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Reliable determination of new lipid peroxidation compounds as potential early Alzheimer Disease biomarkers

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ABSTRACT

Lipid peroxidation plays an important role in Alzheimer Disease, so corresponding metabolites found in urine samples could be potential biomarkers. The aim of this work is to develop a reliable ultra-performance liquid chromatography-tandem mass spectrometry analytical method to determine a new set of lipid peroxidation compounds in urine samples.

Excellent sensitivity was achieved with limits of detection between 0.08 and 17 nmol L⁻¹, which renders this method suitable to monitor analytes concentrations in real samples. The method's precision was satisfactory with coefficients of variation around 5–17% (intra-day) and 8–19% (inter-day). The accuracy of the method was assessed by analysis of spiked urine samples obtaining recoveries between 70% and 120% for most of the analytes. The utility of the described method was tested by analyzing urine samples from patients early diagnosed with mild cognitive impairment or mild dementia Alzheimer Disease following the clinical standard criteria. As preliminary results, some analytes (17(RS)–10-*epi*-SC- Δ^{15} –11-dihomo-IsoF, PGE₂) and total parameters (Neuroprostanes, Isoprostanes, Isofurans) show differences between the control and the clinical groups. So, these analytes could be potential early Alzheimer Disease biomarkers assessing the patients' pro-oxidant condition.

1. Introduction

Alzheimer Disease (AD) is characterized by changes in brain, Mild Cognitive Impairment (MCI), and pathological levels of biomarkers (β -amyloid, total tau, and phosphorylated-tau proteins) in cerebrospinal fluid (CSF) samples [1,2]. However, CSF samples are obtained by invasive techniques [3], so the reliable determination of potential AD biomarkers in non-invasive samples is required.

Oxidative stress plays a major role in the pathogenesis of AD [4–7]. Of note, lipid peroxidation is the most promising oxidative stress process, since brain has a high and specific lipid composition [8–10]. The peroxidation by-products from brain lipids include F₂-isoprostanes and isofurans (F₂-IsoP, IsoF), F₄-neuroprostanes and neurofurans (F₄-NeuroP and NeuroF), and F₂-dihomo-isoprostanes and dihomom-isofurans (dihomo-IsoP and dihomom-IsoF) [11]. Preliminary research shows high levels of IsoPs and NeuroPs in AD brain [12], CSF, urine,

and plasma samples [13–16]. Urine is the most non-invasively achievable biofluid that contains lipid peroxidation metabolites [17]. However, its sample treatment shows some difficulties (low concentrations, interferences, glucuronic acid conjugation), so additional sample treatment steps are required [18,19].

The developed analytical methods to determine lipid peroxidation biomarkers in urine samples are mainly based on gas chromatography (GC) coupled to mass spectrometry (MS) [20], but these methods require a tedious and time-consuming derivatization, and they show low capacity to identify different isomers [21]. Also, immunoassays have been employed [22], with the drawbacks of poor selectivity, and results not comparable to chromatographic results [23].

In contrast, the liquid chromatography coupled to mass spectrometry (LC-MS/MS) provides high sensitivity and selectivity [24]. However, few LC methods have been applied to human urine samples [25], and most of them show high retention times, and long chromatograms [26–28].

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The aim of this work was to develop a reliable analytical method to determine lipid peroxidation biomarkers in urine samples. Then, this method was applied to samples from AD patients. For this, we used simple sample treatment and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). To our knowledge, this is the first LC validated method to determine simultaneously 17 lipid peroxidation biomarkers in urine samples, and it could be applied in further clinical studies.

2. Materials and methods

2.1. Participants, urine collection, and storage

The Ethics Committee (CEIC) at the Health Research Institute La Fe (Valencia) approved the study protocol and informed consent was obtained from all the participants. Patients aged between 50 and 75 years old were recruited from the Neurology Unit in the University and Polytechnic Hospital La Fe, Valencia (Spain). Participants were classified into 3 groups (control, MCI-AD, and mild dementia AD) based on psychological testing, structural neuroimaging (NMR-TAC), and CSF biomarkers as shown in Electronic [Supplementary material Table S1](#) [29,30]. The characteristics of participants in this study are summarized in Electronic [Supplementary material Table S2](#). Questionnaires addressing the neuropsychological assessment (Repeatable Battery for Assessment of Neuropsychological Status-RBANS-MR) [31], the functionality (Functionality Assessment Questionnaire – FAQ) [32] and the clinical dementia (Clinical Dementia Rating-CDR) [33] were conducted to all participants, and urine samples were collected in sterile pots with screw caps. Thereafter, urine samples were aliquoted in tubes and stored at -80°C until analysis.

2.2. Reagents

Isoprostanes' standards of 15(R)–15-F_{2t}-IsoP, 2,3-dinor-15-*epi*–15-F_{2t}-IsoP, 5-F_{2t}-IsoP, 15-keto-15-E_{2t}-IsoP, 15-keto-15-F_{2t}-IsoP, 15-E_{2t}-IsoP, 15-F_{2t}-IsoP, the prostaglandins PGE₂ and PGF_{2α}, as well as the deuterated internal standard (IS) PGF_{2α}-d₄ were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). The standards of 7(RS)-ST-Δ⁸-11-dihomo-IsoF, 10-*epi*–10-F_{4t}-NeuroP, d₄-10-*epi*–10-F_{4t}-NeuroP, 4(RS)-F_{4t}-NeuroP, 17-*epi*–17-F_{2t}-dihomo-IsoP, 17-F_{2t}-dihomo-IsoP, 17(RS)–10-*epi*-SC-Δ¹⁵–11-dihomo-IsoF, Ent-7(RS)–7-F_{2t}-dihomo-IsoP, and 14(RS)–14-F_{4t}-NeuroP were synthesized by Durand's team at the Institute des Biomolécules Max Mousseron (IBMM) (Montpellier, France) (Fig. S1 in Electronic [Supplementary material](#)) [34]. Methanol (LC-MS grade) and n-heptane (analytical grade) were obtained from J.T. Baker (Avantor Performance Materials B.V., The Netherlands). Chlorhydric acid and acetic acid glacial (99.5%) were purchased from Panreac (Castellar del Vallés, Barcelona, Spain). Ammonium acetate, sodium hydroxide, and β-glucuronidase (type H2, from Helix pomatia) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Creatinine assay (MicroVue creatinine EIA) from Quidel Corporation (San Diego, CA, USA).

2.3. Solutions

Individual stock solutions of 15-keto-15-E_{2t}-IsoP, 15-keto-15-F_{2t}-IsoP, PGE₂, 15-E_{2t}-IsoP, 15-F_{2t}-IsoP, PGF_{2α}, PGF_{2α}-d₄, 7(RS)-ST-Δ⁸-11-dihomo-IsoF, 5-F_{2t}-IsoP, 10-*epi*–10-F_{4t}-NeuroP, d₄-10-*epi*–10-F_{4t}-NeuroP, 4(RS)-F_{4t}-NeuroP, 17-*epi*–17-F_{2t}-dihomo-IsoP, 17-F_{2t}-dihomo-IsoP, 17(RS)–10-*epi*-SC-Δ¹⁵–11-dihomo-IsoF, Ent-7(RS)–7-F_{2t}-dihomo-IsoP, and 14(RS)–14-F_{4t}-NeuroP standards at a concentration of 1 mmol L⁻¹ were prepared in H₂O (0.01% v/v CH₃COOH, pH 3):CH₃OH (85:15 v/v) and kept at -20°C . 15(R)–15-F_{2t}-IsoP and 2,3-dinor-15-*epi*–15-F_{2t}-IsoP standards were dissolved in methyl acetate or ethanol, at a concentration of 2800 or 300 μmol L⁻¹, respectively, and

used as stock solutions. A 2.5 μmol L⁻¹ working solution of the set of analytes was prepared in H₂O (0.01% v/v CH₃COOH, pH 3):CH₃OH (85:15 v/v) by mixing appropriate volumes of the above mentioned individual solutions, and kept at -20°C . The calibration curves were prepared daily by serial dilution in H₂O (pH 3):CH₃OH (85:15 v/v) at concentrations ranging from 300 nmol L⁻¹ to 0.004 nmol L⁻¹ of each analyte. Solutions of ammonium acetate (100 mmol L⁻¹, pH7), sodium acetate (100 mmol L⁻¹, pH 4.9), and sodium hydroxide (2.5 mol L⁻¹) were prepared.

2.4. Materials

The Strata X-AW (100 mg, 3 mL) solid phase extraction cartridges from Phenomenex (Torrance, CA, USA) were used for sample solid-phase extraction (SPE). Vortex mixer was from Velp Scientifica (Usmate, Italy). Centrifuge Biocen22R was from OrtoAlresa (Madrid, Spain). Thermomixer HLC from Ditas (Pforzheim, Germany). Speed vacuum concentrator (mi Vac) was from Genevac LTD (Ipswich, United Kingdom). SPE 12-position vacuum manifold from Phenomenex (Madrid, Spain). 96-well sample plates (AcquityUPLC 700 μL) were from Waters (Barcelona, Spain). Microplate reader for 96 well plates (Halo LED 96) was from Dynamica Scientific Ltd. (London, United Kingdom).

2.5. UPLC-MS/MS chromatographic system

The chromatographic system used consisted of a Waters Acquity UPLC-Xevo TQD system (Milford, MA, USA). The conditions employed were: negative electrospray ionization (ESI), capillary voltage 3.5 kV, source temperature 120 °C, desolvation temperature 300 °C, nitrogen cone, and desolvation gas flows were 25 and 680 L h⁻¹, respectively. Dwell time was set to 5 ms to ensure a minimum of 10 data points per peak. Separation conditions were selected to achieve appropriate chromatographic retention and resolution by using an Acquity UPLC BEH C₁₈ column (2.1 × 50 mm, 1.7 μm) and pre-column (2.1 × 5 mm) from Waters. A binary mobile phase CH₃OH (0.01% v/v CH₃COOH):H₂O (0.01% v/v CH₃COOH, pH 3) with gradient elution was used. The flow rate was 0.4 mL min⁻¹, the column temperature was 37 °C and the injection volume was 10 μL. The gradient started with 30% v/v CH₃OH (0.01% v/v CH₃COOH) (i.e. channel B) and from 1 to 4.0 min %B increased up to 90%. Return to initial conditions was achieved at 4.1 min and conditions were maintained for 3.9 min for system re-equilibration.

Mass spectrometric detection was carried out by multiple reaction monitoring (MRM). The MS/MS conditions for the detection of the prostaglandins (PGE₂, PGF_{2α}), isoprostanes (2,3-dinor-15-*epi*–15-F_{2t}-IsoP, 5-F_{2t}-IsoP, 15-keto-15-E_{2t}-IsoP, 15-keto-15-F_{2t}-IsoP, 15-E_{2t}-IsoP, 15-F_{2t}-IsoP, 15(R)-15-F_{2t}-IsoP), the deuterated internal standard (PGF_{2α}-d₄), and total IsoPs, IsoFs, NeuroPs and NeuroFs were obtained from previous works employing the same equipment [35,36]. The MS/MS conditions for the detection of 7(RS)-ST-Δ⁸-11-dihomo-IsoF, 10-*epi*–10-F_{4t}-NeuroP, d₄-10-*epi*–10-F_{4t}-NeuroP, 17-*epi*–17-F_{2t}-dihomo-IsoP, 17-F_{2t}-dihomo-IsoP, 17(RS)–10-*epi*-SC-Δ¹⁵–11-dihomo-IsoF, Ent-7(RS)–7-F_{2t}-dihomo-IsoP, 14(RS)–14-F_{4t}-NeuroP, and 4(RS)-F_{4t}-NeuroP were established from individual 5 μmol L⁻¹ standard solutions by direct infusion in the ESI-MS/MS system. The corresponding MS/MS spectra for these new analytes are shown in Electronic [Supplementary material Fig. S2](#), and the characteristic fragmentations for all the analytes are shown in Electronic [Supplementary material Table S3](#).

The MS/MS acquisition parameters are summarized in [Table 1](#). As can be seen, two MRM transitions per analyte were acquired for quantification and confirmation of 15-keto-15-E_{2t}-IsoP, 15-keto-15-F_{2t}-IsoP, PGE₂, 15-F_{2t}-IsoP, PGF_{2α}, 17-*epi*–17-F_{2t}-dihomo-IsoP, and 2,3-dinor-15-*epi*–15-F_{2t}-IsoP. A single MRM transition was used for 15(R)–15-F_{2t}-IsoP, 15-E_{2t}-IsoP, 5-F_{2t}-IsoP, 7(RS)-ST-Δ⁸-11-dihomo-IsoF, 10-*epi*–10-F_{4t}-NeuroP, d₄-10-*epi*–10-F_{4t}-NeuroP, PGF_{2α}-d₄, 17-

Table 1
Detection parameters.

| Analyte | <i>m/z</i> Parent Ion | Cone [V] | <i>m/z</i> Daughter Ion | | | |
|--|-----------------------|----------|-------------------------|---------|--------------|---------|
| | | | Quantification | CE [eV] | Confirmation | CE [eV] |
| 15(R) – 15-F _{2t} -IsoP | 353 | 45 | 193 | 25 | – | – |
| 2,3-dinor – 15- <i>epi</i> – 15-F _{2t} -IsoP | 325 | 45 | 237 | 10 | 137 | 10 |
| 15-keto – 15-E _{2t} -IsoP | 349 | 45 | 113 | 20 | 161 | 20 |
| 15-keto – 15-F _{2t} -IsoP | 351 | 40 | 315 | 15 | 289 | 15 |
| PGE ₂ | 351 | 40 | 271 | 10 | 189 | 10 |
| 15-E _{2t} -IsoP | 351 | 40 | 189 | 15 | – | – |
| 15-F _{2t} -IsoP | 353 | 45 | 193 | 25 | 165 | 25 |
| PGF _{2α} | 353 | 45 | 193 | 25 | 165 | 25 |
| PGF _{2α} -d ₄ | 357.5 | 45 | 197.3 | 30 | – | – |
| 7(RS)-ST-Δ ⁸ -11-dihomo-IsoF | 397 | 30 | 201 | 20 | – | – |
| 5-F _{2t} -IsoP | 353 | 40 | 115 | 23 | – | – |
| 10- <i>epi</i> – 10-F _{4t} -NeuroP | 377 | 55 | 152.9 | 15 | – | – |
| d ₄ -10- <i>epi</i> – 10-F _{4t} -NeuroP | 381.4 | 55 | 157.2 | 15 | – | – |
| 17- <i>epi</i> – 17-F _{2t} -dihomo-IsoP | 381 | 45 | 221 | 25 | 245 | 25 |
| 17-F _{2t} -dihomo-IsoP | 381 | 45 | 363 | 10 | – | – |
| 17(RS) – 10- <i>epi</i> -SC-Δ ¹⁵ – 11-dihomo-IsoF | 397.4 | 30 | 155 | 22 | – | – |
| Ent-7(RS) – 7-F _{2t} -dihomo-IsoP | 381.6 | 50 | 193.1 | 35 | – | – |
| 14(RS) – 14-F _{4t} -NeuroP | 377 | 50 | 150.8 | 15 | – | – |
| 4(RS)-F _{4t} -NeuroP | 377 | 50 | 271 | 20 | – | – |
| NeuroPs | 377 | 35 | 101 | 20 | – | – |
| IsoPs | 353.2 | 40 | 115 | 20 | – | – |
| NeuroFs | 393 | 35 | 193 | 20 | – | – |
| IsoFs | 369.2 | 45 | 115 | 20 | – | – |

F_{2t}-dihomo-IsoP, 17(RS)-10-*epi*-SC-Δ¹⁵ – 11-dihomo-IsoF, Ent-7(RS) – 7-F_{2t}-dihomo-IsoP, 14(RS) – 14-F_{4t}-NeuroP, and 4(RS)-F_{4t}-NeuroP due to the lack of suitable additional fragmentation.

2.6. Sample treatment

Urine samples were thawed on ice and homogenized, and 5 μL of internal standard (IS) solution (PGF_{2α}-d₄ 10 μmol L⁻¹ and d₄-10-*epi* – 10-F_{4t}-NeuroP 6 μmol L⁻¹) were added to 1 mL of sample. In order to remove the glucuronide and sulfate derivatives, the samples were enzymatically hydrolyzed by adding ~5000 UE mL⁻¹ of β-glucuronidase and sodium acetate buffer (250 μL, 100 mmol L⁻¹, pH 4.9) for 2 h at 37 °C [19]. The reactions were stopped and the enzyme was precipitated with 500 μL of MeOH and 8 μL of HCl (37%, v/v), then the mixture was centrifuged at 14,000g and 4 °C for 10 min.

Thereafter, the supernatants were adjusted at pH 6–7 with sodium hydroxide (2.5 mol L⁻¹) and homogenized. For cleanup and pre-concentration, solid phase extraction (SPE) was carried out. SPE cartridges were conditioned first with 1 mL CH₃OH and then with 1 mL H₂O (pH 6–7). The diluted sample (2 mL) was loaded in the SPE cartridge and it was washed with 1 mL of ammonium acetate (100 mmol L⁻¹, pH 7) and 1 mL of heptane. Elution was carried out with 2 × 500 μL methanol (5% v/v acetic acid). Recovered extracts were evaporated and reconstituted in 100 μL of H₂O (pH 3):CH₃OH (85:15 v/v). Finally, they were injected in the chromatographic system (UPLC-MS/MS).

2.7. Method validation

The bioanalytical method validation followed the US Food and Drug Administration (FDA) guidelines [37]. The analytical characteristics assayed during the validation procedure were linearity range, precision, accuracy, selectivity, limit of detection (LOD), limit of quantification (LOQ), carry-over, as well as the stability of analytes. The accuracy was evaluated by means of the recovery test. Urine samples were spiked at three concentration levels, and they were analyzed each validation day (n = 6). Also, the non-spiked samples were analyzed each validation day in order to quantify the recoveries. The selectivity was studied by analyzing blanks and non-spiked samples with and without internal standards. The precision was estimated from the analysis of standards

and spiked samples at three concentration levels (i.e. low, mid, and high) within one validation batch (intra-day) and among validation batches (inter-day). The LOD and LOQ were established, as the concentrations required generating a signal-to-noise ratio of 3 and 10, respectively. The carry-over was evaluated by the injection of a blank sample after a high concentration sample or standard. Analyte stability in spiked urine samples after three freeze-thaw cycles and the short-term stability (24 h at 4 °C) were assessed by comparison with freshly prepared standard solutions.

2.8. Analysis of urine samples

Morning urine samples (n = 18) were collected as described above, and kept at –80 °C until analysis. The previously described analytical method was applied to these samples. To standardize the results, urinary levels of creatinine were determined using a colorimetric assay kit (MicroVue creatinine EIA) and measuring in a microplate spectrophotometer. In this way, the suitability of the developed method for this kind of samples was assayed, and a first approach to the lipid peroxidation compounds concentrations that can be found in urine samples from patients with MCI-AD and mild dementia-AD was established.

2.9. Statistical analysis

Data analysis was carried out using the Unscrambler 7.6 (Camo, Woodbridge, USA) software. A multivariable regression model based on the oxidative stress biomarkers levels is described to discriminate among healthy, MCI-AD, and mild dementia-AD participants. It consists of partial least squares (PLS). The PLS model was constructed with 21 independent variables (predictors), and 1 dependent variable (response). All the variables were centered and predictors were also auto-scaled. The cross validation was used to validate the model. The predictive ability of the model is expressed by the percentage of validation explained variance.

Univariate statistical analyses were performed using SPSS version 20.0 software (SPSS, Inc., Chicago, IL, USA). For all analyses, the statistical significance was set at *p* < 0.05. Data were presented as mean ± standard deviation (SD), median (inter-quartile range, IQR) or number of cases (n) and percentage (%). Kolmogorov-Smirnov test was

used to test for normal distribution of the data. For comparison of some biomarkers concentrations, nonparametric methods were used. Kruskal-Wallis tests were performed to compare medians among 3 groups, and Mann Whitney tests to compare medians between 2 groups.

3. Results and discussion

3.1. Sample treatment optimization

Several parameters involved in the urine samples treatment to determine the selected lipid peroxidation biomarkers were optimized. First, internal standards were selected taking into account their physical-chemical characteristics (retention time, polarity, structure). Among the different available deuterated compounds, $\text{PGF}_{2\alpha}\text{-d}_4$ and $\text{d}_4\text{-10-epi-10-F}_{4t}\text{-NeuroP}$, accomplished the similarity requirements compared to most of the analytes ($\text{F}_2\text{-IsoPs}$, and $\text{F}_4\text{-NeuroPs}$, respectively), and they were selected for further work. They were added in the first step of sample treatment procedure in order to minimize all the experimental errors.

Secondly, several types of SPE cartridges were assayed to carry out urine sample clean-up and analytes preconcentration. They were based on reversed phase (C18-encapped, C18-not encapped, polymeric) and weak anion exchange and reversed phase (NH_2 , polymeric). The results showed that a mix mode SPE based on weak anion exchange and reversed phase provided better recoveries with a 10-factor preconcentration (data not shown).

Thirdly, some SPE conditions (sample pH, clean-up solvents, elution solvent, and elution volume) were assayed to obtain higher recoveries. The optimum conditions selected were sample at pH 7, a two-step cartridge clean-up with ammonium acetate solution and heptane, and an elution step with 1 mL of methanol containing acetic acid at 5% v/v.

3.2. Analytical performance data

The reliability of the described method was evaluated by employing standards that contained the analytes in the $0.03\text{--}300\text{ nmol L}^{-1}$ concentration range. As shown in Electronic Supplementary material Table S4, the method provided an adequate linearity for all the analytes within the corresponding concentrations ranges, and R^2 ranged between 0.98 and 0.993. The method also provided suitable precision, with intra-day and inter-day coefficients of variation of 5–17% ($n = 5$) and 8–19% ($n = 6$), respectively (at medium concentration level within the linearity interval). The higher irreproducibility corresponded to compounds with lower sensitivity ($14(\text{RS})\text{-14-F}_{4t}\text{-NeuroP}$, $\text{Ent-7}(\text{RS})\text{-7-F}_{2t}\text{-dihomo-IsoP}$) or poor chromatographic resolution ($17\text{-epi-17-F}_{2t}\text{-dihomo-IsoP}$, PGE_2). Also in Table S4 (Electronic Supplementary material), we can see the instrumental LODs obtained for each analyte, they were between 0.08 and 17 nmol L^{-1} . LODs obtained were suitable to detect these analytes in urine human samples, and similar to those previously reported in literature [17,27]. The LOQs were between 0.15 and 35 nmol L^{-1} .

The accuracy of the method was evaluated by analyzing standard solutions containing analytes at different concentrations within the tested concentration ranges. The results obtained are listed at Table 2. In all instances, the proposed method provided values close to the real concentrations. In general, the lowest concentrations showed higher irreproducibility. In addition, quantitative recoveries for urine samples spiked at different concentration levels (low, medium and high) were achieved for most of the analytes (Table 2), only a few of them ($15\text{-E}_{2t}\text{-IsoP}$, $17\text{-F}_{2t}\text{-dihomo-IsoP}$, $17(\text{RS})\text{-10-epi-SC-}\Delta^{15}\text{-11-dihomo-IsoF}$, $7(\text{RS})\text{-ST-}\Delta^8\text{-11-dihomo-IsoF}$) showed recoveries below 80% or above 120%. Matrix effect was evaluated by using a linear regression model to compare the concentrations obtained for each analyte in spiked urine samples using the proposed method (Y) with the added concentrations (X). For all the analytes, slopes were statistically comparable to 1, so matrix effect was not observed at 5% significance level in any analyte.

Carry over in the blank sample following the high concentration standard was lower than 10% of the lower limit of quantification.

As regards total parameters (IsoPs, IsoFs, NeuroPs, NeuroFs), relative contents were determined in urine samples from participants.

3.3. Analyte stability study

The analytes' stability was assayed at long-term (after one, two, or three freeze-thaw cycles) and at short-term (24 h at 4°C). This study was carried out determining the concentrations in a spiked urine sample (at medium concentration level in the interval concentrations) by triplicate. Then, these concentrations were compared to freshly prepared standard solutions. As shown in Electronic Supplementary material Table S5 the recoveries were between 72% and 130%. Hence, no significant deterioration of the analytes was observed.

3.4. Application to urine samples

The reliability of the validated method was tested analyzing 18 urine samples by triplicate. These samples were from healthy, MCI-AD, and mild dementia-AD patients. The analytes $15(\text{R})\text{-15-F}_{2t}\text{-IsoP}$, $5\text{-F}_{2t}\text{-IsoP}$, $17\text{-F}_{2t}\text{-dihomo-IsoP}$, $\text{PGF}_{2\alpha}$, 2,3-dinor-15-epi-15- $\text{F}_{2t}\text{-IsoP}$, and $4(\text{RS})\text{-F}_{4t}\text{-NeuroP}$ were detected in all the samples, indicating the satisfactory sensitivity of the developed method to analyze real samples. However, in a previous work, only $\text{F}_{2t}\text{-dihomo-IsoPs}$ were detected in urine samples from healthy elderly people, reflecting not enough sensitivity to detect $\text{F}_4\text{-NeuroPs}$ [38]. Also, total parameters were found above the LOD (a signal-to-noise ratio of 3), showing changes in the relative levels of IsoPs, IsoFs, NeuroPs, and NeuroFs among the analyzed samples. The values are listed in Table 3. Chromatograms obtained for some analytes ($15(\text{R})\text{-15-F}_{2t}\text{-IsoP}$, $\text{PGF}_{2\alpha}$, 2,3-dinor-15-epi-15- $\text{F}_{2t}\text{-IsoP}$, PGE_2) and total parameters (neuroprostanes, isoprostanes) from the analysis of participants urine samples are shown in Fig. 1.

3.5. Statistical analysis

Due to the low number of samples analyzed, the PLS model allows us to carry out a preliminary study of correlations between predictor variables (biomarkers levels) and response variable (control, MCI-AD, mild dementia-AD), as well as an approximate classification of participants. This model was considered satisfactory in terms of predictive ability.

In this model, 21 independent variables (17 individual biomarkers, 4 total parameters) are spatially distributed in order to improve the separation among the 3 groups of participants (0 = control, 1 = MCI-AD, 2 = mild-dementia-AD). Fig. S3 in Electronic Supplementary material shows that explained variance was around 80% with 3 principal components (PC). Therefore, the maximum number of latent variables selected was 3. In the loadings plot (Fig. S4a in Electronic Supplementary material) we observed that the PC1 correlated with the response variable (group), as well as with 15-keto-15- $\text{F}_{2t}\text{-IsoP}$, 2,3-dinor-15-epi-15- $\text{F}_{2t}\text{-IsoP}$, 15- $\text{F}_{2t}\text{-IsoP}$ and 15- $\text{E}_{2t}\text{-IsoP}$. This suggested that these variables varied together and increased with the PC1. However, an inverse correlation was observed along PC1 between the response variable (group) and some biomarkers ($10\text{-epi-10-F}_{4t}\text{-NeuroP}$, $17(\text{RS})\text{-10-epi-SC-}\Delta_{15}\text{-11-dihomo-IsoF}$, PGE_2) and total parameters (IsoF, IsoP, NeuroP). Therefore, this component might be considered a measure of the AD diagnostic. As regards the scores plot obtained from PC2 vs PC1, a separation of the 3 participants groups was achieved (Fig. S4b in Electronic Supplementary material). Specifically, healthy and mild dementia-AD participants could be classified correctly, but the MCI-AD group separation was less evident.

In order to confirm previous results in multivariable analysis, univariate statistical analysis was carried out. For this, nonparametric methods were used as the variables were not normally distributed. A

Table 2

Accuracy and precision of standard solutions and recoveries obtained from the analysis of spiked urine samples at different concentration levels.

| Analytes | Standard Solutions | | | Spiked urine samples | | |
|--|---|----------------------|----------------------|---|----------------------|-------------------|
| | Standard concentration (nmol L ⁻¹) | [Recovery ± s (%)] | | Sample added concentration (nmol L ⁻¹) | [Recovery ± s (%)] | |
| | | Intra-day (n = 5) | Inter-day (n = 6) | | Intra-day (n = 3) | Inter-day (n = 6) |
| 2,3-dinor – 15- <i>epi</i> – 15-F _{2t} -IsoP | 18.75 | 108 ± 20 | 120 ± 20 | 1.87 | 110 ± 17 | 120 ± 10 |
| | 75 | 105 ± 8 | 99 ± 12 | 7.5 | 92 ± 19 | 112 ± 7 |
| | 150 | 97 ± 19 | 104 ± 20 | 15 | 102 ± 3 | 115 ± 5 |
| 15-keto – 15-F _{2t} -IsoP | 18.75 | 120 ± 5 | 92 ± 8 | 1.87 | 96 ± 11 | 112 ± 14 |
| | 75 | 87 ± 7 | 83 ± 16 | 7.5 | 121 ± 5 | 118 ± 19 |
| | 150 | 95 ± 14 | 120 ± 10 | 15 | 86 ± 5 | 118 ± 17 |
| 15-keto – 15-E _{2t} -IsoP | 18.75 | 115 ± 12 | 117 ± 20 | 1.87 | 109 ± 9 | 109 ± 19 |
| | 75 | 122 ± 8 | 83 ± 14 | 7.5 | 115 ± 9 | 121 ± 12 |
| | 150 | 102 ± 8 | 108 ± 13 | 15 | 102 ± 12 | 95 ± 13 |
| 15(R) – 15-F _{2t} -IsoP | 18.75 | 107 ± 18 | 106 ± 15 | 1.87 | 120 ± 20 | 109 ± 20 |
| | 75 | 97 ± 7 | 94 ± 8 | 7.5 | 85 ± 20 | 87 ± 19 |
| | 150 | 83 ± 3 | 92 ± 20 | 15 | 104 ± 9 | 102 ± 18 |
| PGE ₂ | 18.75 | 111 ± 19 | 112 ± 20 | 1.87 | 119 ± 9 | 120 ± 20 |
| | 75 | 91 ± 16 | 90 ± 16 | 7.5 | 95 ± 20 | 100 ± 20 |
| | 150 | 88 ± 10 | 100 ± 20 | 15 | 101 ± 13 | 110 ± 20 |
| 15-E _{2t} -IsoP | 18.75 | 115 ± 19 | 100 ± 20 | 1.87 | 106 ± 20 | 95 ± 20 |
| | 75 | 109 ± 11 | 100 ± 19 | 7.5 | 70 ± 12 | 69 ± 14 |
| | 150 | 108 ± 7 | 111 ± 7 | 15 | 79 ± 7 | 77 ± 6 |
| 15-F _{2t} -IsoP | 18.75 | 80 ± 18 | 84 ± 19 | 1.87 | 90 ± 20 | 100 ± 20 |
| | 75 | 94 ± 11 | 96 ± 13 | 7.5 | 79 ± 9 | 88 ± 12 |
| | 150 | 121 ± 5 | 120 ± 12 | 15 | 108 ± 13 | 114 ± 17 |
| PGF _{2α} | 18.75 | 120 ± 10 | 111 ± 11 | 1.87 | 111 ± 18 | 107 ± 6 |
| | 75 | 102 ± 7 | 96 ± 15 | 7.5 | 80 ± 10 | 105 ± 20 |
| | 150 | 97 ± 19 | 101 ± 17 | 15 | 111 ± 9 | 120 ± 17 |
| 4(RS)-F _{4t} -NeuroP | 17.7 | 112 ± 18 | 102 ± 20 | 1.77 | 104 ± 14 | 89 ± 19 |
| | 71 | 95 ± 5 | 94 ± 17 | 7.09 | 98 ± 4 | 102 ± 12 |
| | 141.9 | 109 ± 16 | 99 ± 18 | 14.19 | 92 ± 16 | 94 ± 13 |
| 7(RS)-ST-Δ ⁸ -11-dihomo-IsoF | 16.9 | 81 ± 11 | 84 ± 20 | 1.69 | 119 ± 5 | 122 ± 9 |
| | 67.6 | 120 ± 15 | 101 ± 19 | 6.76 | 78 ± 10 | 75 ± 15 |
| | 135.2 | 98 ± 13 | 96 ± 11 | 13.52 | 74 ± 7 | 75 ± 11 |
| 5-F _{2t} -IsoP | 18.8 | 119 ± 20 | 115 ± 13 | 1.88 | 79 ± 4 | 80 ± 10 |
| | 75.2 | 106 ± 5 | 115 ± 20 | 7.52 | 115 ± 5 | 117 ± 9 |
| | 150.3 | 87 ± 8 | 85 ± 19 | 15.03 | 95 ± 12 | 110 ± 20 |
| 10- <i>epi</i> – 10-F _{4t} -NeuroP | 17.7 | 104 ± 11 | 97 ± 18 | 1.77 | 123 ± 12 | 130 ± 20 |
| | 70.9 | 89 ± 8 | 96 ± 9 | 7.09 | 120 ± 19 | 116 ± 20 |
| | 141.9 | 97 ± 3 | 102 ± 7 | 14.19 | 120 ± 14 | 123 ± 19 |
| 17- <i>epi</i> – 17-F _{2t} -dihomo-IsoP | 17.6 | 99 ± 20 | 80 ± 16 | 1.76 | 100 ± 10 | 110 ± 4 |
| | 70.3 | 77 ± 13 | 77 ± 14 | 7.03 | 80 ± 20 | 70 ± 5 |
| | 140.7 | 115 ± 10 | 105 ± 19 | 14.07 | 85 ± 14 | 71 ± 6 |
| 17-F _{2t} -dihomo-IsoP | 17.5 | 119 ± 8 | 111 ± 20 | 1.75 | 130 ± 11 | 130 ± 16 |
| | 70.0 | 113 ± 8 | 98 ± 10 | 7.00 | 81 ± 5 | 96 ± 18 |
| | 140.1 | 108 ± 10 | 108 ± 10 | 14.01 | 107 ± 12 | 108 ± 15 |
| 17(RS) – 10- <i>epi</i> -SC-Δ ¹⁵ – 11-dihomo-IsoF | 16.9 | 77 ± 6 | 74 ± 20 | 1.69 | 120 ± 30 | 130 ± 30 |
| | 67.6 | 84 ± 8 | 75 ± 8 | 6.76 | 101 ± 20 | 101 ± 20 |
| | 135.3 | 98 ± 6 | 92 ± 13 | 13.52 | 130 ± 4 | 132 ± 19 |
| Ent – 7(RS) – 7-F _{2t} -dihomo-IsoP | 17.6 | – | – | 1.76 | – | – |
| | 70.3 | 118 ± 12 | 106 ± 19 | 7.03 | 113 ± 20 | 78 ± 14 |
| | 140.7 | 120 ± 19 | 119 ± 20 | 14.07 | 114 ± 4 | 104 ± 18 |
| 14(RS) – 14-F _{4t} -NeuroP | 17.7 | – | – | 1.77 | – | – |
| | 70.9 | 111 ± 14 | 105 ± 19 | 7.09 | 86 ± 4 | 78 ± 12 |
| | 141.9 | 118 ± 20 | 110 ± 19 | 14.19 | 108 ± 10 | 96 ± 20 |

comparison of each analyte among groups using Kruskal-Wallis test revealed that the medians for NeuroP ($p = 0.017$), IsoP ($p = 0.010$), IsoF ($p = 0.013$), and PGE₂ ($p = 0.029$) were significantly different among groups (Table 3). Also, the comparison of each analyte between each two groups of participants using Mann Whitney test indicated that medians were different between control and MCI-AD groups for PGE₂ and NeuroP, and between control and mild dementia-AD groups for NeuroP, IsoP, and IsoF. Therefore, PGE₂ and NeuroP can be considered potential AD biomarkers at MCI phase, while IsoP and IsoF can be potential AD biomarkers at mild dementia phase. These differences can be explained by the different origin of these molecules. In fact, PGE₂ is generated by the action of cyclooxygenase enzymes and its function is related to inflammation [39], while the other compounds derived from lipid peroxidation by free radicals. Specifically, IsoP and IsoF derived

from arachidonic acid, and NeuroP derived from docosahexaenoic acid (abundant in brain grey matter) [11]. No statistically significant differences were obtained for the other compounds. In Fig. 2 we can see that for some biomarkers obtained by free-radical mediated oxidation (NeuroP, IsoP, IsoF, 17(RS) – 10-*epi*-SC-Δ¹⁵ – 11-dihomo-IsoF) their levels decreased along the disease as it was observed in the multivariable regression model. It could be explained by the temporary nature of some oxidized metabolites, which continue their oxidation towards other compounds of lower molecular weight along the disease development [40]. Another explanation could be the overexpression of antioxidant enzymes in patients at early AD stages, it would be corroborated by previous studies showing that individuals at risk of AD may suffer reductive stress [41].

Table 3
Results obtained from the analysis of urine samples (n = 18).

| Analyte | Concentrations found (ng mg ⁻¹ creatinine) (median (Q1-Q3)) | | | Kruskal-Wallis Test χ^2 (d.f.), p-value | Mann-Whitney Test p-value |
|--|--|---------------------|--------------------------|---|--|
| | Control (n = 5) | MCI-AD (n = 5) | Mild dementia-AD (n = 8) | | |
| 15(R) – 15-F _{2t} -IsoP | 0.39 (0.28–0.95) | 0.39 (0.23–0.66) | 0.25 (0.11–0.54) | 1.48 (2), 0.476 | 0.841 ^a 0.284 ^b 0.435 ^c |
| 2,3-dinor – 15-epi – 15-F _{2t} -IsoP | 2.23 (1.77–6.03) | 2.44 (0.55–3.21) | 2.41 (1.33–4.0) | 0.166 (2), 0.920 | 0.841 ^a |
| 15-keto – 15-E _{2t} -IsoP | 0.35 (0–0.95) | 1.38 (0.59–7.19) | 1.26 (0.30–2.42) | 3.22 (2), 0.200 | 0.943 ^v 0.833 ^c 0.151 ^a 0.171 ^b 0.622 ^c |
| PGE ₂ | 2.03 (1.78–5.11) | 0.16 (0.06–0.61) | 1.58 (0.14–2.68) | 7.10 (2), 0.029 [*] | 0.008 ^{b,*} 0.171 ^b 0.171 ^c |
| 15-E _{2t} -IsoP | 0.10 (0.03–0.29) | 0.28 (0.08–0.61) | 0.11 (0.02–0.50) | 5 (2), 0.079 | 0.310 ^a 0.622 ^b 0.524 ^c |
| 15-F _{2t} -IsoP | 0.02 (0–0.03) | 0.00 (0–0.01) | 0.005 (0–0.017) | 2.07 (2), 0.355 | 0.222 ^a 0.524 ^b 0.435 ^c |
| PGF _{2α} | 3.85 (3.18–5.36) | 4.03 (2.08–5.05) | 2.97 (1.51–3.80) | 2.65 (2), 0.266 | 0.841 ^a 0.171 ^b 0.284 ^c |
| 4(RS)-F _{4t} -NeuroP | 0.53 (0.33–0.77) | 0.44 (0.12–1.96) | 0.29 (0.05–1.05) | 1.69 (2), 0.429 | 0.548 ^a 0.284 ^b 0.524 ^c |
| 7(RS)-ST- Δ^8 -11-dihomo-IsoF | 0.72 (0.33–0.90) | 0.03 (0.01–0.56) | 0.20 (0.03–0.57) | 2.44 (2), 0.296 | 0.222 ^a 0.222 ^b 0.833 ^c |
| 5-F _{2t} -IsoP | 0.61 (0.27–1.48) | 1.21 (0.45–3.33) | 0.62 (0.08–1.08) | 2.02 (2), 0.363 | 0.421 ^a 0.622 ^b 0.222 ^c |
| 10-epi – 10-F _{4t} -NeuroP | 0.49 (0.16–1.01) | 0.45 (0.19–0.62) | 0.14 (0–0.32) | 4.16 (2), 0.125 | 0.690 ^a 0.093 ^b 0.127 ^c |
| 17-epi – 17-F _{2t} -dihomo-IsoP | 0.00 (0–0.99) | 0.80 (0–0.87) | 0.38 (0.04–0.50) | 0.093 (2), 0.955 | 1.0 ^a 0.724 ^b 0.724 ^c |
| 17-F _{2t} -dihomo-IsoP | 1.31 (0.71–2.18) | 0.53 (0.35–1.44) | 0.70 (0.34–1.38) | 2.08 (2), 0.353 | 0.222 ^a 0.284 ^b 0.943 ^c |
| 17(RS) – 10-epi-SC- Δ^{15} – 11-dihomo-IsoF | 1.70 (1.01–2.12) | 0.63 (0.08–1.15) | 0.54 (0.25–0.86) | 4.51 (2), 0.105 | 0.056 ^a 0.093 ^b 0.943 ^c |
| Ent – 7(RS) – 7-F _{2t} -dihomo-IsoP | 0.28 (0.05–0.46) | 0.24 (0–0.84) | 0.04 (0–0.10) | 1.68 (2), 0.433 | 1.0 ^a 0.222 ^b 0.524 ^c |
| 14(RS) – 14-F _{4t} -NeuroP | 0.0 (0–3.16) | 2.05 (0–13.5) | 0.0 (0–0.44) | 1.87 (2), 0.392 | 0.421 ^a 0.833 ^b 0.284 ^c |
| NeuroPs ^d | 37.0 (23.4–54.5) | 10.25 (8.11–15.11) | 4.96 (2.31–14.0) | 8.09 (2), 0.017 [*] | 0.016 ^{a,*} 0.019 ^{b,*} 0.222 ^c |
| IsoPs ^d | 161.5 (139–174.4) | 106.9 (71.9–262.6) | 66.4 (59.4–84.0) | 9.28 (2), 0.010 [*] | 0.548 ^a 0.002 ^{b,*} 0.065 ^c |
| NeuroFs ^d | 4.0 (3.1–5.8) | 4.3 (2.6–37.5) | 0.90 (0.49–6.43) | 4.10 (2), 0.128 | 0.548 ^a 0.171 ^b 0.093 ^c |
| IsoFs ^d | 64.1 (59.2–125.7) | 43.15 (23.22–66.11) | 32.9 (25.6–39.8) | 8.71 (2), 0.013 [*] | 0.095 ^a 0.002 ^{b,*} 0.435 ^c |

Q1: quartile 1; Q3: quartile 3.

* P < 0.05.

^a Comparison between control and MCI-AD groups.

^b Comparison between control and mild dementia-AD groups.

^c Comparison between MCI-AD and mild dementia-AD groups.

^d Total parameters results are expressed as (intensity of signal units x (internal standard concentration, mg L⁻¹)) mg⁻¹ of creatinine.

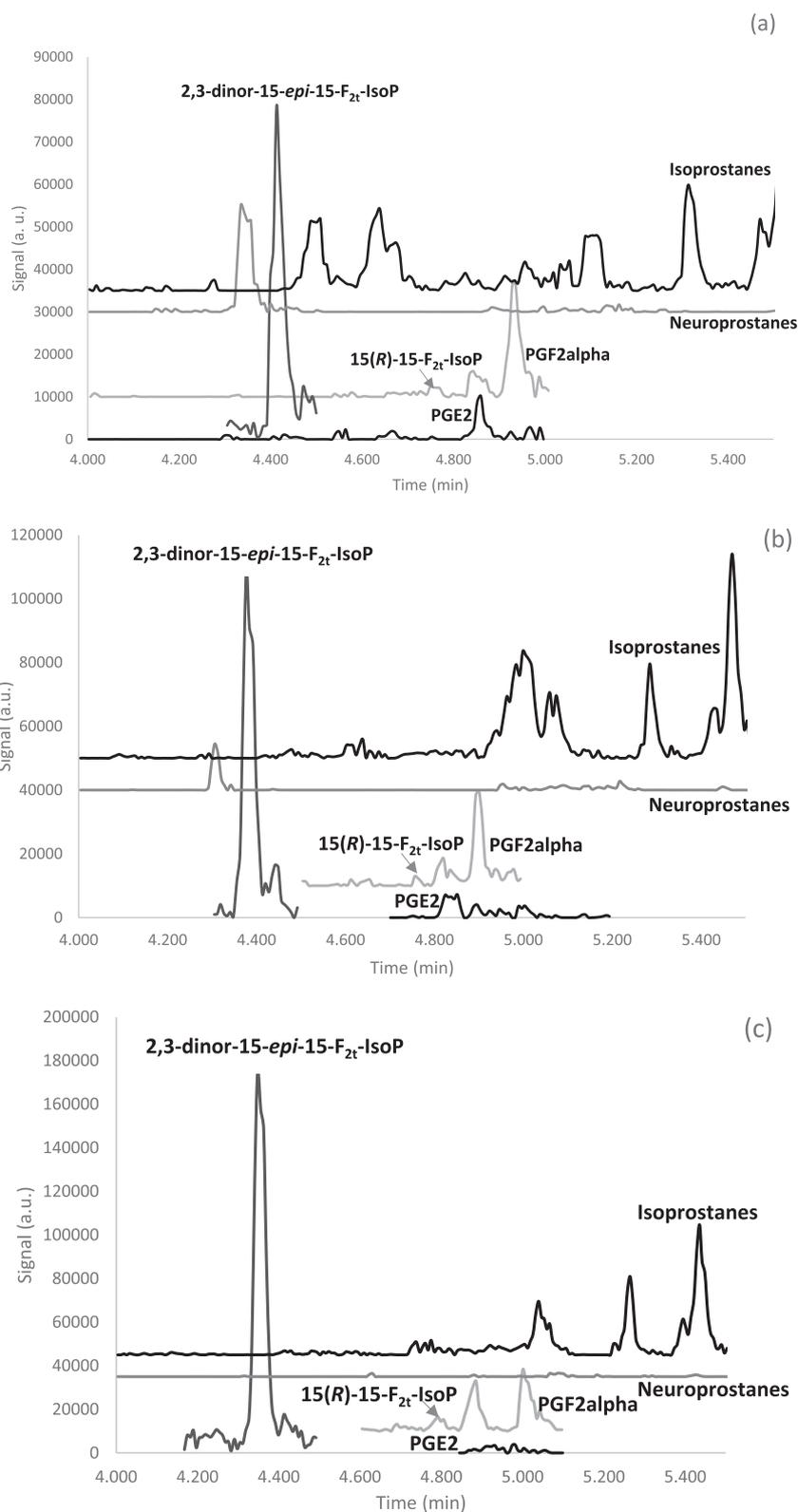


Fig. 1. Chromatograms obtained from urine samples analysis for: (a) control participants, (b) MCI-AD patients, and (c) mild dementia-AD patients.

4. Conclusions

The present work describes a reliable and sensitive UPLC-MS/MS method for the simultaneous determination of 17 analytes (F_2 -IsoPs, prostaglandins, F_4 -NeuroPs, F_2 -dihomo-IsoPs, dihomo-IsoFs) and 4 total parameters (IsoP, NeuroP, IsoF, NeuroF) in urine samples. Of note, most of these analytes have not been measured in previous works found in

literature [38,42], and the lack of validated liquid chromatographic methods to determine these new set of lipid peroxidation biomarkers in urine human samples adds relevance to this new method. The described UPLC-MS/MS method can be considered a simple alternative to previously published methods, which employ chemical derivatization and gas chromatography.

The developed method provided suitable accuracy, sensitivity, and

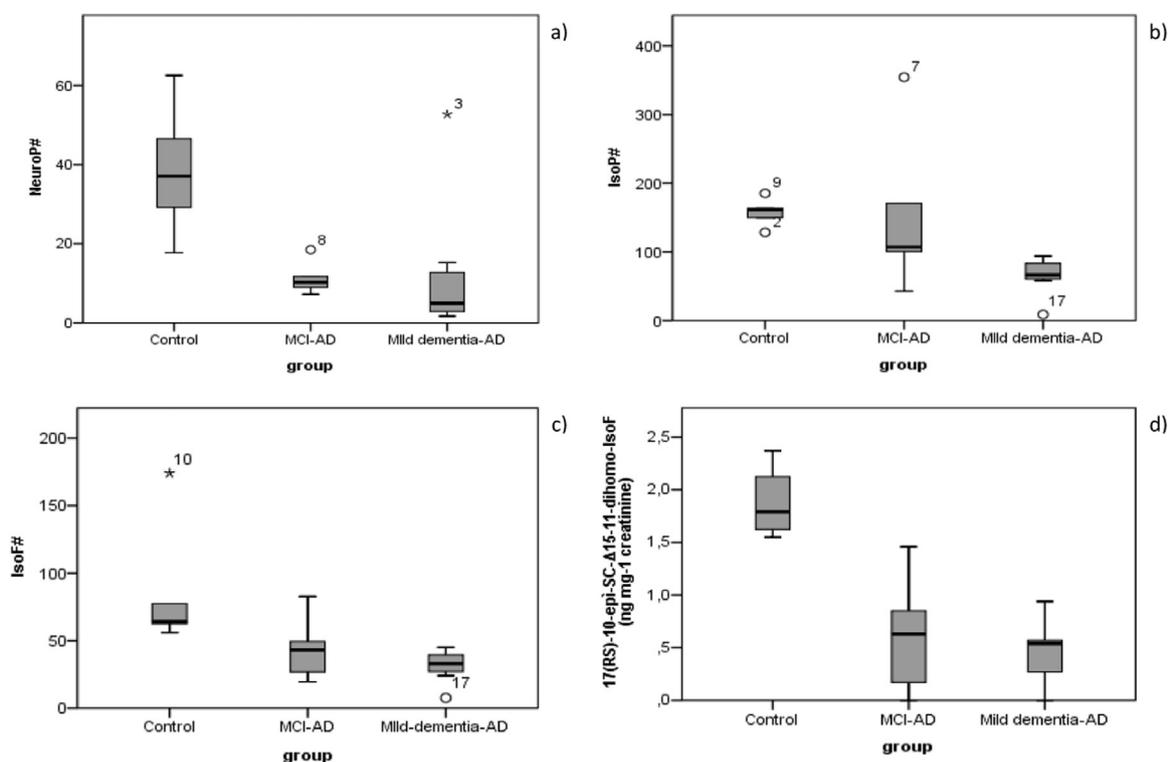


Fig. 2. Box plot graphs representing the concentrations of: a) NeuroP; b) IsoP; c) IsoF; d) 17(RS)–10-epi-SC-A15-11-dihomo-IsoF, in the three groups of participants. Boxes represent the 1st and the 3rd quartiles, the black lines the median, and whiskers encompass from the 9th to 91st percentiles. # Total parameters are expressed as (intensity of signal units x (internal standard concentration, mg L⁻¹)) mg⁻¹ of creatinine.

high throughput to be applied to urine samples from MCI and mild dementia AD patients. Some analytes showed statistically significant lower concentrations for the MCI-AD and mild dementia-AD groups. Nevertheless, further analysis employing the developed analytical method and a greater number of samples is required to corroborate these preliminary results. Therefore, the described analytical method constitutes a useful tool for subsequent clinical studies in order to validate these compounds as potential early AD biomarkers.

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Conflict of interest

The authors report no conflict of interest.

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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.2018.03.002>.

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