Journal of Chromatography B, 877 (2009) 3937–3945

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development of an on-line weak-cation exchange liquid chromatography–tandem mass spectrometric method for screening aldehyde products in biological matrices

Mark Eggink[a,1], Ségolène Charret[a], Maikel Wijtmans[b], Henk Lingeman[a], Jeroen Kool[a], Wilfried M.A. Niessen[a], Hubertus Irth[a,*]

[a] VU University Amsterdam, Faculty of Science, Section of Analytical Chemistry and Applied Spectroscopy, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands
[b] VU University Amsterdam, Faculty of Science, Pharmacochemistry, Section of Medicinal Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 9 July 2009
Accepted 28 September 2009
Available online 14 October 2009

Keywords:
On-line weak-cation exchange solid phase extraction
Liquid chromatography
Mass spectrometry
4-APC
Derivatization
Aldehydes
Lipid peroxidation

ABSTRACT

This paper focuses on the development and optimization of an on-line weak-cation exchange SPE (WCXE) coupled to gradient HPLC with tandem MS detection. The system enables the selective purification and re-concentration of the in-vial derivatized aldehydes from plasma and urine samples. Aldehydes are important as biomarkers for oxidative stress. Using a derivatization cocktail consisting of 4-(2-(trimethylammonio)ethoxy)benzenaminium dibromide (4-APC) and $NaBH_3CN$ in the screening and detection of known and unknown aldehyde biomarkers, one can take advantage of the specific fragmentation characteristics of this derivatization reagent in MS/MS. The WCXE column gives the advantages of direct injection of the sample after protein precipitation and centrifugation into the WCXE-LC–MS/MS system. Injection volumes up to 50 μl can be injected without overloading the WCX column. Detection limits of 0.5 nM can be reached for the detection of the derivatized aldehydes. The system is robust with low intra-/inter-day variation in retention time and peak area. An *in vitro* model shows how derivatized aldehydes in human and rat plasma are detected. Finally, plasma treated with radical inducer shows elevated aldehyde species compared to untreated plasma.

1. Introduction

Oxidative stress occurs when there is an imbalance between the production of reactive oxygen and a biological system losing the ability to detoxify the reactive intermediates or repair the resulting damage. These reactive oxygen species (ROS) include oxygen ions, free radicals and peroxides and can lead to damage to DNA, proteins, and cell membranes (lipid peroxidation). This eventually results in cell death but can also be involved in the development of chronic diseases such as atherosclerosis [1], parkinson disease [2], and the pathologies caused by diabetes mellitus [3–5], rheumatoid arthritis [6] and alzheimer disease [7,8]. In many diseases, it is unclear if the oxidants trigger the disease, or if they are produced as a result of the disease and cause some of the disease symptoms.

Because of the short lifetimes [9] of the ROS and the lack of sufficiently sensitive analytical detection techniques, the direct detection of radicals in biological systems is difficult to perform. For this reason, there is a great need for biomarkers of radical damage, which can be used to monitor the involvement of this damage in the pathogenesis of diseases or in the toxicity of xenobiotics causing ROS.

Lipid peroxidation (LPO) is probably the most widely investigated process induced by free radicals. The presence of membrane phospholipids at the sites where the ROS are produced provides easily affected endogenous targets. In particular, the group of polyunsaturated fatty acids (PUFAs) is highly vulnerable to reactions with free radicals. Aldehydes like hexanal, malondialdehyde, heptanal, nonanal, 4-hydroxyhexenal or 4-hydroxynonenal are frequently used as biomarkers [10]. They indicate the extent of free radical damage to different PUFAs. They are excreted and thus analyzed in, for example, exhaled breath condensate, plasma or urine. Aldehydes have been analyzed using various methods [11–14], including GC–MS [11,13] and, more recently, LC–MS [14]. In order to analyze aldehydes by GC–MS or LC–MS, derivatization is required. The most widely used derivatization agent for aldehydes is 2,4-dinitrophenylhydrazine (DNPH) [14–17]. Alternatively, pentafluorophenyl-, 2-chloro-, 2,4-dichlorophenylhydrazine and cyclohexanedione are applied as derivatization reagents [13,18]. These reagents were typically designed for UV–vis and fluorescence detection but are currently also used in combination with MS detec-

* Corresponding author. Tel.: +31 20 598737; fax: +31 20 5987543.
E-mail address: Irth@few.vu.nl (H. Irth).
[1] Present address: Synthon BV, Microweg 22, 6503 GN Nijmegen, The Netherlands.

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.09.043

tion, for example, for the detection of aldehyde-DNPH derivatives in nM range by means of negative-ion atmospheric-pressure chemical ionization (APCI) [16,19].

In previous work, we presented the development of a novel derivatizing agent, 4-(2-(trimethylammonio)ethoxy)benzenaminium halide (4-APC), designed for the selective determination of aldehydes in biological samples by positive-ion electrospray ionization mass spectrometry (ESI-MS) [20]. The derivatization of aldehydes by a cocktail of 4-APC and $NaBH_3CN$ occurs rapidly at mild pH and temperature, and is characterised by an irreversible reduction leading to stable secondary amines as final products. Because of high selectivity of the cocktail, ketones do not derivatize under the used conditions.

To analyze known aldehydes and screen for unknown ones, an additional purification and concentration step must be implemented. This is useful to clean-up plasma or urine samples and especially important to analyze the low abundant aldehydes and to reduce matrix effects. Most used derivatization agents like DNPH need labour-intensive sample pre-treatment to extract the derivatives [15,17], with off-line liquid–liquid extraction or solid phase extraction (SPE) on C18 cartridges. The automation of this process by on-line coupling of the SPE to HPLC results in methods that are generally faster, less expensive and less prone to errors, thus resulting in better reproducibility [21–28]. Our novel selective derivatization agent, 4-APC, possesses a permanent positive charge, and therefore gives us the ability to use a more selective on-line SPE-HPLC system based on ion-exchange processes rather than the more generic C18-based SPE-HPLC systems.

In the present paper, we describe the development and optimization of an on-line weak-cation exchange SPE (WCXE) coupled to gradient HPLC with tandem MS detection. The system enables us to selectively purify and re-concentrate the in-vial derivatized aldehydes from plasma and urine samples. A key advantage of this on-line WCXE-LC–MS/MS setup over a conventional RP-SPE-LC–MS/MS setup is that samples with high contents of organic modifier can be injected directly; additional dilution or evaporation is not required. The system is used for both screening and quantitative analysis of aldehydes in biological matrices. We demonstrate the use of on-line WCXE-LC–MS/MS for the determination of derivatized aldehydes in both urine and plasma. Furthermore, the power of the derivatization reagent 4-APC is demonstrated in screening for and detection of known and unknown aldehyde biomarkers by taking advantage of the specific fragmentation characteristics of the derivatization reagent in MS/MS. Human and rat plasma were derivatized and the aldehyde profiles were compared. Additionally, human plasma treated with AAPH was monitored for the levels of 11 aldehydes in time.

2. Experimental

2.1. Chemicals and reagents

4-(2-(Trimethylammonio)ethoxy)benzenaminium dibromide (4-APC, **1**) was synthesized through a major improvement of our previously reported procedure [20], synthetic details of which can be found in the Supplementary Material. Butanal, pentanal, *trans*-2-pentenal, hexanal, heptanal, octanal, nonanal, decanal, cyclohexylcarboxaldehyde, 1,1,3,3-tetramethoxypropane (TMP), hydrochloric acid (HCl), sodium cyanoborohydride ($NaBH_3CN$), benzoylcholine chloride, ammonium bicarbonate, ammonium acetate, 2,2′-azobis(2-amidopropane) dihydrochloride (AAPH), lyophilized human plasma and rat plasma were all purchased from Sigma–Aldrich, Zwijndrecht, the Netherlands. 4-Hydroxyhexenal (4-HHE) and 4-hydroxynonenal (4-HNE) were purchased from Bio-Connect, Huissen, The Netherlands. Methanol (MeOH), acetonitrile (ACN) and formic acid (FA) were purchased from Biosolve, Valkenswaard, The Netherlands. Urine was obtained from 5 healthy volunteers.

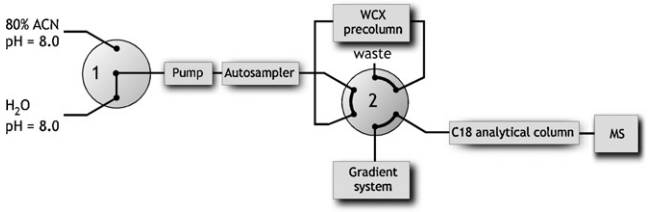

Fig. 1. Schematic diagram of the WCXE-LC–MS/MS system. 1 is the solvent select valve and 2 is the two way switch valve for loading, washing and equilibration.

2.2. Preparation of MDA standard and aldehyde solution

1,1,3,3-Tetramethoxypropane (TMP) was used to prepare a malondialdehyde (MDA) stock solution. A volume of 17 μl was dissolved in 10 ml 0.1 M HCl. This solution was incubated at 40 °C for 60 min to hydrolyze TMP to MDA (final concentration ca. 10 mM). The concentration of MDA was determined by measuring its absorbance at 245 nm (ε = 31,800 mol l^{-1} cm^{-1}) according to Esterbauer and Cheeseman [29]. For each aldehyde, a stock solution of 10 mM in MeOH was prepared and stored in the freezer at −20 °C. The stock solution was diluted with H_2O to the final working solution.

2.3. On-line weak-cation exchange-HPLC system

A schematic diagram of the setup of the on-line WCXE LC–MS/MS system used in this study is given in Fig. 1. A solvent-select valve coupled to a Shimadzu 10-ADVP pump ('s Hertogenbosch, The Netherlands) was used to direct either a washing solvent (30 mM ammonium carbonate, 80% ACN, pH 8) or a loading/conditioning solvent (30 mM ammonium carbonate, pH 8) at a flow rate of 150 μl/min to an Agilent 1100 autosampler with a 100 μl injection loop (thermostated at 10 °C). The autosampler was connected to an in-house build weak-cation exchange column via a six-port two-way switch valve was controlled by in-house written software. A slurry of 100 mg/ml CBA (ISOLUTE) SPE material, particle size 40 μm, Biotage, Uppsala, Sweden in acetone was prepared. The slurry was transferred in a 10 mm × 2.1 mm column by applying 0.1 atmospheres at the end of the column. The bottom was sealed with a 0.2 μm PEEK frit. When the column was filled, the top was sealed with a similar screen. The column was placed in an in-house build cartridge holder. A gradient system consisting of two Agilent 1100 HPLC pumps (Agilent Technologies, Amstelveen, The Netherlands) controlled by Chemstation Rev B.01.09 was connected to the same switching valve to perform the elution and separation. A Waters Atlantis dC18 reversed-phase column (C18, 150 mm × 2.1 mm, 3 μm) was used at 45 °C and with a flow rate of 200 μl/min for the separation of the derivatized aldehydes standards and biological samples.

The gradient elution was programmed as follows: after the injection (5–100 μl), the sample was flushed on the WCXE column with the loading/conditioning buffer at a flow rate of 150 μl/min. After 1 min, the solvent-select valve was switched for 1 min to the washing buffer (80% of ACN). Subsequently, the solvent-select valve was switched back for the loading/conditioning buffer for reconditioning. After 2.5 min, the six-port two-way switching valve was switched to put the WCXE column on-line with the LC–MS system and the gradient elution program started. Trapped derivatives are eluted from the WCX column with the gradient system. Mobile phase A (99% H_2O + 1% ACN + 0.2% formic acid) was maintained for 5 min and then solvent B (5% H_2O + 95% ACN + 0.2% formic acid) was

increased from 0 to 90% in 10 min with a 5-min hold at 90% B. After this, the column was reconditioned for 10 min with 100% mobile phase A. The effluent from the LC column was directed to ESI-MS.

2.4. Mass spectrometer

A Micromass (Wythenshawe, Manchester, UK) Q-TOF2 quadrupole–time-of-flight hybrid mass spectrometer equipped with a Micromass Z-spray ESI source was used for detection. MassLynx software (version 3.5) running under Windows NT was used for control of the system and data acquisition. The TOF analyzer was operated at a 20-kHz frequency with a spectrum integration time of 1 s in "full spectrum" MS in the positive-ion mode in the range m/z 150–450 ("interscan" time, 0.1 s). The ESI source conditions for the HPLC analysis were as follows: source temperature 100 °C, desolvation temperature 325 °C, capillary voltage 2.5 kV. The cone voltage was 20 V. Nitrogen (99.999% purity; Praxair, Oevel, Belgium) was used with flow rates of 20 l/h for nebulization, 50 l/h for cone gas, and 350 l/h for desolvation. Argon (99.9995% purity; Praxair) was used in the collision cell. For the determination of the optimal collision energy in the MS/MS experiments, derivatized aldehyde solutions were fragmented with a collision energy in the range of 15 and 25 V.

2.5. Sample pre-treatment and derivatization

For the optimization of the derivatization reaction, 200 μl of 0.5 mg/ml 4-APC dibromide in 150 mM ammonium acetate buffer pH 5.7, 50 μl 0.75 mg/ml $NaBH_3CN$ in methanol and 250 μl of aldehyde standard were mixed and vortexed for 60 s. The derivatization was carried out at 10 °C in the thermostated Agilent 1100 autosampler. After 3 h, the first sample was injected in the LC–MS/MS for analysis. Compared to our previous report [20], we have reduced the amount of 4-APC by a factor of four. This is still more than a 100 times excess of derivatization agent. As such, the reaction kinetics were not significantly influenced by this reduction. The main reason for the decreasing of the 4-APC concentration is to reduce the amount of cations loaded onto the WCX column in order to achieve a higher loadability for the aldehyde derivatives.

2.6. Pre-treatment and derivatization of urine and plasma samples

Urine samples of 5 healthy volunteers were collected, pooled and stored at −20 °C. The urine samples were centrifuged at 13,600 rpm for 15 min at 10 °C before derivatization. 25–250 μl of urine was mixed with 200 μl of 4-APC (0.5 mg/ml) in 300 mM ammonium acetate buffer pH 5.7, 50 μl $NaBH_3CN$ (0.75 mg/ml in methanol) and 50 μl spiked aldehyde.

The lyophilized rat and human plasma samples were dissolved in water at a concentration of 1 mg/ml and stored at −20 °C. After thawing, 50 μl of plasma was mixed 50 μl of cold ACN, vortexed for 1 min and centrifuged for 20 min at 13,600 rpm at 4 °C. For the derivatization, 50 μl of sample was mixed with 40 μl of 4-APC (0.5 mg/ml) in ammonium acetate buffer (300 mM) and 10 μl $NaBH_3CN$ (0.75 mg/ml in methanol). After a minimum of 4 h, the samples were analyzed. To the human plasma samples, an aldehyde standard mixture with a final concentration of 80 nM was added.

2.7. In vitro oxidation in human plasma

200 μl of human plasma was mixed with 50 μl AAPH solution (final concentration 1 mM) and incubated at 37 °C. At 3.5 and 20 h, 50 μl was taken from the solution and mixed with cold ACN, vortexed for 1 min and centrifuged for 20 min at 13,600 rpm at 4 °C. For the derivatization, 50 μl of sample was mixed with 40 μl of 4-APC (0.5 mg/ml) in ammonium acetate buffer (300 mM) and 10 μl $NaBH_3CN$ (0.75 mg/ml in methanol). The control experiment was performed similarly with the exception that 50 μl of H_2O was added instead of AAPH, without any incubation time.

2.8. Method validation

For the method development of the on-line WCXE-LC–MS/MS setup, different parameters were optimized. Comparison between direct injection on the reversed-phase column and the complete on-line WCXE-LC–MS/MS system was done with derivatized aldehydes mixtures (10 μl). The breakthrough volume was determined by injecting 50 μl of sample followed by washing steps with aqueous and organic buffers, respectively. The waste outlet was monitored for the elution of 4-APC. For the determination of the effect of buffer concentration of the injected sample in the presence of urine, a mixture of pre-derivatized aldehydes was spiked in urine. The final ammonium acetate buffer concentration was varied between 4 and 150 mM. Matrix effects of urine were determined by diluting urine 10, 5 and 2 times with a mixture of pre-derivatized aldehydes. The maximum injection volume was determined by injecting 10, 25, 50, 75, or 100 μl of derivatized aldehydes in the WCXE-LC–MS/MS system. To evaluate the intra-day precision, a 250 nM derivatized aldehyde solution was injected four times on the same day. The inter-day precision was determined by analyzing the same sample in triplicate another day ($n = 7$). The linearity, relative standard deviations of retention time and peak area in the extracted ion chromatogram, and the limit of detection of 12 aldehydes were determined by five point calibration series ranging from 0 to 125 nM.

3. Results and discussion

Screening for aldehyde biomarkers of oxidative stress in biological matrices can be a complex task. Sample purification and re-concentration procedures are mainly required to improve selectivity and facilitate finding these biomarkers. We have reported the development of a novel derivatizing agent 4-APC with specific MS and tandem MS characteristics [20]. In combination with $NaBH_3CN$, the use of 4-APC allowed the rapid, highly sensitive and selective determination of aldehydes in biological matrices.

In the current research, not only (un)saturated aldehydes and malondialdehyde were analyzed but also unsaturated hydroxyaldehydes like 4-HNE and 4-HHE, which are important and often used biomarkers for monitoring the radical damage of PUFA's [30–32]. Aldehyde biomarkers are generally analyzed in urine. However, being degradation products of PUFA's, aldehyde biomarkers are more abundantly present in plasma [33]. Before they reach the urine, side reactions can occur which lower the amount of aldehyde products. However, with the analysis of aldehydes in plasma samples, more elaborate sample clean-up is needed, in particular involving the precipitation of proteins. The latter is mainly done with cold acetonitrile, ethanol or perchloric acid [34], followed by centrifugation. After a treatment with acetonitrile, the supernatant consists of up to 50% of acetonitrile. Before injection of this supernatant in RP-LC or RP-SPE, evaporation or prior extraction with an apolar solvent like hexane [17] and subsequent evaporation and finally redissolution in a small quantity of LC-eluent in required. However, if 4-APC is used as derivatizing agent in combination with an on-line WCXE, these steps are not required. A typical WCX sorbent is CBA (a carboxy propyl phase) material which contains a silica-based backbone with carboxylic groups. These groups are completely deprotonated at a pH of 6.8 and higher, and completely protonated at a pH of 2.8 and lower. This makes them ideally suited

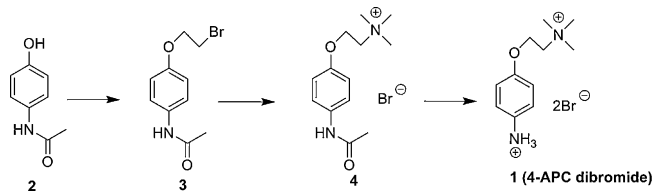

Fig. 2. Synthesis pathway of 4-(2-(trimethylammonio)ethoxy)benzenaminium dibromide (4-APC).

for trapping and reconcentrating of 4-APC derivatives from biological samples. Additionally, ESI-MS compatible buffers can be used in these pH ranges.

3.1. Improvement of the synthesis of 4-APC

In our previous publication on 4-APC [20], we reported the synthesis of 4-APC as a chloride bromide salt ($C_{11}H_{20}Br_{1.57}Cl_{0.43}N_2O$). In that report, we had already briefly described how the last step can be more conveniently carried out with aq. HBr instead of with aq. HCl. Use of aq. HBr gives rise to 4-APC as a dibromide salt in a higher yielding deprotection protocol. This dibromide salt behaves identically in all respects to the previously reported Cl, Br salt [20] and it was used throughout the current paper. In the Supplementary Material, the full procedure for the improved multi-gram synthesis of 4-APC dibromide (**1**) is presented (Fig. 2) together with stability data for the compound. Note also that, compared to our previous procedure [20], an unnecessary recrystallisation of intermediate **4** has been omitted, resulting in a higher yield for the second step.

3.2. Identification with MS and tandem MS

For the highly sensitive and selective determination of aldehydes in biological matrices by ESI-MS, 4-APC as derivatizing agent has some important characteristics: it has a permanent positive charge and it does not react with other carbonyl compounds. In addition, it provides characteristic neutral losses in MS/MS analysis.

Fig. 3 shows the extracted ion chromatograms of a standard mixture of twelve aldehyde derivatives: 4-HHE, 4-HNE, MDA, butanal, *trans*-2-pentenal, pentanal, hexanal, heptanal, cyclohexylcarboxaldehyde, octanal, nonanal, and decanal. Comparison between the extracted ion chromatograms from the WCXE-LC–MS/MS system to previously reported chromatograms without the on-line WXCE system [20] indicates that there is no deterioration of chromatographic performance. It should be clarified at this point, that the WCXE column was not implemented to improve the LC separation of the aldehyde derivatives, but to achieve a more efficient and reliable analysis of biological samples. In that respect, the WCXE column provides sample clean-up, analyte preconcentration, and reduction of matrix effects, as demonstrated below. Consistent with earlier findings, all derivatized aldehydes examined showed an M^+ ion [20]. In the homologous series of aldehydes (butanal through decanal), the increasing length of the carbon chain resulted in equal retention time shifts in the chromatogram. As expected, the additional hydroxyl group in 4-HHE and 4-HNE resulted in shorter retention time compared to the saturated and non-hydroxylated analogues, hexanal and nonanal, respectively. Fig. 4 shows characteristic mass spectra of 4-APC derivatized aldehydes in MS/MS. Different fragmentation patterns are detected between the hydroxyl alkenals, the aliphatic alkanals and the dialkanals. For 4-APC derivatized aliphatic aldehydes (Fig. 4A and

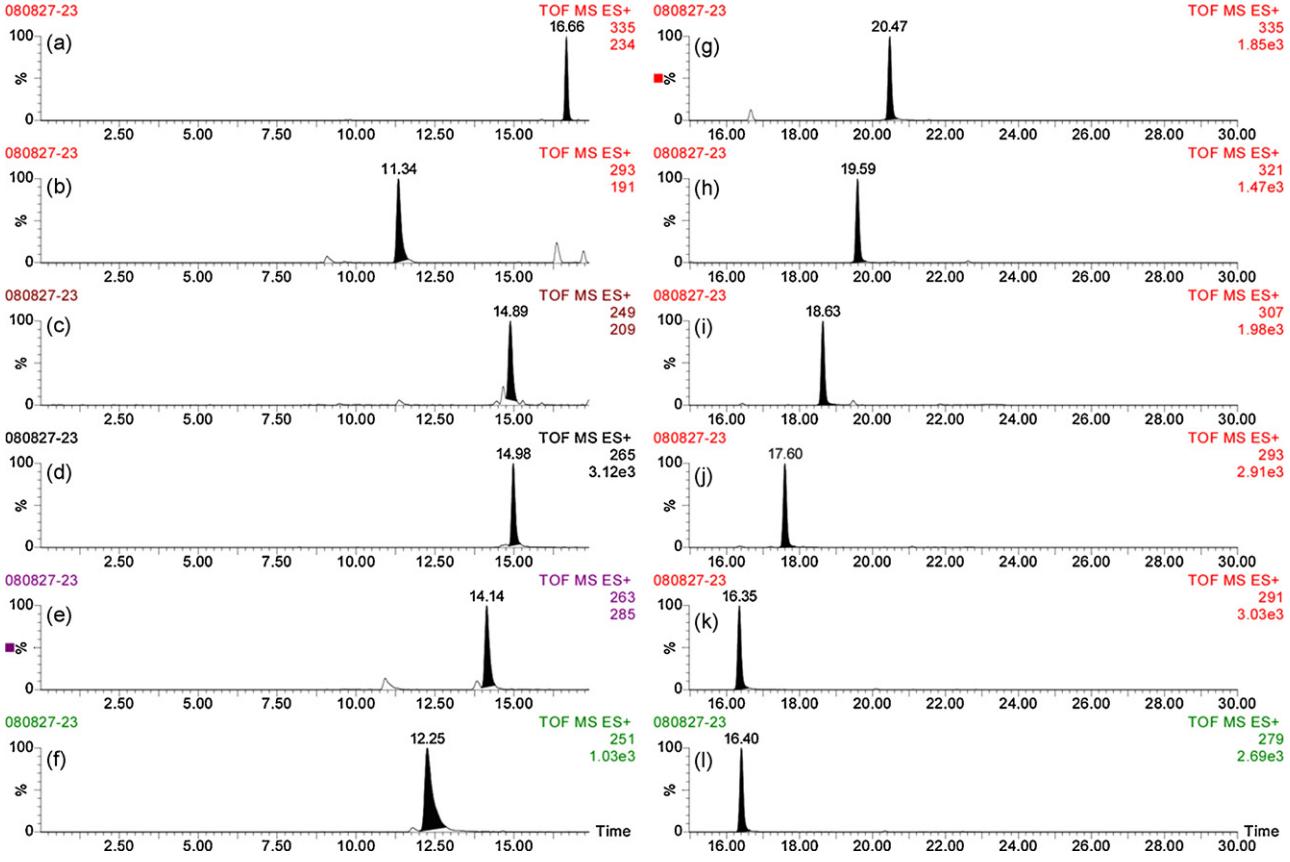

Fig. 3. Extracted ion chromatograms of the twelve used aldehyde derivatives: (a) 4-HNE, (b) 4-HHE, (c) malondialdehyde, (d) pentanal, (e) *trans*-2-pentenal, (f) butanal, (g) decanal, (h) nonanal, (i) octanal, (j) cyclohexylcarboxyaldehyde, (k) heptanal and (l) hexanal.

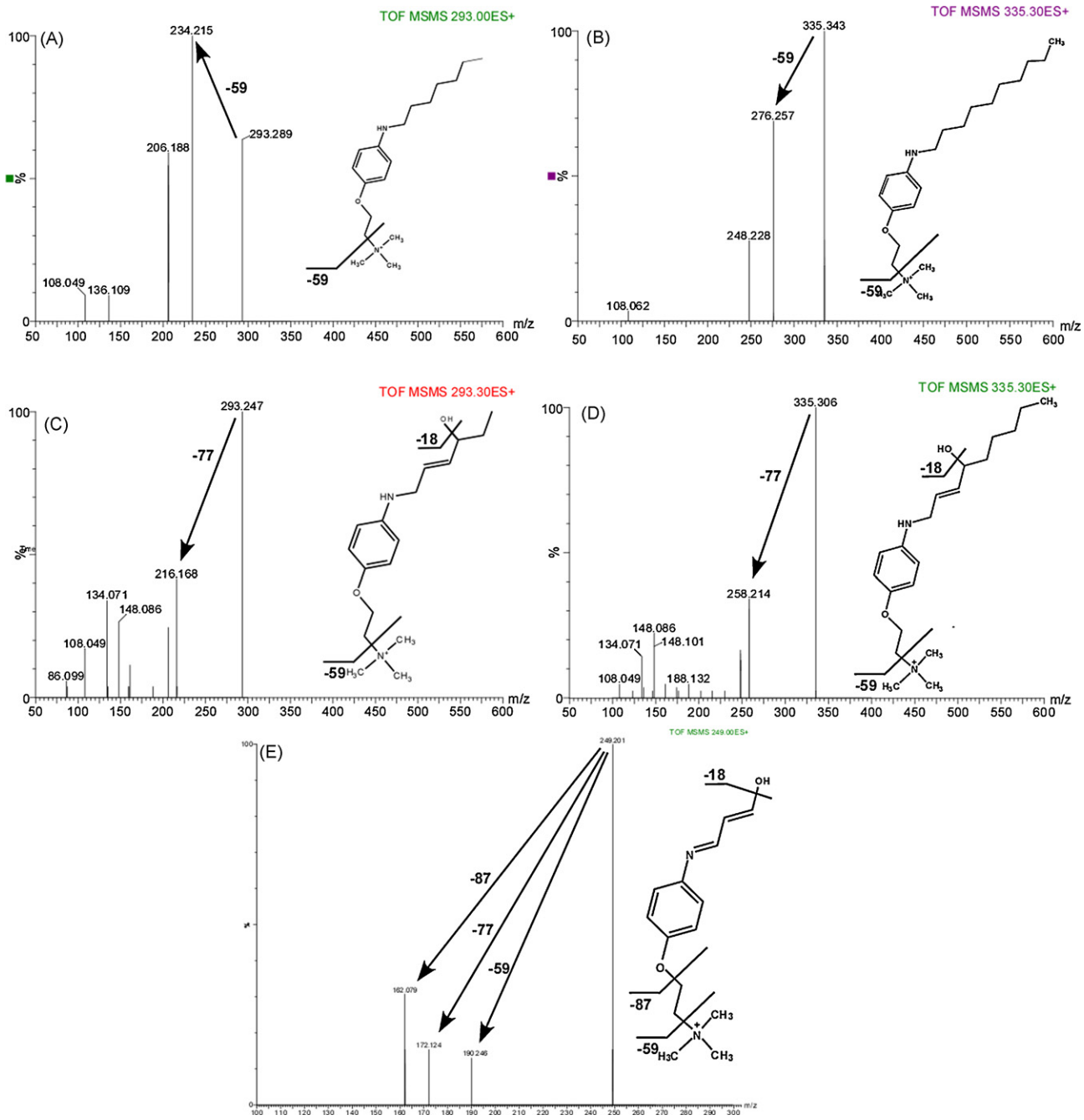

Fig. 4. MS/MS spectra of heptanal (A), decanal (B), 4-HHE (C), 4-HNE (D) and malondialdehyde (E). A typical neutral loss of 77 for a hydroxy-alkenal instead of 59 for the aliphatic alkanals is observed.

B), characteristic losses of 59 and 87 Da are observed, consistent with losses of C_3H_9N and $C_5H_{13}N$ (most likely due to subsequent losses of C_3H_9N and C_2H_4), respectively, as already described earlier [35] for heptanal and decanal. For 4-HHE (Fig. 4C) and 4-HNE (Fig. 4D), characteristic losses of 77 and 87 Da are observed, with the loss of 77 Da being consistent with the loss of C_3H_9N and H_2O (dehydration) [34]. In the case of MDA (Fig. 4E), which forms an imine with 4-APC but does not undergo subsequent reduction as can be seen from the structure in Fig. 4E, losses of 59, 77 and 87 Da are observed [20]. Possibly, because the hydroxyl group of derivatized MDA is located at an unsaturated carbon, as opposed to the situation with the hydroxy-alkenals, a neutral loss of 59 Da is still observed to a large extent accompanied by only a minor loss of H_2O. Next to the retention time and the precursor *m*/*z*, this type of information is useful for the characterization of a derivatized aldehyde detected. The *m*/*z* of a particular hydroxy-alkenal is isobaric with that of the alkanal with one additional CH_2 group (*m*/*z* difference is 38 mDa). Thus, in complex samples containing unknown aldehyde biomarkers analyzed by single MS, high-resolution MS would be required for confirmation of identity, whereas the differences in the fragmentation pattern in our MS/MS data immediately provides a mean for unambiguous confirmation of identity without the need for high-resolution MS. To optimize the sensitivity of the method, the optimal fragmentation energy and the signal loss in the Q-TOF2 were determined. Although the fragmentation sites of the derivatized aldehydes are similar, different collision energies could be necessary to achieve the highest MS/MS signal for each individual aldehyde derivative. The breakdown curves of the derivatized

aldehydes, *i.e.*, the intensities of M^+ and the two main fragments corresponding to 59-Da and 87-Da losses as a function of the collision energy, were determined to optimize the MS/MS settings. Some of the results are shown in the supplementary data, Fig. S6.

We performed all our studies using a Q-TOF instrument. The Q-TOF provides the ability to apply accurate-mass determination for confirmation of identity of the derivatized aldehydes, which is especially useful in screening for unknown aldehyde biomarkers. However, the studies could also have been performed on a triple quadrupole instrument. In that case, selected reaction monitoring (SRM) with transitions between M^+ and the $[M^+-59]$-fragment for aldehyde derivatives (and between M^+ and the $[M^+-77]$-fragment for the hydroxy-alkenal derivatives) at compound-dependent optimized collision energies would be applied in targeted analysis of specific aldehyde biomarkers. The use of the triple–quadrupole instrument also provides the possibility to use the constant neutral-loss analysis mode (fixed neutral loss of 59 and/or 77 Da) for the non-targeted screening of samples for unknown aldehyde biomarkers.

3.3. Method development and optimization

The performance of the proposed on-line sample pre-treatment method was evaluated by determining the recovery of the extraction procedure, the breakthrough volume of the WCXE column, the matrix effects in urine and plasma, the influence of buffer concentration on the trapping efficiency of derivatives in spiked urine and plasma and in standard solutions, the repeatability, the maximum injection volume, the linearity of the calibration curve and the quantification limits.

First, the optimal time settings of the WCXE-LC–MS/MS system were determined. The washing and elution steps were optimized by varying the washing times between each step. The breakthrough volume was determined to be 6 min with an injection volume of 50 μl and a flow rate of 150 μl/min. After 6 min, the first trace of 4-APC could be detected in the extracted ion chromatogram of the ion with *m*/*z* 195. The optimal loading and washing times were both determined to be 1 min. Reconditioning of the WCXE column and changing the eluent in the system after the washing step was found to be a critical step in the procedure. When the reconditioning time is too short, retention times in the analytical separation were unstable. This was the result of the dead volumes in the system. Therefore, the reconditioning time was 3.5 min, which results in a total duration of the on-line WCXE procedure of 5.5 min. To keep a reliable system, the WCXE cartridges were exchanged every 50 injections.

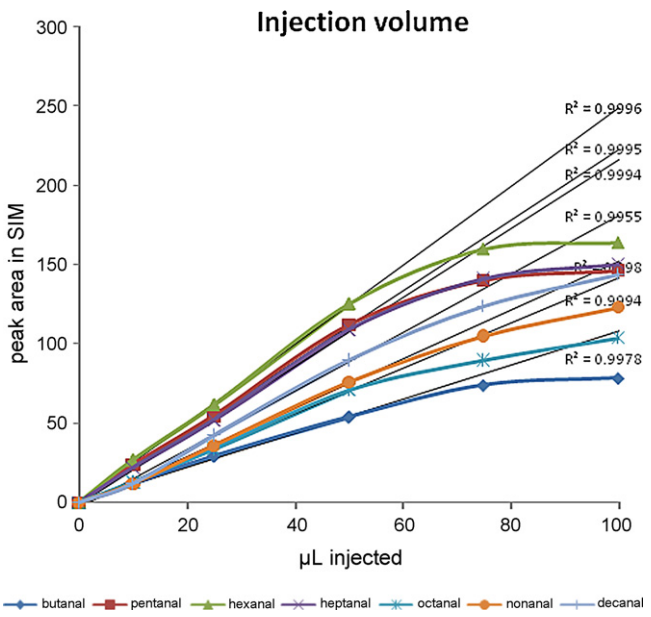

Fig. 5. Optimization of injection volume of the WCXE-LC-MS/MS system. 400 μl of 0.5 mg/ml 4-APC dibromide in 150 mM ammonium acetate buffer pH 5.7, 100 μl 0.75 mg/ml $NaBH_3CN$ in methanol and 500 μl of 100 nM aldehyde standard (final concentration 50 nM). The maximum injection volume was determined by injecting 10, 25, 50, 75, or 100 μl of the prior derivatized aldehydes.

The recovery of the sample pre-treatment was tested by comparing the peak areas between a direct injection of a standard mixture of derivatized aldehydes in a reversed-phase C18 system connected to LC–MS/MS and in the WCXE-LC–MS/MS system (Fig. S7 shows the corresponding chromatograms). The 4-APC derivatives of hexanal, heptanal, octanal, nonanal and decanal showed recoveries of better than 93%, indicating no major losses occurring in the on-line WCXE sample pre-treatment. The repeatability of the WCXE-LC–MS/MS system is tested by studying intra-day and inter-day variation. The maximum intra-day variations ($\%RSD_{intra}$) of the retention time and the peak area of hexanal, heptanal, octanal, nonanal and decanal were 0.03%, and 2.12%, respectively. The maximum inter-day variations ($\%RSD_{inter}$) of the retention time and the peak area were 0.5%, and 3.9%, respectively, for the same compounds.

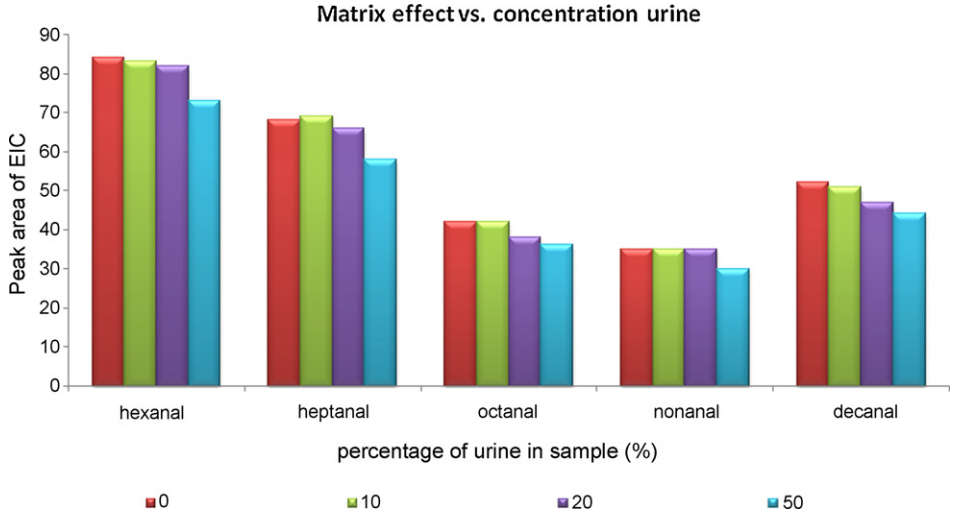

Fig. 6. Determination of the effect of urine matrix on the measured peak area of prior derivatized aldehydes.

Although the 4-APC derivatives can be efficiently and reproducibly trapped onto the WCXE column, the loadability of the WCXE column is an important factor, especially because 4-APC itself is also trapped on the WCXE column and is present in a large excess. The loadability was tested using different injection volumes in the range between 1 and 100 μl. Fig. 5 shows a linear relation between response and injection volume for the seven tested aldehydes derivatives up to 55 μl and significant deviation from linearity at higher injection volumes. Therefore, an injection volume of 50 μl was set for the system.

3.4. Analytical performance of the WCXE-LC–MS/MS

Table 2 shows analytical data for twelve derivatized aldehydes in standard buffer solution. All aldehyde derivatives tested showed a good linearity ($r^2 \geq 0.992$) in a dynamic range of almost three orders of magnitude. The limit of detection, defined as three times the signal-to-noise ratio, of these derivatives was determined at 0.5 nM for all compounds except for *trans*-2-pentenal which was 2.5 nM. The RSDs of the retention times were within 1.5% which indicates a robust separation system and no distortion by the online WCXE part of the system. The average RSDs of the peak areas ($n = 3$) were within 4.9% for all compounds which indicates an excellent repeatability of the derivatization and the WCXE trapping. Comparing these results to our previous work, the LODs decreased with an factor of 6 for most aldehydes derivatives [20].

3.5. Extraction from biological matrices (of "urine and plasma")

To mimic and study the effect of the different salt concentrations in urine samples on the trapping efficiency of the derivatized aldehydes on the WCXE column, 2.5 times diluted urine samples were spiked with a mixture of pre-derivatized aldehydes in different buffer concentrations ranging from 4 to 150 mM ammonium acetate. The concentration of the injection buffer does not significantly influence the trapping efficiency of the derivatives (data not shown). With a 4 mM ammonium acetate buffer pH 5.7, some deviation in the peak areas of the hexanal and heptanal derivatives was observed, but between 9 and 150 mM ammonium acetate, peak areas of all derivatives remained constant and unaffected by the buffer concentration.

The matrix effect of urine was estimated by comparing the response for five aldehyde derivatives between a solvent standard and three diluted urine samples (1:1, 1:5 and 1:10) spiked with pre-derivatized aldehydes. Fig. 6 shows that an only modest loss of response (12.3–15.4%) occurs with increasing urine concentration in the injected samples. Because this loss of response is similar for the tested derivatives which are eluting on different time points, the matrix effect appears to be independent of the retention time and the compound. A possible explanation is that the aldehydes react with other components in urine, *e.g.*, imine formation with amine groups present, before they are derivatized.

Table 1
Recovery of spiked aldehydes in human plasma.

Derivatized aldehydes	Recovery (%)
Malondialdehyde	80.0
Butanal	110.8
trans-2-Pentenal	99.7
Pentanal	109.2
Hexanal	105.6
Cyclohexylcarboxaldehyde	103.7
Heptanal	106.3
Octanal	99.6
Nonanal	70.3
Decanal	39.9
4-Hydroxyhexenal	113.1
4-Hydroxynonenal	106.6

To determine the extraction recovery, or more accurately the overall process efficiency [33], in human plasma, various experiments were performed. In each case, comparison of peak areas between standard solution and spiked plasma samples was made.

In one experiment, 17 nM benzoylcholine was added as internal standard to two human plasma sample lots. After protein precipitation and centrifugation, the plasma samples were derivatized for 4 h either with or without 4-APC and $NaBH_3CN$. Subsequently, the samples were analyzed by WCXE-LC–MS/MS. The two samples showed a recovery of 97% and 101% for benzoylcholine relative to the benzoylcholine standard solution. As the extraction recovery is better than 99% (see above), this result indicates that no matrix effect was present after the WCXE step. This experiment also shows the advantage of WCXE over C18-RP-SPE. Due to need for protein precipitation, the plasma samples contained 40% ACN. In C18-RP-SPE, this would have adversely affected the extraction recovery, unless an additional evaporation or dilution step would have been applied to reduce the ACN content.

In another experiment, aldehyde recovery from plasma was determined by comparing peak areas of a 50 μl injection of 80 nM derivatized aldehyde mixture with twelve aldehydes and human plasma spiked with the same aldehyde mixture (final concentration 80 nM). After protein precipitation and centrifugation, plasma samples were derivatized for 4 h. The results of recovery experiments with plasma are summarized in Table 1. The internal standard benzoylcholine showed 99% recovery. After the blank correction (based on the analysis of a non-spiked derivatized plasma), 4-hydroxyhexenal, 4-hydroxynonenal, butanal, *trans*-2-pentenal, pentanal, hexanal, heptanal, cyclohexylcarboxaldehyde and octanal showed recoveries between 99.7% and 113%. Malondialdehyde, nonanal and decanal showed recoveries of 80%, 70.3% and 39.9%, respectively. For malondialdehyde, the derivatization time could be the limiting factor. For nonanal and decanal, which are

Table 2
Linear dynamic range, correlation coefficients (r^2), relative standard deviations of retention times, peak areas, limits of detection (LODs) and the absolute amount of aldehyde derivative.

	Linear range (nM)	r^2	RSD_{area} (%)	Average t_R	RSD t_R (%)	LOD (nM)	Inj. amount (fmol)
Malondialdehyde	2.5–125	0.992	4.86	14.89	0.49	0.5	25
Butanal	2.5–125	0.999	2.10	12.24	0.45	0.5	25
trans-2-Pentenal	12.5–125	0.995	4.73	14.20	1.47	2.5	125
Pentanal	2.5–125	0.995	1.82	14.99	0.10	0.5	25
Hexanal	2.5–125	0.997	1.40	16.41	0.12	0.5	25
Heptanal	2.5–125	0.998	2.70	17.60	0.23	0.5	25
Cyclohexylcarboxaldehyde	2.5–125	0.995	1.32	16.35	0.17	0.5	25
Octanal	2.5–125	0.998	2.13	18.63	0.16	0.5	25
Nonanal	2.5–125	0.997	1.35	19.57	0.15	0.5	25
Decanal	2.5–125	0.997	4.13	20.47	0.06	0.5	25
4-Hydroxyhexenal	2.5–125	0.999	2.59	11.34	0.94	0.5	25
4-Hydroxynonenal	2.5–125	0.999	4.04	16.75	0.42	0.5	25

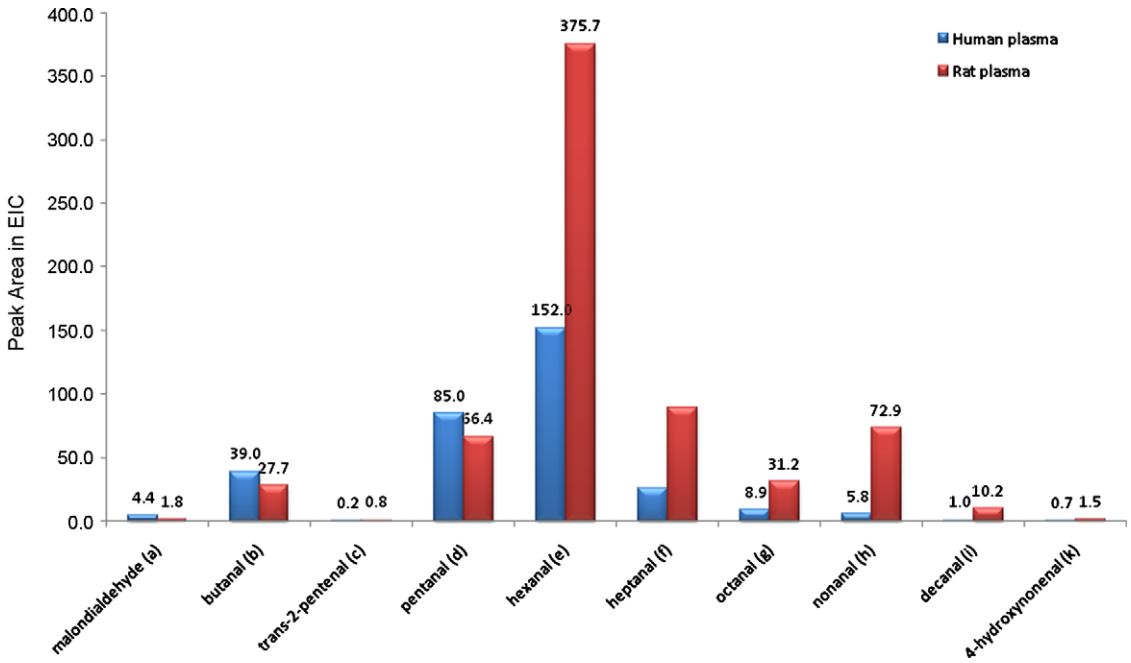

Fig. 7. Comparison of 4-APC derivatized human and rat plasma, the detected peaks are named with (a) till (k) and represent the aldehydes.

the most non-polar aldehydes in the aldehyde mixture, side reaction with plasma could result in a lower recovery, although despite the lower recovery the results are repeatable. Although the loss in recovery of the long chained aldehydes (nonanal and decanal), a comparison can still be made for quantification in plasma due to a repeatable system. At the quantification limits the lower recoveries will probably negatively influence the results; therefore conclusions must be carefully drawn (Table 2).

3.6. Application: detection of derivatized aldehydes in plasma

In order to demonstrate the applicability of our approach consisting of derivatization in bioanalysis, various experiments were performed with plasma samples. First, the same absolute amount of human and rat plasma was derivatized with 4-APC and analyzed under the same conditions. Fig. 7 shows the difference in aldehyde profile observed for rat and human plasma. Hexanal, heptanal, octanal, nonanal, decanal and 4-HNE were observed at a higher level in rat plasma, whereas MDA, butanal and pentanal were found at a higher level in human plasma. This result suggests the potential of screening the aldehyde profiles in large sets of plasma samples.In a second series of experiments, AAPH, a water-soluble azo compound which is extensively applied as a free radical generator, was used to enhance lipid peroxidation in plasma samples. Decomposition of AAPH results in molecular nitrogen and 2 carbon radicals. The carbon radicals may combine to produce stable products or react with molecular oxygen to give oxygen radicals, which in turn can damage crucial cellular molecules, including membrane lipids [36]. As such, AAPH can be used for *in vitro* oxidation in plasma, thereby mimicking oxidative stress *in vivo*.

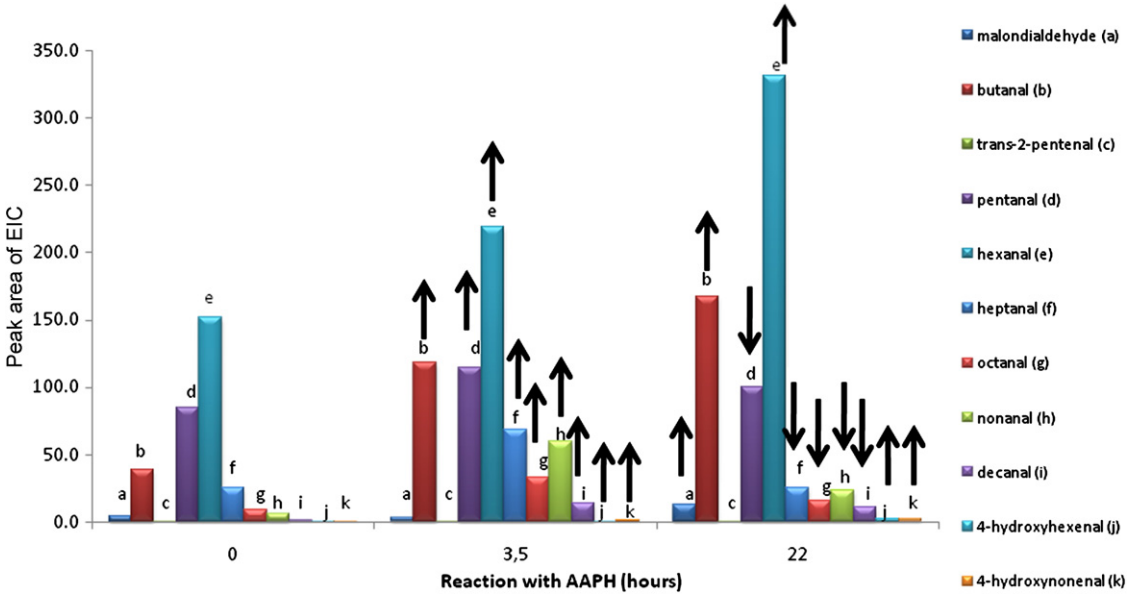

Fig. 8. Formation of aldehydes in time after *in vitro* lipid peroxidation in human plasma.

Fig. 8 shows a time-effect curve (0, 3.5 and 22 h) for induction of lipid peroxidation in plasma by means of AAPH. At the three different time points, 0, 3.5 and 22 h, AAPH-treated plasma was derivatized with 4-APC and subsequently analyzed with WCXE-LC–MS/MS. The induction of aldehyde formation by AAPH is clearly demonstrated. The concentration of MDA, hexanal, butanal, 4-HHE and 4-HNE increased with time, whereas the concentration of pentanal, heptanal, octanal and decanal first increase but decline after 3.5 h. This may indicate additional reactions of the aldehydic products, such as peroxyaldehydes, as have been reported by Brash et al. [31]. Another possible explanation could be that the aldehydes react slowly in time with proteins via an imine formation with lysine [37].

4. Conclusion

Screening for aldehyde biomarkers in urine or plasma of free radical damage to lipids is an important non-invasive tool to detect a possible relationship between radical damage and diseases. By back-tracking the initial radical activator, an organ or cells could be discovered to get more information on the typical diseases.

The present WCXE-LC–MS/MS protocol is capable to reveal the differences in aldehyde profiles and, therefore, to screen for known and unknown aldehyde biomarkers after derivatization with a cocktail of 4-APC label and $NaBH_3CN$. The characteristic fragmentation of the 4-APC-derivatized aldehydes enables confirmation of identity with known aldehydes and allows differentiating between hydroxyl-aldehydes and aldehydes.

Our method especially facilitates the analysis of aldehyde biomarkers in plasma. The on-line solid phase extraction (WCXE) column gives the advantages of direct injection of the sample after protein precipitation and centrifugation before WCXE-LC–MS/MS analysis. Injection volumes up to 50 μl can be injected without overloading the WCX column and detection limits of 0.5 nM can be reached for the detection of the derivatized aldehydes. The system is robust with low intra-/inter-day variation in retention time and peak area.

Furthermore, *in vitro* oxidation with AAPH showed elevated aldehyde species in human and rat plasma, which indicates that the presented system could be used for screening aldehydes resulting from *in vivo* oxidative stress.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.09.043.

References

[1] B. Halliwell, Free Radic. Biol. Med. 5 (2009) 531.
[2] S.J. Chinta, J.K. Andersen, Biochim. Biophys. Acta 1780 (2008) 1362.
[3] J. Wu, X. Han, J. Zhou, Hunan Yi Ke Da Xue Xue Bao 24 (1999) 543.
[4] J.P. Freitas, P.M. Filipe, F.G. Rodrigo, Diabetes Res. Clin. Pract. 36 (1997) 71.
[5] P. Vajdovich, T. Gaal, A. Szilagyi, Acta Physiol. Hung. 81 (1993) 317.
[6] V.A. Ramos, P.A. Ramos, M.C. Dominguez, J. Pediatr. (Rio J.) 76 (2000) 125.
[7] E.E. Tuppo, L.J. Forman, B.W. Spur, R.E. Chan-Ting, A. Chopra, T.A. Cavalieri, Brain Res. Bull. 54 (2001) 565.
[8] E.E. Tuppo, L.J. Forman, J. Am. Osteopath. Assoc. 101 (2001) S11.
[9] K.H. Cheeseman, T.F. Slater, Br. Med. Bull. 49 (1993) 481.
[10] L.L. de Zwart, J.H. Meerman, J.N. Commandeur, N.P. Vermeulen, Free Radic. Biol. Med. 26 (1999) 202.
[11] R.A. Wheatley, Trends Anal. Chem. 19 (2000) 617.
[12] H. Esterbauer, H. Zollner, Free Radic. Biol. Med. 7 (1989) 197.
[13] X.P. Luo, M. Yazdanpanah, N. Bhooi, D.C. Lehotay, Anal. Biochem. 228 (1995) 294.
[14] S. Kolliker, M. Oehme, C. Dye, Anal. Chem. 70 (1998) 1979.
[15] K. Nagy, F. Pollreisz, Z. Takats, K. Vekey, Rapid Commun. Mass Spectrom. 18 (2004) 2473.
[16] R. Andreoli, P. Manini, M. Corradi, A. Mutti, W.M.A. Niessen, Rapid Commun. Mass Spectrom. 17 (2003) 637.
[17] G. Zurek, U. Karst, J. Chromatogr. A 864 (1999) 191.
[18] I.C. O'Brien-Coker, G. Perkins, A.I. Mallet, Rapid Commun. Mass Spectrom. 15 (2001) 920.
[19] O. Korchazhkina, C. Exley, S.A. Spencer, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 794 (2003) 353.
[20] M. Eggink, M. Wijtmans, R. Ekkebus, H. Lingeman, I.J. Esch, J. Kool, W.M. Niessen, H. Irth, Anal. Chem. 80 (23) (2008) 9042.
[21] A. Holm, E. Storbraten, A. Mihailova, B. Karaszewski, E. Lundanes, T. Greibrokk, Anal. Bioanal. Chem. 382 (2005) 751.
[22] S. Huq, M. Garriques, K.M. Kallury, J. Chromatogr. A 1135 (2006) 12.
[23] E. Rozet, R. Morello, F. Lecomte, G.B. Martin, P. Chiap, J. Crommen, K.S. Boos, P. Hubert, J. Chromatogr. B 844 (2006) 251.
[24] Y. Sato, E. Yamamoto, S. Takakuwa, T. Kato, N. Asakawa, J. Chromatogr. A 1190 (2008) 8.
[25] X. Wei, G. Yang, L. Qi, Y. Chen, Talanta 77 (2009) 1197.
[26] X. Wei, J. Yin, G. Yang, C. He, Y. Chen, J. Sep. Sci. 30 (2007) 2851.
[27] G. Yang, H. Liu, Y. Zhang, S. Wang, J. Yin, B. Yin, Y. Chen, J. Chromatogr. A 1129 (2006) 231.
[28] H.J. Zhang, J.F. Huang, B. Lin, Y.Q. Feng, J. Chromatogr. A 1160 (2007) 114.
[29] H. Esterbauer, K.H. Cheeseman, Methods Enzymol. 186 (1990) 407.
[30] M. Guichardant, B. Chantegrel, C. Deshayes, A. Doutheau, P. Moliere, M. Lagarde, Biochem. Soc. Trans. 32 (2004) 139.
[31] C. Schneider, N.A. Porter, A.R. Brash, J. Biol. Chem. 283 (2008) 15539.
[32] F.J.G.M. Vankuijk, L.L. Holte, E.A. Dratz, Biochim. Biophys. Acta 1043 (1990) 116.
[33] M. Yazdanpanah, X. Luo, R. Lau, M. Greenberg, L.J. Fisher, D.C. Lehotay, Free Radic. Biol. Med. 23 (1997) 870.
[34] S. Souverain, S. Rudaz, J.L. Veuthey, J. Pharm. Biomed. Anal. 35 (2004) 913.
[35] P. Uutela, R. Reinila, P. Piepponen, R.A. Ketola, R. Kostiainen, Rapid Commun. Mass Spectrom. 19 (2005) 2950.
[36] S.M. Culbertson, N.A. Porter, Free Radic. Res. 33 (2000) 705.
[37] M. Pournamdari, A. Saadi, E. Ellis, R. Andrew, B. Walker, D.G. Watson, Anal. Chim. Acta 633 (2009) 216.