids [39].

Mobile phases used for HILIC are semi-aqueous, whereas the aqueous eluent is considered to be the strongest. Therefore, starting with a low percentage of the aqueous phase and increasing it to 80% during the run, compounds are eluted beginning with the non-polar and ending with the highly polar ones. Therefore, the elution order is inverted to RP chromatography where highly polar compounds have very short retention times [40]. HILIC is very well suited for coupling with MS-detection and sensitivity of these methods often achieves a 10–1000 fold increase in sensitivity compared to RP-HPLC.

As EBC contains only little matrix and the volume available is limited, we decided to use lyophilisation of the samples as the only step of sample preparation and concentration of the analytes. As the analytes are non-volatile amino acids (salts), significant losses during this step are not to be expected and sample preparation is facilitated. As shown in Fig. 1, the chromatograms are very clean and the analyte peaks are clearly resolved from any interferences. The detection limits of 0.05 μg/l for Pro and Tyr and 5 ng/l for *t*-Hyp and NT are superior to previous methods for the determination of NT in EBC [4,13–16].

Precision data were excellent regarding the low concentrations used, varying from 2.3 to 7.0% for the within-day precision and from 2.6 to 13.2% for the between-day precision. The data for accuracy in individual EBC were also very good with an accuracy of 104.2% for Pro, 115.8% for *t*-Hyp, 88.9% for Tyr and 101.9% for NT. This illustrates the good compensation of possible matrix effects by the use of labelled internal standards. Regarding the good precision and accuracy data for *t*-Hyp, this is also applicable for the use of *cis*-Hyp as the internal standard.

To our knowledge, the developed method described here is the first to allow for the simultaneous determination of 3-nitrotyrosine, tyrosine, *trans*-hydroxyproline and proline in exhaled breath condensate. The volume of sample material needed is 1 ml, which is comparatively low for the simultaneous analysis of four substances in this matrix. The peaks obtained with the HILIC-column were sharp and retention times were reproducible with no notable shift, even after several chromatographic batches. The zwitter-ionic properties of the ZIC-HILIC® column used in this study make it especially suitable for the determination of amino acids, as already shown in our previous investigation [26].

The results obtained from our preliminary study investigating the EBC of 27 healthy subjects provide a promising basis for further, larger studies on persons suffering from allergic airway diseases or fibrotic diversifications in the lung. The most interesting result of this investigation was the high degree of correlation between the amino acids tyrosine and proline in EBC, proving that a certain portion of the epithelial lining fluid of the airways is carried away with the airflow. In our opinion, the accurate determination of both tyrosine and proline might be an excellent measure for the extent of the dilution of the epithelial lining fluid in collected EBC samples. While the levels of Pro and Tyr in EBC varied immensely from subject to subject, the ratio of Pro/Tyr remained almost constant in all samples investigated (cf. Fig. 2), regardless of the volume of EBC collected.

Another interesting result was the correlation between the levels of *t*-Hyp and its precursor Pro. As *t*-Hyp is almost exclusively incorporated in collagen or its precursors, this might indicate that collagen or its precursors are decomposed to a small percentage even in the lungs of healthy persons not suffering from fibrosis or other lung diseases. In contrast, no significant correlation was found between the levels of NT and its precursor Tyr in EBC, indicating that the formation (and exhalation) of NT is most likely influenced by exogenous factors that have not been assessed in this preliminary study.

Both NT and *t*-Hyp represent biomarkers of different effects taking place in the airways: while NT is described as a biomarker of nitrosative stress or allergic/inflammative response, *t*-Hyp is supposed to be a biomarker of collagen turnover. The simultaneous determination of both compounds in EBC of persons with different lung diseases might help to clarify the quality of both markers in the future. Moreover, the additional quantification of proline and tyrosine might serve as a parameter for the standardisation of EBC results that is more meaningful than the EBC volume collected or the breath volume exhaled during collection.

5. Conclusion

We have developed and validated a highly sensitive, accurate and specific new LC/MS/MS-method for the simultaneous detection of the biomarkers 3-nitrotyrosine and *trans*-hydroxyproline as well as their precursors, the amino acids tyrosine and proline in EBC. Precision and accuracy data were excellent and possible matrix effects could be compensated for by the use of labelled internal standards. Due to its simple and rapid sample preparation, the method is very practicable and suitable even for the examination of larger series.

The data for accuracy and precision can be described as excellent, even at very low concentrations. The limit of detection of 5 ng/l for 3-nitrotyrosine and *trans*-hydroxyproline and 0.05 μg/l for tyrosine and proline was sufficient for the detection of the analytes in all samples of a preliminary study. As the levels of tyrosine and proline showed a strong correlation in EBC regardless of EBC volume, we conclude that these parameters could be very suitable to compensate for possible dilution effects that can occur during sampling of EBC.

Acknowledgement

The authors would like to thank Ms. Kathy Bischof for the kind review of this manuscript.

References

[1] A. Antczak, P. Gorski, Int. J. Occup. Med. Environ. Health 15 (2002)317.
[2] S.A. Kharitonov, P.J. Barnes, Chest 130 (2006) 1541.
[3] C. Gessner, H. Kuhn, H.J. Seyfarth, H. Pankau, J. Winkler, J. Schauer, H. Wirtz, Pneumologie 55 (2001) 414.
[4] T. Göen, A. Müller-Lux, P. Dewes, A. Musiol, T. Kraus, J. Chromatogr. B 826 (2005) 261.
[5] M. Phillips, K. Gleeson, J.M.B. Hughes, J. Greenberg, R.N. Cataneo, L. Baker, W.P. McVay, Lancet 353 (1999) 1930.
[6] A. Antczak, P. Montuschi, S. Kharitonov, P. Gorski, P.J. Barnes, Am. J. Respir. Crit. Care Med. 166 (2002) 301.
[7] A. Caglieri, M. Goldoni, O. Acampa, R. Andreoli, M.V. Vettori, M. Corradi, P. Apostoli, A. Mutti, Environ. Health Persp. 114 (2006) 542.
[8] S. Kwiatkowska, M. Luczynska, I. Grzelewska-Rzymowska, D. Nowak, M. Zieba, Respir. Med. 101 (2007) 574.
[9] R. Andreoli, P. Manini, M. Corradi, A. Mutti, W.M. Niessen, Rapid Commun. Mass. Spectrom. 17 (2003) 637.
[10] R.H. Liu, J.H. Hotchkiss, Mutat. Res. 339 (1995) 73.
[11] J.A. De Andrade, J.P. Crow, L. Viera, C. Bruce Alexander, K. Randell Young, D.C. McGiffin, G.L. Zorn, S. Zhu, S. Malton, R.M. Jackson, Am. J. Respir. Crit. Care Med. 161 (2000) 2035.
[12] D.A. Kaminsky, J. Mitchell, N. Carroll, A. James, R. Soultanakis, Y. Janssen, J. Allergy Clin. Immunol. 104 (1999) 747.
[13] M. Larstad, A.S. Soderling, K. Caidahl, A.C. Olin, Nitric Oxide 13 (2005) 134.
[14] A. Bodini, D.G. Peroni, F. Zardini, M. Corradi, R. Alinovi, A.L. Boner, G.L. Piacentini, Mediators Inflamm. 2006 (2006) 1.
[15] E. Baraldi, G. Giordano, M.F. Pasquale, S. Carraro, A. Mardegan, G. Bonetto, C. Bastardo, F. Zacchello, S. Zanconato, Allergy 61 (2006) 90.
[16] S. Celio, H. Troxler, S.S. Durka, J. Chladek, J.H. Wildhaber, F.H. Sennhauser, C.W. Heizmann, A. Moeller, Nitric Oxide 15 (2006) 226.
[17] H. Nishino, T. Tanaka, J.K. Shiroishi, J.S. Sato, Y. Naruse, S. Kagamirnori, Bull. Environ. Contam. Toxicol. 47 (1999) 609.
[18] R. Smith, Clin. Sci. 59 (1980) 215.
[19] L. Hyldstrup, P. McNair, G. Finn Jensen, H. Rist Nielsen, I. Transbøl, Calcif. Tissue Int. 36 (1984) 639.
[20] S.M. Mohammed, S.A. Suleiman, S.K. Addae, S.H. Annobil, F.K. Adzaku, O.F. Kadoummi, J.T. Richards, Clin. Chim. Acta 203 (1991) 285.
[21] S.C. Hopkins, G.M. Palmieri, H.B. Niell, M. Moinuddin, M.S. Soloway, Cancer 53 (1984) 117.
[22] O. Hagemeyer, H. Otten, T. Kraus, Int. Arch. Occup. Environ. Health 79 (2006) 613.
[23] S. Mas, X. Casterad, N. Laso, M.J. Lafuente, R. Panades, A. Calleja, S. Hernandez, D. Turuguet, R. Deulofeu, A. Ballesta, C. Ascaso, A. Lafuente, Am. J. Ind. Med. 45 (2004) 186.
[24] R.M. Effros, K.W. Hoagland, M. Bosbous, D. Castillo, B. Foss, M. Dunning, M. Gare, W. Lin, F. Sun, Am. J. Respir. Crit. Care Med. 165 (2002) 663.
[25] I. Horvath, J. Hunt, P.J. Barnes, K. Alving, A. Antczak, E. Baraldi, G. Becher, W.J. van Beeurden, M. Corradi, R. Dekhuijzen, R.A. Dweik, T. Dwyer, R. Effros, S. Erzurum, B. Gaston, C. Gessner, A. Greening, L.P. Ho, J. Hohlfeld, O. Jöbsis, D. Laskowski, S. Loukides, D. Marlin, P. Montuschi, A.C. Olin, A.E. Redington, P. Reinhold, E.L. van Rensen, I. Rubinstein, P. Silkoff, K. Toren, G. Vass, C. Vogelberg, H. Wirtz, Eur. Respir. J. 26 (2005) 523.
[26] T. Schettgen, A. Tings, C. Brodowsky, A. Müller-Lux, A. Musiol, T. Kraus, Anal. Bioanal. Chem. 387 (2007) 2783.
[27] E. Schwedhelm, J. Sandmann, D. Tsikas, J. Labelled Radiopharm. 41 (1998) 773.
[28] P.A. Biondi, L.M. Chiesa, M.R. Storelli, P. Renon, J. Chromatogr. Sci. 35 (1997) 509.
[29] H. Inoue, H. Iguchi, A. Kouno, Y. Tsuruta, J. Chromatogr. B 757 (2001) 369.
[30] H. Inoue, Y. Date, K. Kohashi, H. Yoshitomi, Y. Tsuruta, Biol. Pharm. Bull. 19 (1996) 163.
[31] M. Badadani, S.V. Babu, K.T. Shetty, Ann. Clin. Biochem. 44 (2007) 164.
[32] M. Delport, S. Maas, S.W. van der Merwe, J.B. Laurens, J. Chromatogr. B 804 (2002) 345.
[33] T. Langrock, P. Czihal, R. Hoffman, Amino Acids 30 (2006) 291.
[34] A.J. Alpert, J. Chromatogr. 449 (1990) 177.
[35] A.J. Alpert, M. Shukla, A.K. Shukla, L.R. Zieske, S.W. Yuen, M.A.J. Ferguson, A. Mehlert, M. Pauly, R. Orlando, J. Chromatogr. A 676 (1994) 191.
[36] H. Lindner, B. Sarg, C. Meraner, W. Helliger, J. Chromatogr. A 743 (1996) 137.
[37] H. Lindner, B. Sarg, W. Helliger, J. Chromatogr. A 782 (1997) 55.
[38] H. Schlichtherle-Cerny, M. Affolter, C. Cerny, Anal. Chem. 75 (2003) 2349.
[39] M. Godejohann, J. Chromatogr. A 1156 (2007) 87.
[40] H. Idborg, L. Zamani, P.O. Edlund, I. Schuppe-Koistinen, S.P. Jacobsson, J. Chromatogr. B 828 (2005) 9.