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A new and fast methodology to assess oxidative damage in cardiovascular diseases risk development through eVol-MEPS–UHPLC analysis of four urinary biomarkers

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ABSTRACT

In this work, a new, fast and reliable methodology using a digitally controlled microextraction by packed sorbent (eVol®-MEPS) followed by ultra-high pressure liquid chromatography (UHPLC) analysis with photodiodes (PDA) detection, was developed to establish the urinary profile levels of four putative oxidative stress biomarkers (OSBs) in healthy subjects and patients evidencing cardiovascular diseases (CVDs). This data was used to verify the suitability of the selected OSBs (uric acid—UAc, malondialdehyde —MDA, 5-(hydroxymethyl)uracil—5-HMUra and 8-hydroxy-2′-deoxyguanosine—8-oxodG) as potential biomarkers of CVDs progression. Important parameters affecting the efficiency of the extraction process were optimized, particularly stationary phase selection, pH influence, sample volume, number of extraction cycles and washing and elution volumes. The experimental conditions that allowed the best extraction efficiency, expressed in terms of total area of the target analytes and data reproducibility, includes a 10 times dilution and pH adjustment of the urine samples to 6.0, followed by a gradient elution through the C8 adsorbent with 5 times 50 μL of 0.01% formic acid and 3 × 50 μL of 20% methanol in 0.01% formic acid. The chromatographic separation of the target analytes was performed with a HSS T3 column (100 mm × 2.1 mm, 1.7 μm in particle size) using 0.01% formic acid 20% methanol at 250 μL min^{-1}. The methodology was validated in terms of selectivity, linearity, instrumental limit of detection (LOD), method limit of quantification (LOQ), matrix effect, accuracy and precision (intra-and inter-day). Good results were obtained in terms of selectivity and linearity ($r^2 > 0.9906$), as well as the LOD and LOQ, whose values were low, ranging from 0.00005 to 0.72 μg mL^{-1} and 0.00023 to 2.31 μg mL^{-1}, respectively. The recovery results (91.1–123.0%), intra-day (1.0–8.3%), inter-day precision (4.6–6.3%) and the matrix effect (60.1–110.3%) of eVol®-MEPS/UHPLC-PDA method were also very satisfactory. Finally, the application of the methodology to the determination of target biomarkers in normal subjects and CVDs patients' revealed that the DNA adducts 5-HMUra and 8-oxodG levels are much more abundant in CVDs patients while no statistic differences were obtain for MDA and UAc. This result points to the importance of 5-HMUra and 8-oxodG as biomarkers of CVDs risk progression and further epidemiological studies are needed to explore the importance of this correlation.

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of death in humans. According to World Health Organization statistical data, 17.3 million people died from CVDs in 2008 and it is estimated that by 2030, this number will reach approximately 23.6 million deaths, mainly from heart disease and stroke [1]. There are several risk factors contributing for the CVDs development, but accumulating evidences points to the influence of oxidative stress damage, also known as oxidative damage, in key aspects of several disorders as cancer, different neurodegenerative diseases and particularly different CVDs [2–5]. Oxidative damage is produced whenever the antioxidant defenses are unable to cope with the deleterious effects caused by high levels of reactive oxygen species (ROS). These highly instable molecules can easily react and damage several cellular constituents, namely nucleic acids, proteins, and lipids [4,6]. This has driven several studies exploring the use of dietary antioxidants against oxidative damage effects and particularly in CVDs, the effect of antioxidants on

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endothelial dysfunction in patients with coronary heart disease or other CVDs disorders have been investigated [7]. Although the results obtained were not very conclusive and further studies are needed to clarify this issue, it is nowadays consensual that oxidative damage is a hallmark of several diseases, including CVDs. However, the ability of different OSBs to particularly predict CVDs remains to be established [2]. In the last decades, the scientific and clinical interests in oxidative damage and the possible parameters that can be used to quantify it have increased exponentially and multiple compounds have been studied as potential OSBs. Here we will focus in the urinary analysis of UAc, MDA and the DNA adducts 5-HMUra and 8-oxodG (Fig. 1).

UAc is the final oxidation product of purine catabolism and several studies pointed that there is a clear correlation between increased concentrations of UAc and oxidative stress, endothelial dysfunction, inflammation, subclinical atherosclerosis and an increased risk of cardiovascular events [3,8,9]. This data makes elevated UAc levels a putative OSB and particularly a potential independent CVDs risk biomarker. MDA is another OSB candidate as it is a specific byproduct of ROS-dependent membrane lipids peroxidation, mainly the polyunsaturated fatty acids arachidonic and linoleic acids (reviewed in [10]). Moreover, MDA presents cytotoxic and genotoxic effects and was found elevated in several degenerative diseases associated with oxidative damage, namely CVDs [10–16]. Oxidative damage can induce hundreds of modifications in DNA, being 8-oxodG one of the most abundant [17,18]. This DNA adduct is potentially mutagenic and is used to assess oxidative damage in tissues and urine [6,17,19]. 5-HMUra has been less explored as potential biomarker of oxidatively damaged DNA, although its levels were clearly shown to correlate with diet and oxidation status [20]. Traditional analytical approaches for preparation of urine samples include extractions with solvents (buffers and acetonitrile), clean-up and further fractionation by liquid–liquid extraction (LLE), or solid phase extraction (SPE) [19,21]. However, these procedures are time consuming and require relatively high volumes of sample/solvent which is not reasonable for many routine analyzes. Microextraction in packed syringe (MEPS) is a solid-phase extraction improvement using small sample and solvent volumes which facilitate the enrichment of the analytes, enabling fast and reliable sample extraction procedures [22,23]. Moreover, it allows full automation and is quite more simple and inexpensive than that of conventional methods [22,24–28]. Very recently a semiautomatic eVol® syringe allowing for low void volume sample manipulations and the fully automation of the sample processing methodologies was introduced by SGE Analytical Science (Australia). This syringe is suitable to use with the MEPS™ fibers and this combination is a major breakthrough in sample extraction, offering several additional improvements at the level of sample extraction reliability through the procedures automatization and minimization of user intervention that it allows. The aim of this work was to optimize and validate a fast and sensitive eVol®-MEPS/UHPLC-PDA method for the simultaneous separation and quantification of four potential OSBs, namely UAc, MDA, 8-oxodG and 5-HMUra, using Creat as a normalization factor. The developed method was then applied to a group of cardiovascular risk patients in order to evaluate its suitability in the early diagnosis of CVDs at the clinical environment. This methodology is a valid alternative to the current unreliable immunoassays or expensive mass spectrometry detection approaches used for the quantification of the oxidatively

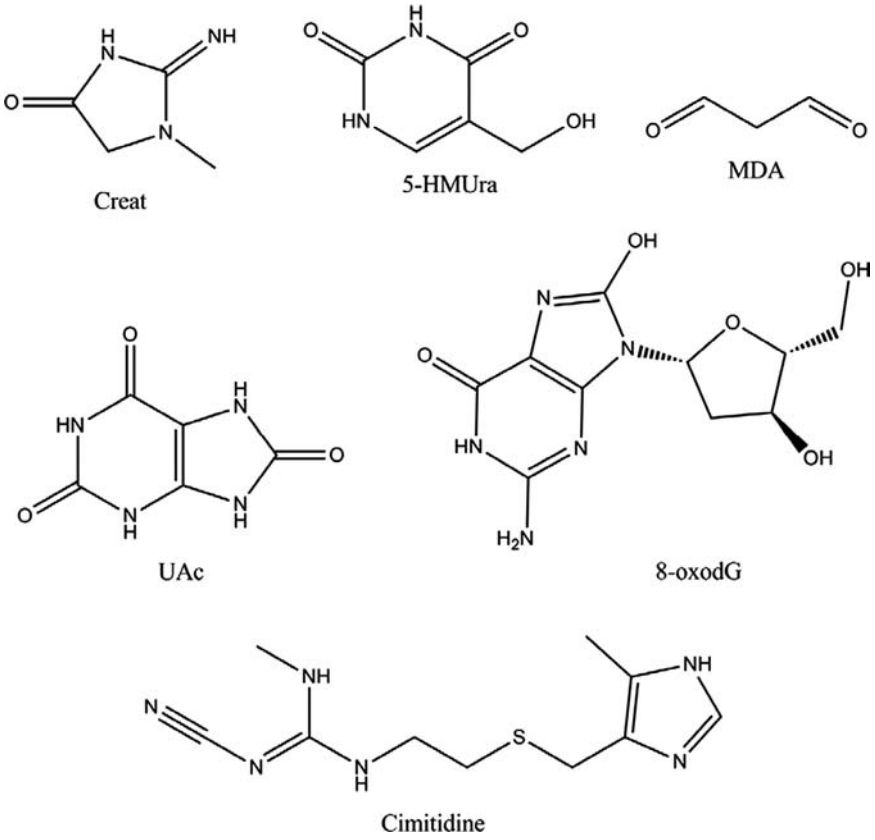

Fig. 1. Chemical structure of the target OSBs (Creat—creatinine; 5-HMUra—5-hydroxymethyluracil; MDA—malonaldehyde; UAc—uric acid; 8-oxodG—8-hydroxy-2′-deoxyguanosine and Cimitidine—internal standard).

modified DNA biomarkers [17,18], as well as the unspecific thiobarbituric acid reactive substances (TBARS) techniques used in the MDA quantification (reviewed in [29]).

2. Experimental

2.1. Reagents, standards and materials

All chemicals used were of analytical grade. Methanol (99.9% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA), formic acid from Merck (Darmstadt, Germany) all of HPLC grade, sodium hydroxide from Panreac (Barcelona, Spain) dimethylsulfoxide (DMSO) from LabScan Analyticalsciences (Dublin, Ireland), and sulfuric acid from Riedel-de-Haën (München, Germany). The biomarkers of oxidatively damaged DNA 5-HMUra and 8-oxodG, and the MDA precursor 1,1,3,3-tetraethoxypropane, as well as creatinine anhydrous and the internal standard (IS) cimitidine (purity of these three standards greater than 95%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). UAc was purchased from Fluka (Madrid, Spain). Ultrapure water (18 MΩ cm at 23 °C) was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA). All the eluates were filtered through 0.22 μm PTFE membrane filters (Millipore, Milford, MA, USA). The eVol® hand-held automated analytical syringe and stationary phases used for MEPS extraction were purchased from SGE Analytical Science (Melbourne, VIC, Australia). The Waters Acquity UPLC HSS T3 analytical column (100 mm × 2.1 mm, 1.8 μm particle size) was purchased from Waters (Milford, MA, USA).

2.2. Subjects and samples collection

The subjects used in this study were divided into normal controls and CVDs patients. The normal controls ($n=12$, age $=40.5 \pm 14.7$) were selected from among the blood donors at the Dr. Nélio Mendonça Hospital (Funchal, Portugal) and had no clinical history of CVDs. The CVDs patients (diagnosed with mild hypercholesterolemia and hypertension) of the study ($n=10$, age $=50.4 \pm 9.0$) were indicated by the Laboratory of Clinical Pathology of the same hospital. All subjects signed an informed consent to participate in the study, and the research was approved by the Ethics Committee of the referred hospital. Each individual (either patient or healthy volunteer) provided a sample of morning urine in a 20 mL sterile PVC container. The samples were immediately frozen at −80 °C and kept until being processed. Before extraction, the pH values of the urine samples were adjusted to 6 with 0.1 M sodium hydroxide solution or 0.1% formic acid.

2.3. Standard preparation and urine samples

Analytical standard stocks of Creat, 5-HMUra and cimitidine were prepared individually in ultrapure water (1000 μg mL^{-1}). UAc and MDA were prepared as described in Zuo et al. [30] and Sim et al. [31], respectively. The stock solution of 8-oxodG (400 μg mL^{-1}) was prepared in DMSO. All stocks were aliquoted in 4 mL vials, and stored at −20 °C. Synthetic urine (SU) was prepared according to Uppuluri et al. [32], excepted for Creat that was not used. Working standard solutions containing the OSBs were prepared daily from the individual stock solutions by diluting them in the SU solution. Urine and SU samples were diluted (1:10), pH adjusted and filtered through 0.22 μm membrane PTFE filters. The concentration ranges (Table 1) were selected according to the sensitivity of UHPLC-PDA for each biomarker (the physical–chemical characteristics of each compound affect the analytical signal and higher concentrations were used for some compounds in order to allow their detection). Creat was used as normalizing factor and cimitidine (10 μg mL^{-1}) as IS. All solutions were analyzed in triplicate ($n=3$).

2.4. Optimization of MEPS extraction using eVol®

The MEPS procedure was carried out with an eVol® semi-automatic syringe (SGE Analytical Science, Melbourne, Australia), loaded with a 500 μL gas-tight syringe with a removable needle. The syringe was fitted with a BIN (barrel insert needle) containing 4 mg of the sorbent material through which samples and solutions were discharged. A SU sample spiked with the ML concentrations of each OSB (indicated in Table 1) was used to optimize the MEPS procedure, which include best sorbent selection, pH, volume sample loading, number of extraction cycles, washing and elution volumes. The performance of the five MEPS sorbents commercially available at the time, C2, C8, C18, SIL (silica) and M1 (mix of C8 plus SCX—strong cation exchanger), was tested and compared in order to select the best sorbent for the different biomarkers. The pH influence was evaluate by tested the extraction efficiency at pH 2, 6 and 8. The optimal number of extraction cycles as well as sample volumes were also considered by assaying SU aliquots of 50 μL, 100 μL, 250 μL and 500 μL, loaded up and down once, 5 and 10 times. Except for the preliminary assays, each sample aliquot was pumped only once through the MEPS-BIN and the sample portion extracted was discarded into waste before the next aliquot was loaded. Different elution volumes (50 μL and 100 μL of 0.01% formic acid in Elution I and 30 μL and 50 μL of 20% methanol with 0.01% formic acid in Elution II) were tested. The flow rate during aspiration was limited to 20 μL s^{-1} to prevent cavitation and increase the interaction time of the analyte with the sorbent. All procedures were performed in triplicate ($n=3$). Before using MEPS, sorbent conditioning was performed with 100 μL of methanol followed by another 100 μL of 0.01% formic acid. This step activates the sorbent and ensures reproducibility in the retention of the analytes from the extraction by reducing the carry-over effect [22]. In all measurements (standards and samples) cimetidine was used as the IS and added after the eVol MEPS extraction.

2.5. UHPLC-PDA analysis and operating conditions

The analysis of OSBs was carried out on a Waters Ultra Performance Liquid Chromatographic Acquity system (UPLC, Acquity H-Class) (Milford, MA, USA) combined with a Waters Acquity quaternary solvent manager (QSM), an Acquity sample manager (SM), a column heater, a PDA detector, and a degassing system. The whole configuration was driven by Empower software v2.0 (Waters Corporation). Optimum separation was achieved with a mobile phase of 0.01% formic acid and methanol, with a constant flow rate of 250 μL min^{-1}, giving a maximum back pressure of 6.000 psi, which is within the capabilities of the UPLC™. The extracts (2 μL) were injected into the UPLC system, equipped with an Acquity UPLC™ strength silica HSS T3 analytical column (100 mm × 2.1 mm, 1.8 μm particle size) protected with an Acquity UPLC™ HSS T3 Van Guard™ Pre-column (Waters, Milford, USA). The column temperature was thermostated at 30 °C and the samples were kept at 15 °C in the sample manager. The sample analysis was performed during 3.50 min followed by a re-equilibration time of 3 min. All solvents and samples were filtered through 0.22 μm PTFE filters (Millipore, Milford, USA), before use. For quantification purposes the PDA detection was conducted by using four distinct channels that were set to the maximum absorbance wavelength of each biomarker (Table 1). They were identified by comparing the retention time and spectral characteristics of their peaks.

Table 1
Analytical data obtained after MEPS/UHPLC-PDA methodology.

Biomarkers		Creat	5-HMUra	UAc	MDA	8-oxodG
Peak number		1	2	3	4	5
RT (min)		0.827	1.021	1.134	1.435	1.653
λ_{max}[a] (nm)		215	215	285	246	295
Analytical performance						
Conc. range (μg mL^{-1})		5–350	0.0005–0.01	5–250	1–60	0.1–5
Regression equation		0.0124 × +0.1324	1277.5 × +0.8318	0.0302 × −0.1681	0.0096 × +0.0213	0.0724 × +0.0052
r^2[b]		0.9913	0.9906	0.9951	0.9920	0.9946
LOD[c] (μg mL^{-1})		0.01	0.00005	0.29	0.72	0.04
LOQ[d] (μg mL^{-1})		2.02	0.00023	2.31	1.57	0.13
% Matrix effect		63.50	60.11	110.29	71.54	82.21
Fortification level (μg mL^{-1})	LL[e]	5	0.0005	5	1	0.1
	ML[e]	100	0.0025	100	15	1.5
	HL[e]	350	0.01	250	60	5.0
Accuracy (%)	LL	93.77	91.06	91.04	94.50	94.68
	ML	102.04	95.50	100.40	98.21	96.84
	HL	123.02	104.68	102.05	102.32	103.50
Intra-day ($n=7$) RSD (%)	LL	5.18	5.67	4.09	5.74	8.34
	ML	5.71	4.24	5.71	2.43	4.75
	HL	3.65	0.95	1.67	5.62	2.03
Inter-day ($n=25$) RSD (%)	LL	5.04	7.68	4.58	6.07	6.12
	ML	5.37	7.69	6.08	7.37	5.79
	HL	5.41	6.14	6.49	6.33	4.95

[a] Maximum absorbance values obtained in the PDA system detection.
[b] Correlation coefficient; it gives an estimating how well the experimental points fit a straight line.
[c] Limit of detection.
[d] Limit of quantification. Values obtained from ordinary least-squares regression data.
[e] Concentration levels used: LL-low level; ML-medium level and HL-high level.

2.6. Method validation design

In order to validate the proposed eVol®-MEPS/UHPLC-PDA methodology for determination of urinary OSBs, selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and matrix effect were assessed as previously described [24]. These parameters were calculated for each biomarker using concentrations usually found in human urine (Table 1). Three calibration curves using six levels of concentration for each compound spiked in SU were built for each compound and the respective correlation coefficients were calculated by least-squares linear regression analysis of the peak area of each analyte. The biomarkers concentration was then determined by interpolation of the peak area of each individual compound in the calibration curves equations previously obtained from least-squares regression data. The sensitivity of the method was assessed by determining the limits of detections (LODs) and limits of quantifications (LOQs). Remain analytical parameters were determinate by spiking SU with three different concentration levels, corresponding to the lowest level (LL), medium level (ML) and highest level (HL) of the six points calibration curve of each biomarker. The method precision was evaluated with six replicates ($n=6$) of spiked SU in analyzed in the same day to obtain intra-day precision (repeatability) and daily through three different days ($n=24$) for inter-day precision (reproducibility). The accuracy of the method was assessed through a recovery study carried out with triplicates of SU spiked previously to the extraction. In the extraction efficiency determination, one set of triplicates ($n=3$) was spiked previously to the extraction, while a second set of SU aliquots was spiked only after. The matrix effect was evaluated by the percentage of the quotient between the slopes of the standards in blank matrix (SU) and those obtained by spiking urine (standard addition method).

2.7. Statistical analysis

Data statistical analysis was performed using the SPSS 19.0 (SPSS Inc., Chicago IL, USA). Significant differences among the groups were assessed with a one-way analysis of variance (ANOVA). The least square difference (LSD) test (p-value < 0.05) and principal component analysis (PCA) were used to compare the means and to verify the distribution of the variables for the two groups analyzed, respectively. PCA is a tool for data exploration that allows the reduction of the data dimensionality, thus facilitating inter-technique relationships analysis. New variables, the principal components, are obtained to explain the greater part of total variance with a minimum of information loss [28]. Figures and tables were generated using the Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA, USA).

3. Results and discussion

3.1. Optimization of eVol®MEPS procedure

In the optimization of the eVol®MEPS procedure, the selection of the sorbent, sample pH, number of extraction cycles, washing and elution conditions, were carefully investigated, as they affect the MEPS performance [33]. Five commercial eVol®MEPS sorbents have been evaluated in terms of extraction efficiency and reproducibility. These fibers possess different properties and affinities for polar and non-polar compounds which will determine their suitability for the method developed. As can be observed in the Fig. 2A, the highest extraction efficiency, measured in therms of extraction recovery (%), was achieved with the C8 fiber and the same result was obtained for each single analyte except for UAc (Fig. S1). This is due to the fact that these biomarkers

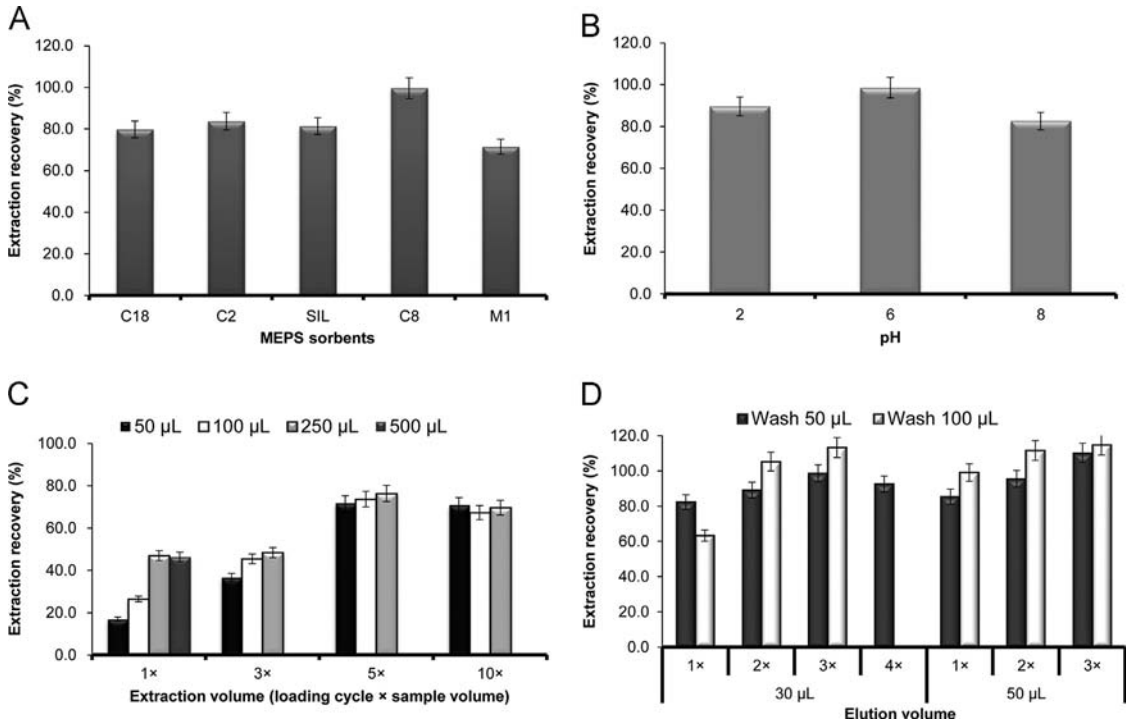

Fig. 2. (A) Comparison of the performance of five different MEPS sorbents; (B) effect of pH; (C) influence of the number of extraction cycles (extraction–discard) and sample volume; and (D) influence of different elution volumes on the extraction efficiency of target OSBs from urine of control and CVDs subjects, by eVol® MEPS. Values expressed as mean ± standard deviation ($n=3$), numbers above bars indicate the total elution volume (µL).

(Creat, 5-HMUra, MDA and 8-oxodG) have intermediate polarities, being C8 the resin that possess higher affinity for these type of analytes polarities. UAc, however, has a polar nature and therefore interacts better with C2 which is more polar than C8 and C18. Overall, C8 was the most suitable fiber for the extraction methodology proposed and was used in more than 100 extractions without loss of efficiency (evaluated by UHPLC-PDA analysis, data not shown). To assess the pH influence on the extraction efficiency, samples pH was adjusted to 2, 6 and 8 with 0.1 M NaOH or 0.1% formic acid. As seen in Fig. 2B no significant differences were obtained between the three conditions, although medium extraction recovery (%) is slightly higher at pH 6. This condition is also the more consensual to all compounds when they are considered individually (Fig. S2). Moreover, peak resolution was better for pH 6 (data not shown), and the physiological pH of urine samples varies between 6 and 7 [34]. Therefore, pH 6 was chosen as the optimal sample pH to perform the extraction procedure.

Retention of analytes on the stationary phase is also affected by the number of cycles and the velocity that sample components, namely the target analytes, contact the resin during the aspiration/dispension steps. In practical terms, sample aliquots were charged up and down through the eVol® syringe one or more times (cycles) at the minimum flow rate of 20 µL s^{-1} (to prevent cavitation and favor the interaction between the target analytes and the resin) and the dispensed solution discarded or not. Multiple extraction cycles were assayed and were obtained better results in terms of extraction efficiency and peak resolution when the dispensed solution was discarded. We observed that when the solution was not discarded, we were favoring the binding of unspecific analytes to the stationary phase and peak resolution was highly affected (data not shown). The influence of the number of cycles of extraction and sample volume in extraction efficiency for each of the biomarkers can be analyzed in Fig. 2C. As can be observed, the competition for adsorption of the target analytes in the active sites of the C8 adsorbent is favored by multiple loadings (up to 5) of small sample volumes (50 µL). This result is also valid for Creat, 5-HMUra, UAc, but not for MDA and the 8-oxodG, which revealed higher efficacy with higher loading volumes (Fig. S3). Since the purpose of this work was the simultaneous quantification of the four OSBs, we select as the best condition five loading cycles of 50 µL sample each. This experimental condition presents additional advantages because urine is a complex matrix and could easily saturate the resin. Moreover, by using small and 10 times diluted (see Section 2) sample volumes, we are able to obtain better peak resolution with less interference of unspecific urinary analytes and simultaneously preserve the lifetime of the resin. To complete the extraction procedure optimization, two other critical steps, washing and elution, were assayed. Washing the resin after sample application enables the removal of interfering analytes, namely sugars. We performed the washing step with 50 µL or 100 µL of 0.01% formic acid and observed that a significantly amount of the target analytes were washed away, affecting the yield of the following elution step. As we were using small and diluted sample volumes, the level of urinary interferents was also very low and so we decided to perform a gradient elution using 0.01% formic acid (elution I) followed by 20% methanol 0.01% formic acid (elution II). In elution II, 30 µL or 50 µL of 20% methanol 0.01% formic acid, loaded up to 3 times were assayed. As can be depicted from Fig. 2D and Fig. S4, in which the elution conditions assayed are compared by compound, there are no significant differences in using 50 µL or 100 µL in the elution I, but the increase in the loading times in elution II clearly improved the recovery of the target analytes. Overall, the best conditions were obtained using a first elution with 50 µL of 0.01% formic acid followed by three cycles of 30 µL of 20% methanol 0.01% formic acid, obtaining a final elution volume of 140 µL.

3.2. Method validation

To study the selectivity of the method, the chromatograms of SU and processed urine from different individuals were compared. As can be observed in Fig. 3A, the method is highly sensitive for

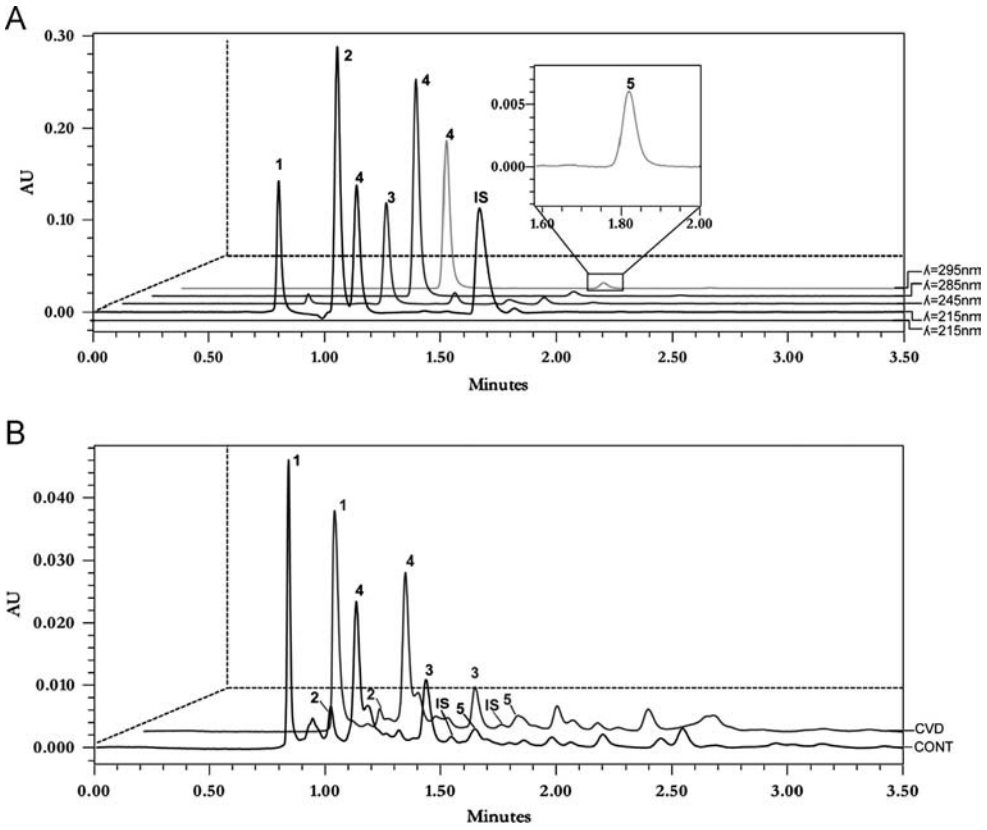

Fig. 3. Typical profile of eVol®MEPS/UHPLC-PDA chromatogram of a (A) synthetic urine and synthetic urine spiked with the target SOBs (the concentration levels spiked were the ML levels reported in Table 1) and (B) urine from control and CVDs patients (for peak identification see Table 1).

the target biomarkers, allowing the separation of the analytes of interest from the other components present in the matrix.

Another parameter evaluated was the linearity, which can be defined as the ability to provide analytical results proportional to the concentration of analyte present in the sample in a concentration range [24]. This was obtained by using the IS approach as urine is a complex and instable matrix. This method allows a correct evaluation of the response due to sample manipulations (e.g. concentration, extraction methodology, sample preparation). Cimetidine has been previously used as IS in the quantification of Creat and UAc by Zuo et al. [30] and therefore was chosen to our method development. Linear responses over the studied range of concentrations were obtained in the UHPLC-PDA analysis as well as high correlation coefficients using the least-squares linear regression data processing methodology ($r^2 > 0.9906$). Sample quantification was then performed using the means of the calibration curves equations obtained from least-squares regression data as previously described to interpolate the peak areas measured in the chromatograms at the maximum absorption wavelengths for each compound. The results obtained, indicated in Table 1, present low detection limits, ranging between 0.00005 μg mL^{-1} (5-HMUra) and 0.72 μg mL^{-1} (MDA), while LOQs ranged from 0.00023 μg mL^{-1} (5-HMUra) to 2.31 μg mL^{-1} (UAc).

As we are working with biological samples (urine), it was expected to have some degree of matrix effect and in fact, it is present in all biomarkers, except UAc. This is probably due to the fact that UAc is a very abundant compound in urine and will compete with the binding of other matrix interferents. In order to assess the accuracy of the method, a recovery study was conducted by fortifying SU with known amounts of the biomarkers in three concentration levels (LL, ML and HL) that include the range of concentrations that can be found in real samples (see Table 1). The results obtained show a good accuracy of the method, particularly for the medium concentrations levels (ML), ranging from 95.5% to 100.4%. For the low concentrations levels (LL), accuracy values are slightly lower, ranging from 91.0% to 94.7%. Regarding the higher concentration levels (HL), the accuracy obtained for creatinine was a little high (123.0%). However this is not very problematic as the creatinine concentrations obtained are in the ML range, 100 μg creatinine/mL of plasma, which is only about 30% of the HL concentration used, 350 μg creatinine/mL of plasma (data not shown). The development of analytical method is subject to random variations that can be estimated through precision. This parameter is usually expressed in terms of intra-day and inter-day precision, and the calculation is performed by estimating the relative standard deviation (RSD), also known as coefficient of variation (CV), and the recommended values should be above 15%. The intra-day precision was measured by comparing the RSD of spiked SU (LL, ML and HL) samples analyzed on the same day ($n=7$) and the inter-day precision was determined by the RSD obtained for spiked SU samples analyzed for three alternate days ($n=21$). The results obtained for both parameters were satisfactory with medium RSD values around 5.2% (maximum value of 8.3%).

3.3. Quantification of OSBs by eVol®MEPS/UHPLC-PDA

After the validation of analytical method, a total of 22 urine samples (12 from control subjects and 10 from CVDs subjects) were analyzed in triplicate. All biomarkers were identified by the retention time and the respective wavelength corresponding to the maximum of absorption. Separation of the OSBs standards mixture prepared in SU was achieved in a 3.50 min run (Fig. 3A). Fig. 3B shows a chromatogram of a control and a CVDs group urine sample. As can be seen, a good resolution with minimal interference of other matrix components was obtained.

Furthermore, the chromatographic profiles of the different urine samples are similar (data not shown), although the clinical conditions and biomarker concentrations differs among samples. To determine the biomarkers concentrations, results were normalized with Creat. All the data produced can be analyzed with more detail in Supplementary Table 1.

As can be observed in Fig. 4, the UAc and MDA levels on control and CVDs patients groups are very similar. This result is unexpected because accumulating epidemiological and clinical evidences clearly show an association between increased UAc levels and oxidative stress and several forms of CVDs (reviewed in [3,8,9]). Regarding MDA, since it is a byproduct of lipid peroxidation which is a key event triggering the development of CVDs, it was expected to obtain an increment of its levels in the CVDs group. This association is strongly suggested by several reports pointing to a positive correlation between MDA levels and CVDs progression (reviewed in [35]). The fact that this assay covers a small number of patients and little information was available about their diet, medication and disease status, certain contribute for these discrepancies that affect UAc and MDA, but not 5-HMUra and 8-oxodG levels. In fact, the levels of these two DNA adducts are significantly higher in the CVDs patients group (2 times higher for 5-HMUra and almost 3 times higher for8-oxodG). According to Wu et al. [36] and Martinet et al. [37] oxidatively damaged DNA and its repair increase significantly along the formation of atherosclerotic plaque. Additionally, significant increase in biomarkers of oxidatively damaged DNA were observed in patients with coronary heart disease and in patients with risk factors such as diabetes *mellitus*, hypercholesterolemia (reviewed in [38]). Therefore, our results are in agreement with the literature reports and contribute to support the use of 8-oxodG and 5-HMUra as biomarkers of CVDs progression.

It should be noted that the simultaneous quantification of these four biomarkers in the same experimental procedure, involving five orders of magnitude (from 10^{-1} μg UAc/μg Creat and μg MDA/μg Creat to 10^{-3} μg 8-oxodG and 10^{-6} μg 5-HMUra/μg Creat, see Fig. 4 for details) is reported for the first time.

The comparison of our results with the data available in the literature was a little cumbersome because there are few reports using chromatographic techniques (to allow a more consistent data comparison) and even those revealed several inconsistencies in the presentation of the results. Moreover, to our knowledge, there are not available reference concentrations ranges established for these biomarkers. Therefore we normalized the data obtained in the studies presented in Table 2 to the same units used in this report (μg of the biomarker by μg Creat). As can be observed, the concentrations of UAc and 5-HMUra obtained are similar to the ones referred by Jen et al. [39] and Chen et al. [40], respectively. However, concerning to MDA and 8-oxodG, the concentrations reported in the literature present high dispersion, ranging from the 10^{-1} μg MDA/μg Creat (this work) to the 10^{-5} μg MDA/μg Creat reported by Agarwal et al. [41] using smokers and no-smokers patients with diabetic nephropathies.

The concentrations reported for 8-oxodG also present a wide range of variation (from 10^{-3} μg 8-oxodG/μg Creat from our study to 10^{-9} of Serdar et al. [42] report using patients with type 2 diabetes mellitus nephropathy). In both cases, the divergence in the type of samples and diseases associated to the patients used and the different methodologies used to assess the biomarkers, certainly contribute to these discrepancies.

3.4. Statistical analysis

The experimental data obtained was processed with one-way ANOVA ($P_{values} < < 0.05$) for both groups (control and CVDs) using SPSS, version 19.0. Statistically significant differences were obtained between the groups analyzed for 5-HMUra and 8-oxodG. PCA analysis was then applied to the DNA adducts (2 variables and 20 cases-matrix 2×20) and the two main components were extracted. This analysis allowed a good separation of the CVDs and control groups and explains 83.1% of the total variance of the initial set of results. Finally, as can be observed in Fig. 5, separation of 5-HMUra along the first principal component (PC1) using and 8-oxodG in the second main component (PC2), allow a perfect clustering of control and CVDs groups, although a greater dispersion is observed in the patients group. This fact is certainly due to the limit number of samples analyzed and little information available about patient's diet and medication.

4. Conclusions

This work presents a powerful methodology able to simultaneously quantify four urinary OSBs, UAc, MDA and the DNA adducts 5-HMUra and 8-oxodG. The extraction process using an eVol®MEPS semi-automatic syringe is simple and efficient,

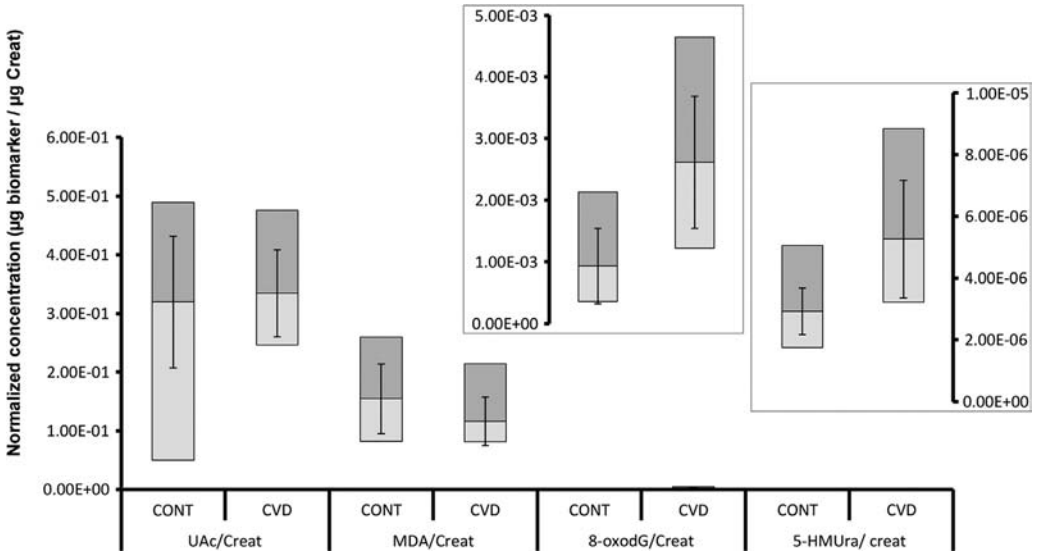

Fig. 4. Modified box plots of the normalized OSB concentrations in normal subjects (CONT) and CVDs patients (CVDs). The references values indicated are the minimum concentration, average concentration, maximum concentration and the standard deviation.

Table 2
Comparison of the results obtained in this study with other reports in the literature.

Biomarkers	Method of analysis	Mean concentrations (μg biomarker/μg Creat)	No. of samples	Clinical condition	Refs.
UAc/Creat	UHPLC-PDA	3.2×10^{-1}	12	Healthy	This study
		3.4×10^{-1}	10	CVD	
	HPLC-PDA	4.8×10^{-1}	3	Healthy	[39]
MDA/Creat	UHPLC-PDA	1.3×10^{-1}	12	Healthy	This study
		$1{,}1 \times 10^{-1}$	10	CVD	
	HPLC-FLR	1.4×10^{-4}	10	Normotensive	[29,43][a]
	HPLC-FLR	1.2×10^{-2}	10	Healthy	[44]
	HPLC-FLR	2.5×10^{-5}	35	Smokers and no-smokers with diabetic nephropathy	[41]
5-HMUra/Creat	UHPLC-PDA	2.9×10^{-6}	12	Healthy	This study
		5.2×10^{-6}	10	CVD	
	GC-NICI/MS	1.1×10^{-5}	21	Smokers and no-smokers	[40]
8-oxodG/Creat	UHPLC-PDA	9.3×10^{-4}	12	Healthy	This study
		2.8×10^{-3}	10	CVD	
	HPLC-MS/MS	7.2×10^{-6}	246	Healthy controls and workers occupationally exposed to diesel exhaust	[19]
	LC–MS/MS	7.37×10^{-9}	20	Patients with type 2 diabetes mellitus	[42]
		8.76×10^{-9}	32	Patients with type 2 diabetes mellitus nephropathy	
	HPLC-EC	4.85×10^{-6}	70	No-smokers	[45]
	HPLC-EC	3.82×10^{-6}	1500	Healthy	[46]
	UHPLC–ESI–MS/MS	2.8×10^{-6}	15	Healthy	[47]

[a] The authors misquoted the data obtained by Agarwal et al. [41].

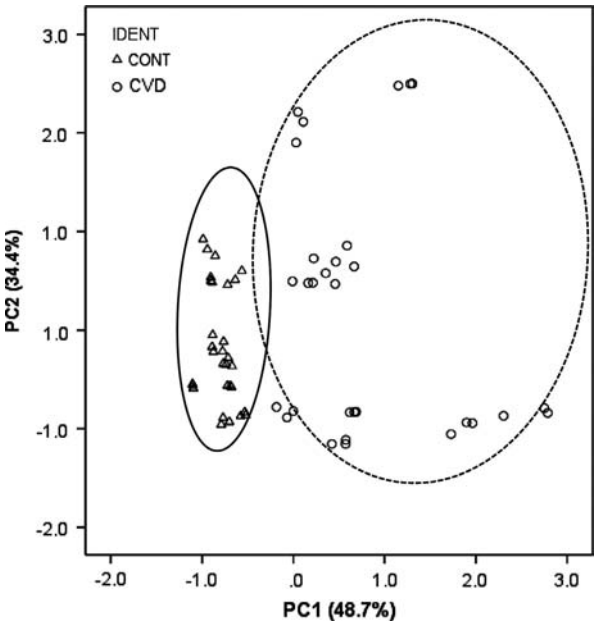

Fig. 5. Principal component analysis (PCA) scores scatter plot for CVDs patients and normal subjects.

allowing excellent recoveries. The following sample analysis with a UHPLC-PDA system was optimized to a high resolution 3.5 min run. Overall, the methodology is very sensitive, fast, reliable and robust, being able to quantify urinary biomarkers differing five orders of magnitude in their concentrations. Moreover, combination of eVol®MEPS using low sample and extracting volumes with a short running time and low mobile phases flows UHPLC analysis is much more environment-friendly than other options available. Finally, the validated method showed a good performance with regard to selectivity, LODs, LOQs, linearity, accuracy, matrix effect and intra/inter-day precisions.

The results obtained from the application of the methodology to different samples, allowed a clearly definition of the CVDs patients group when compared to control samples for the DNA adducts 5-HMUra and 8-oxodG, demonstrating that this is a promising alternative for the fast diagnose of CVDs progression and eventually others diseases in which oxidatively damaged DNA is abnormally present.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.04.064.

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