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Validated analytical method to determine new salivary lipid peroxidation compounds as potential neurodegenerative biomarkers

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A B S T R A C T

Lipid peroxidation is closely related to neurodegenerative diseases since brain shows high lipid composition and oxygen consumption. The determination of lipid peroxidation compounds in non-invasive biological samples would help to monitor the patients' oxidative stress status. A new analytical method based on ultrasound-assisted liquid-liquid semi-microextraction (UA-LLsME) followed by Ultra Performance Liquid Chromatography coupled to tandem Mass Spectrometry was developed to determine 18 lipid peroxidation biomarkers in saliva samples. The variables affecting the UA-LLsME efficiency were systematically studied. Under the optimum conditions, the methodology was validated and showed high-throughput, high sensitivity (limits of detection 0.02-2 nmol L⁻¹), and satisfactory precision (coefficients of variation 2-11% (intra-day) and 5-12% (inter-day)). The reliability of the described method was assessed analysing spiked saliva samples, and the recoveries were between 80% and 120% for most of the analytes. Then, the method suitability was demonstrated by analysing saliva samples (n = 30) from elderly people with neurodegenerative diseases. To conclude, the new developed analytical method is a useful tool to determine salivary lipid peroxidation compounds as potential biomarkers in further clinical studies in which oxidative stress plays an important role.

Keywords:

Liquid-liquid microextraction
saliva
Isoprostanes
Lipid peroxidation
Mass spectrometry

1. Introduction

Oxidative stress is a physiological process that increases with aging and under some pathological conditions [1]. Specifically, lipid peroxidation is closely related to neurodegenerative diseases since brain shows high oxygen consumption and high lipid composition [2,3]. Although blood is considered the best biofluid to evaluate it at systemic level, the employment of non-invasively obtained samples would help to monitor the oxidation status [4]. So saliva is considered a promising matrix in clinical studies [5]. In this sense, the validation of an analytical method to determine some lipid peroxidation biomarkers in saliva samples is required.

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Recent studies have used saliva sample to determine oxidative stress biomarkers related to some systemic diseases [6,7]. The clinical relevancy of saliva is based on the fact that its components can be found in plasma [8,9]. However, salivary compounds levels may be altered by circadian rhythm and oral conditions [10,11], and few correlation studies between blood and saliva levels can be found in literature [12,13].

Some analytical methods have been developed to study salivary oxidative stress biomarkers (glutathione peroxidase, catalase, protein carbonyls, 8-hydroxy-2'-deoxyguanosine), but few of them determined lipid peroxidation compounds, such as malondialdehyde (MDA) [14-16], and prostanoids (e.g. 15-F_{2t}-IsoP) [10]. In general, these methods are based on colorimetric assays and immunoassays [4,17-19], but also liquid chromatography (LC) coupled to mass spectrometry (MS) [10,20], or fluorescence detection methods [18] are used. Nevertheless, they have not been validated, so there is a lack of reliable analytical methods.

Suitable saliva sample treatment is crucial in order to obtain an analytical method with satisfactory sensitivity and selectiv-

Table 1
Demographic and clinical characteristics of the studied population (n = 30).

Age (years)	69 ± 6	
Gender (female, n (%))	18 (53 %)	
Studies level (n (%))	Basic	11 (32%)
	Primary	8 (24%)
	Secondary	6 (18%)
Alcohol consumption (Yes, n (%))	Academic	9 (26%)
	Yes	5 (15 %)
Smoking status (n (%))	Yes	7 (21%)
	Former smoker (more than 10 years)	5 (15%)
	Antiaggregants	1 (3%)
	Corticoids	1 (3%)
	Acetyl cholinesterase inhibitors	4 (12%)
Medications (n, (%))	Two or more	10 (29%)
	Psychotropic drugs	2 (6%)
	Others	7 (21%)
	None	9 (26%)
Depression (Yes, n (%))	None	4 (12%)
	None	7 (205%)
	Dyslipidaemia	4 (12%)
Comorbidity (n (%))	Arterial hypertension	7 (205%)
	Heart disease	1 (3%)
	Two or more	10 (20%)
	Others	5 (15%)
	Leukocytes (x10 ³ cells mm ⁻³)	7.08 ± 2.29
Lymphocytes (x10 ³ cells mm ⁻³)	2.49 ± 1.26	

Data are expressed as mean ± standard deviation, or number of cases (percentages). CSF: cerebrospinal fluid sample.

ity. Among potential techniques, the liquid-liquid microextraction (LLME) allows simultaneous pre-concentration and clean-up steps, with the advantages of low solvent volume consumption, simplicity, and low cost. Nevertheless, few works have applied this technique to saliva samples [21]. The main LLME modes are single-drop microextraction [21], hollow-fiber liquid microextraction [22], and dispersive liquid-liquid microextraction [23]. However, their disadvantages are drop instability, solvents toxicity and non-exhaustive extractions. Recent efforts to solve these drawbacks were based on ultrasound, vortex or salt assisted extraction [24,25], but they have not been applied to saliva samples.

In this work, we have developed a reliable analytical method to determine a new set of salivary compounds reflecting lipid peroxidation. For this, a new sample treatment based on ultrasound-assisted liquid-liquid semimicroextraction (UA-LLSME) and the conditions for ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) were optimized. Therefore, this validated analytical method would constitute a useful tool for lipid peroxidation evaluation in further clinical studies.

2. Materials and methods

2.1. Samples collection, and storage

Saliva samples from participants diagnosed with neurodegenerative diseases (frontotemporal dementia, vascular dementia, Alzheimer's disease) (n = 30) were taken at the Neurology Unit (University and Polytechnic Hospital La Fe, Valencia (Spain)) [26]. The participants were between 50 and 75 years old. The study protocol and informed consent from all the participants were approved by the Ethics Committee (CEIC) at the Health Research Institute La Fe (Valencia). Table 1 shows the demographic and clinical characteristics of participants.

Saliva samples were collected by spitting into sterile bottles (between 10 and 12 a.m.). Participants rinsed their mouth before saliva collection. Then, the samples were aliquoted into 1.5 mL tubes, and those with visible blood contamination were excluded from the study. Finally, samples were stored at -80 °C until analysis.

2.2. Reagents

Some compounds were acquired from Cayman Chemical Company (Ann Arbor, Michigan, USA) (PGE₂, PGF_{2α}, 1a,1b-dihomo-PGF_{2α}, 5-F_{2t}-IsoP, 15-E_{2t}-IsoP, 15-F_{2t}-IsoP, 15-keto-15-E_{2t}-IsoP, 15-keto-15-F_{2t}-IsoP, 15(R)-15-F_{2t}-IsoP, 2,3-dinor-15-*epi*-15-F_{2t}-IsoP, PGF_{2α}-D₄) and used as analytes, as in a previous study [27]. Other compounds nor available commercially were synthesized ((4(RS)-F_{4t}-NeuroP, 7(RS)-ST-Δ⁸-11-dihomo-IsoF, *Ent*-7(RS)-7-F_{2t}-dihomo-IsoP, 10-*epi*-10-F_{4t}-NeuroP, 14(RS)-14-F_{4t}-NeuroP, 17-*epi*-17-F_{2t}-dihomo-IsoP, 17-F_{2t}-dihomo-IsoP, 17(RS)-10-*epi*-SC-Δ¹⁵-11-dihomo-IsoF, D₄-10-*epi*-10-F_{4t}-NeuroP) [28], and they were used as analytes, as in previous studies [29,30].

Methanol (CH₃OH, HPLC grade), acetonitrile (CH₃CN, HPLC grade), and glacial acetic acid were from J.T. Baker (Avantor Performance Materials B.V., The Netherlands). Ethyl acetate (analytical grade) was from Panreac (Barcelona, Spain). Protein assay kit (Pierce™ BCA) was purchased from Thermo Scientific (Rockford, USA).

2.3. Solutions

Stock standard solutions of each compound were prepared at 1 mmol L⁻¹ in H₂O (CH₃COOH 0.01%, v/v):CH₃OH (85:15 v/v). A mix working solution was prepared in H₂O (CH₃COOH 0.01%, v/v):CH₃OH (85:15 v/v) (2.5 μmol L⁻¹ each analyte). The calibration curves were prepared in H₂O (CH₃COOH 0.01%, v/v):CH₃OH (85:15 v/v) (0.004 nmol L⁻¹ - 300 nmol L⁻¹, each analyte) as described in a previous work [29].

2.4. Materials

The ultrasonic bath (Sonorex Digitec) was from Bandelin (Berlin, Germany), the centrifuge (multiSPIN) from Cleaver Scientific Ltd. (Warwickshire, United Kingdom), and the vortex mixer from Velp Scientifica (Usmate, Italy). The thermomixer HLC was from Ditabis (Pforzheim, Germany), the speed vacuum concentrator (mi Vac) from Genevac LTD (Ipswich, United Kingdom), and the spectrophotometer (Halo LED 96) from Dynamica Scientific Ltd. (London, United Kingdom).

2.5. UPLC-MS/MS chromatographic system

The chromatographic system used consisted of an Acquity UPLC system coupled to a Xevo TQ-S mass spectrometer (MS) (Waters, UK). The MS detector consisted of an electrospray ionization source working in the negative mode (ESI-) and multiple reaction monitoring (MRM) mode. The UPLC-MS/MS system conditions were described in a previous work [30]. Briefly, the LC conditions were an Acquity UPLC BEH C18 column, H₂O and CH₃CN with 0.01% v/v CH₃COOH as mobile phases, 55 °C and 4 °C as column and autosampler temperatures, 8 μL as injection volume, and 0.45 mL min⁻¹ as flow rate; and the detection conditions were 2.0 kV as capillary voltage, 150 °C and 395 °C as source and desolvation temperatures, 150 L h⁻¹ and 800 L h⁻¹ as nitrogen cone and desolvation gas flows, and 10 ms as dwell time.

2.6. Saliva sample treatment

Saliva samples were thawed on ice, homogenized and centrifuged at 3500g (10 min, 4 °C) in order to determine analytes in supernatant. The saliva sample treatment was optimized from a previous work with new-borns' saliva samples [31]. First, 150 μL of saliva sample were diluted with 450 μL of H₂O (0.01% (v/v) acetic acid), and 5 μL of internal standard (IS) solution (PGF_{2α}-D₄ 2 μmol L⁻¹ and D₄-10-*epi*-10-F_{4t}-NeroP 1.2 μmol L⁻¹) were added to each

Table 2
Mass spectrometry detection parameters.

Analyte	m/z Precursor Ion	Cone [V]	m/z Product Ion			
			Quantification	CE [eV]	Confirmation	CE [eV]
15(R)-15-F _{2t} -IsoP	353.2	40	193.1	25	309.2	20
2,3-dinor-15-epi-15-F _{2t} -IsoP	325.2	20	237	10	–	–
15-keto-15-E _{2t} -IsoP	349.2	20	331	15	235	15
15-keto-15-F _{2t} -IsoP	351.2	30	315.1	15	289	20
PGE ₂	351.2	40	271	20	315.1	15
15-E _{2t} -IsoP	351.2	40	271	20	315.1	15
15-F _{2t} -IsoP	353.2	40	193.1	25	309.2	20
PGF _{2α}	353.2	40	309.2	20	193.1	25
5-F _{2t} -IsoP	353.2	30	114.8	20	309.2	20
1a,1b-dihomo-PGF _{2α}	381.2	40	337	20	319.1	25
PGF _{2α} -D ₄	357.2	20	313.1	20	–	–
7(RS)-ST-Δ ⁸ -11-dihomo-IsoF	397.2	20	200.9	25	148.8	25
10-epi-10-F _{4t} -NeuroP	377.1	10	152.9	20	109.8	20
D ₄ -10-epi-10-F _{4t} -NeuroP	381.2	20	157	20	–	–
17-epi-17-F _{2t} -dihomo-IsoP	381.2	30	337	20	319.1	20
17-F _{2t} -dihomo-IsoP	381.2	30	337	20	319.1	20
17(RS)-10-epi-SC-Δ ¹⁵ -11-dihomo-IsoF	397.2	10	154.9	30	220.9	25
Ent-7(RS)-7-F _{2t} -dihomo-IsoP	381.2	30	142.9	20	363.2	15
14(RS)-14-F _{4t} -NeuroP	377.1	40	205	15	271	20
4(RS)-F _{4t} -NeuroP	377.1	20	100.8	20	271	20
NeuroPs	377	35	101	20	–	–
IsoPs	353.2	40	115	20	–	–
NeuroFs	393	35	193	20	–	–
IsoFs	369.2	45	115	20	–	–

sample. Then, 600 μL of ethyl acetate were added to each sample and it was sonicated for 10 min. After that, samples were placed at –20 °C for 30 min to improve both phases separation. The organic phase was collected in a new tube and the UA-LLSME was repeated to enhance the analytes extraction from the aqueous phase. Finally, both ethyl acetate layers (600 μL×2) were joined and evaporated to dryness in the speed vacuum concentrator. The residues were reconstituted in 100 μL of H₂O:CH₃OH (85:15 (v/v), 0.01% acetic acid) and injected in the chromatographic system (UPLC-MS/MS).

The same sample treatment was applied to standards (prepared in H₂O containing 0.01% (v/v) acetic acid) in order to take into account the procedure effect over some analytes obtaining a reliable quantification in real samples.

2.7. Method validation

For the method validation some analytical characteristics were evaluated for each analyte (limits of quantification (LOQ), limits of detection (LOD), linearity, precision, accuracy, matrix effect, stability). The LODs and LOQs, defined as the standard concentrations generating a signal-to-noise ratio of 3 and 10, respectively, as well as the linearity were evaluated in a previous study [30]. Then, saliva samples non-spiked and spiked at three concentration levels (i.e. low, mid, and high) were analyzed at three different days to evaluate the precision and accuracy. The matrix effect for each analyte in saliva was evaluated comparing the added concentrations (X) with the found concentrations using the proposed method (Y) by means of a linear regression. Finally, the analytes' stability in spiked saliva samples was assayed under different storage conditions (one, two or three freeze-thaw cycles, and 24 h at 4 °C).

2.8. Analysis of saliva samples

Collected saliva samples were kept at –80 °C until analysis. The suitability of the developed analytical method was evaluated by analysing these real samples. In this way, the concentrations ranges obtained for each analyte in saliva samples from patients were determined. To standardize the results in saliva samples, protein levels were determined using a colorimetric protein assay kit (Pierce™ BCA) and measuring in a spectrophotometer.

2.9. Statistical analysis

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 $p < 0.05$.

3. Results and discussion

3.1. Optimization of the UA-LLSME procedure

The main challenge of saliva pretreatment is the efficient extraction of target analytes and the subsequent clean-up step to remove matrix interferences. In this sense, the developed saliva treatment procedure allows carrying out simultaneously the extraction and clean-up steps. It consists of UA-LLSME, in which a low-density extraction solvent is dispersed into aqueous phase using ultrasonication. Some parameters involved in extraction efficiency were systematically studied, including the sample volume, sample pH, volume of extraction solvent, number of liquid-liquid extraction steps, ultrasonication time and salting-out effect.

First, taking into account the limited saliva volumes available in neurodegenerative disease patients, small sample volumes were assayed between 50 and 150 μL of spiked saliva sample (100 nmol L⁻¹ for each analyte). Satisfactory recoveries were obtained in all the cases. However, according to the low concentrations found for 5-F_{2t}-IsoP and 15-F_{2t}-IsoP in saliva [10], 150 μL was selected as the optimum volume.

The effect of sample pH was evaluated between 3 and 7 using 150 μL of spiked sample. Higher recoveries were obtained at pH 3. It was achieved by diluting the sample with 450 μL of H₂O (0.01% (v/v) acetic acid), since the analytes were in their neutral form ($pK_a > 3$) and their extraction into a non-polar solvent was more efficient. Consequently, the sample pH was adjusted to 3 before the analysis.

As regards the extraction solvent, a non-polar and low density solvent was required in order to improve the analytes extraction efficiency minimizing the interferences. Among the potential solvents and taking into account their toxicity, ethyl acetate was considered a suitable extraction solvent and all further experiments were carried out with it.

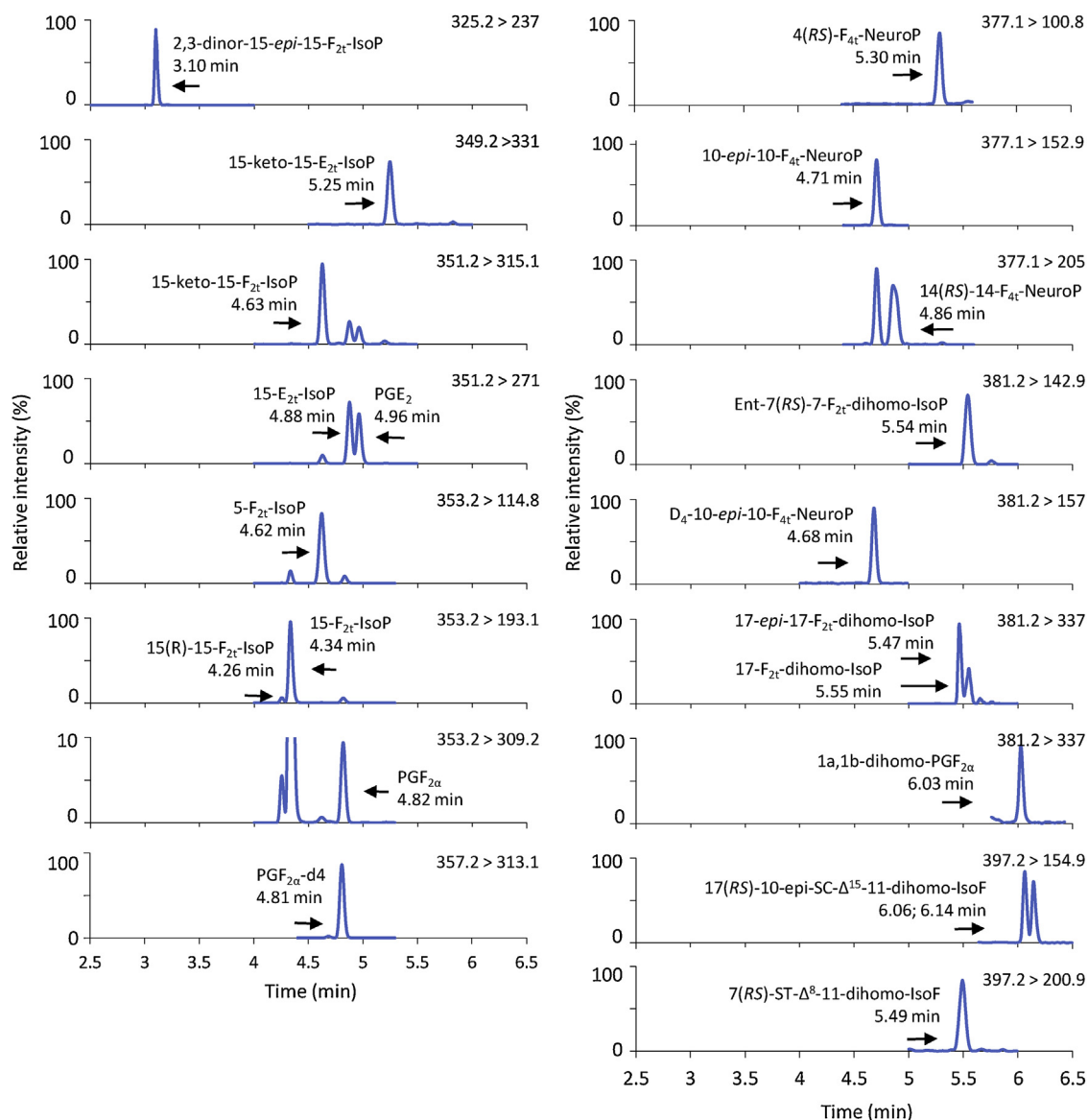


Fig. 1. Chromatograms (MRM transition used for quantification) obtained by the UPLC-MS/MS method for a mix standard solution of 100 nmol L^{-1} of each analyte.

After that, the influence of the extraction solvent volume ($600\text{--}1200 \mu\text{L}$) and the number of liquid-liquid extraction steps (1–2) were evaluated simultaneously. For this, $150 \mu\text{L}$ of spiked saliva (100 nmol L^{-1} of each compound), adjusted to pH 3, and 10 min of sonication were used. Recoveries were close to 100% for most of the analytes by means of two LLE steps and $1200 \mu\text{L}$ ($2 \times 600 \mu\text{L}$) of ethyl acetate, therefore these conditions were selected as optimum.

Different sonication times in the 1–15 min interval were tested under the previous selected conditions. In general, all the analytes increased their analytical signal by extending the sonication time up to 10 min, little improvement was observed for longer times. Therefore, a sonication time of 10 min was set for each liquid-liquid extraction step.

Finally, the salting out effect was assayed by adding a sodium chloride solution (2 mol L^{-1}) to the aqueous phase to improve the analytes extraction into the organic phase [32]. Nevertheless, recoveries were similar to those obtained without the salt addition, so it was not added in further experimental work.

In summary, the optimum saliva treatment conditions were $150 \mu\text{L}$ of sample volume, adjusted at pH 3, a two-step liquid-

liquid extraction with $2 \times 600 \mu\text{L}$ of ethyl acetate as extraction solvent total volume, and 10 min of sonication time. Under optimized conditions, the extraction yield was considered quantitative for most of the analytes, with recoveries ranging from 80 to 120%.

As regards the sample treatment effect over analytes in standards prepared in H_2O (0.01% (v/v) acetic acid), a significant signal increase was observed for 17-epi-17-F_{2t}-dihomo-IsoP, 17-F_{2t}-dihomo-IsoP, 17(RS)-10-epi-SC- Δ^{15} -11-dihomo-IsoF and 1a,1b-dihomo-PGF_{2 α} . Probably, these analytes which are characterized by higher *m/z* parent ion could experiment a significant enhancement of ionization under the higher concentration of acetate ions provided by sample treatment [33]. Therefore, the concentrations of these analytes in saliva samples were quantified using the calibration equations obtained with standard solutions subjected to the same sample treatment.

3.2. Optimization of the UPLC-MS/MS conditions

Transitions, cone voltages, and collision energies for the detection were automatically tuned for each analyte using the

Table 3
Analytical characteristics of the UPLC-MS/MS developed method.

Analyte	Retention time ^a (min)	Linearity		LOD ^c (nmol L ⁻¹)	LOQ ^c (nmol L ⁻¹)
		Linear interval (nmol L ⁻¹)	Calibration equation ^b $y = a+bx$		
2,3-dinor-15- <i>epi</i> -15-F _{2t} -IsoP	3.093 ± 0.004	0.07-150	$a \pm s_a = 1.04 \pm 0.8$; $b \pm s_b = 1.41 \pm 0.01$ $R^2 = 0.999$ (n = 10)	0.02	0.07
15-keto-15-F _{2t} -IsoP	4.631 ± 0.003	0.14-150	$a \pm s_a = -12.469 \pm 9.613$; $b \pm s_b = 2.247 \pm 0.149$ $R^2 = 0.990$ (n = 8)	0.05	0.14
15(R)-15-F _{2t} -IsoP	4.263 ± 0.004	0.07-150	$a \pm s_a = 0.284 \pm 0.334$; $b \pm s_b = 0.913 \pm 0.005$ $R^2 = 0.999$ (n = 10)	0.02	0.07
15-keto-15-E _{2t} -IsoP	5.254 ± 0.005	0.28-150	$a \pm s_a = 0.075 \pm 0.185$; $b \pm s_b = 1.205 \pm 0.003$ $R^2 = 0.999$ (n = 8)	0.1	0.3
PGE ₂	4.957 ± 0.004	0.28-150	$a \pm s_a = -0.912 \pm 0.570$; $b \pm s_b = 1.442 \pm 0.009$ $R^2 = 0.999$ (n = 8)	0.1	0.3
15-E _{2t} -IsoP	4.876 ± 0.003	0.28-150	$a \pm s_a = -0.643 \pm 0.4$; $b \pm s_b = 1.794 \pm 0.005$ $R^2 = 0.999$ (n = 8)	0.1	0.3
15-F _{2t} -IsoP	4.34 ± 0.01	0.07-150	$a \pm s_a = 167.37 \pm 38.68$; $b \pm s_b = 18.56 \pm 0.33$ $R^2 = 0.998$ (n = 10)	0.02	0.07
PGF _{2α}	4.816 ± 0.004	0.07-150	$a \pm s_a = 6.5 \pm 2.911$; $b \pm s_b = 3.06 \pm 0.025$ $R^2 = 0.999$ (n = 10)	0.02	0.07
7(RS)-ST-Δ ⁸ -11-dihomo-IsoF	5.491 ± 0.003	0.12-135	$a \pm s_a = -0.826 \pm 0.549$; $b \pm s_b = 0.540 \pm 0.009$ $R^2 = 0.999$ (n = 8)	0.04	0.12
5-F _{2t} -IsoP	4.616 ± 0.004	0.07-150	$a \pm s_a = 2.042 \pm 0.642$; $b \pm s_b = 0.754 \pm 0.005$ $R^2 = 0.999$ (n = 9)	0.02	0.07
10- <i>epi</i> -10-F _{4t} -NeuroP	4.71 ± 0.01	0.1-142	$a \pm s_a = -7.667 \pm 2.525$; $b \pm s_b = 2.615 \pm 0.023$ $R^2 = 0.999$ (n = 9)	0.03	0.1
17- <i>epi</i> -17-F _{2t} -dihomo-IsoP	5.47 ± 0.01	0.13-140	$a \pm s_a = -0.123 \pm 0.939$; $b \pm s_b = 2.855 \pm 0.008$ $R^2 = 0.999$ (n = 10)	0.04	0.13
17-F _{2t} -dihomo-IsoP	5.549 ± 0.003	0.13-140	$a \pm s_a = -0.308 \pm 1.029$; $b \pm s_b = 1.763 \pm 0.009$ $R^2 = 0.999$ (n = 7)	0.04	0.13
17(RS)-10- <i>epi</i> -SC-Δ ¹⁵ -11-dihomo-IsoF	6.117 ± 0.04	2-135	$a \pm s_a = -1.213 \pm 0.457$; $b \pm s_b = 0.861 \pm 0.004$ $R^2 = 0.999$ (n = 9)	0.7	2
Ent-7(RS)-7-F _{2t} -dihomo-IsoP	5.536 ± 0.004	0.07-140	$a \pm s_a = -1.639 \pm 1.083$; $b \pm s_b = 1.097 \pm 0.018$ $R^2 = 0.999$ (n = 8)	0.02	0.07
14(RS)-14-F _{4t} -NeuroP	4.86 ± 0.03	8-142	$a \pm s_a = 0.178 \pm 0.334$; $b \pm s_b = 0.139 \pm 0.003$ $R^2 = 0.997$ (n = 9)	2	8
1a,1b-dihomo-IsoP	6.03 ± 0.01	0.28-150	$a \pm s_a = -7.786 \pm 2.388$; $b \pm s_b = 1.094 \pm 0.02$ $R^2 = 0.998$ (n = 9)	0.1	0.28
4(RS)-F _{4t} -NeuroP	5.296 ± 0.005	8-142	$a \pm s_a = -4.11 \pm 1.355$; $b \pm s_b = 0.56 \pm 0.012$ $R^2 = 0.997$ (n = 8)	2	8

QuanOptimizer software (Waters) and individual 0.5 μg mL⁻¹ standard solutions (Table 2). The data station operating software used was MassLynx 4.1 (Waters).

Regarding chromatographic optimization the mobile phase composition was firstly studied. The modifier acetic acid at 0.01% (v/v) was used in the mobile phase in order to improve the analytes ionization in the MS detector. Moreover, acetonitrile and methanol were tested as organic modifiers obtaining better results with acetonitrile. Different combinations of flow rates (from 0.3 to 0.5 mL min⁻¹), column temperatures (from 40 °C to 60 °C) and gradients were tested in order to obtain a satisfactory chromatographic resolution in a run-time as short as possible. The injection volume was studied at two levels (4 and 8 μL) and better sensitivity without loss in resolution was obtained when injecting 8 μL.

Under the optimum conditions, described in Section 2.5, a good chromatographic separation was achieved for the 18 analytes in less than 8 min, as can be seen in Fig. 1.

3.3. Analytical performance data

Standards with concentrations between 0.03 and 300 nmol L⁻¹ were used to evaluate the analytical method performance. As shown in Table 3, all compounds showed adequate linearity with R² between 0.990 and 0.999, as well as suitable LODs (0.02–2 nmol L⁻¹) and LOQs (0.07–8 nmol L⁻¹) [30]. In general, our results match with those from works in literature [12], allowing the quantification of these analytes at levels normally found in saliva samples [10].

Regarding precision, it was satisfactory for standard solutions (Table 4), and the analysis of spiked saliva samples provided coefficients of variation between 2.5 and 18% for most of the analytes, higher values corresponded to the inter-day analysis (Table 4). These values were satisfactory (<20%) taking into account the several steps required in the sample treatment procedure. The accuracy of the method was evaluated by analyzing saliva samples spiked at three concentration levels (low, medium, and high). They were quantified using external calibration equations and satisfactory recoveries were obtained for all the analytes (Table 4), with the exception of 17-*epi*-17-F_{2t}-dihomo-IsoP, 17-F_{2t}-dihomo-IsoP, 17(RS)-10-*epi*-SC-Δ¹⁵-11-dihomo-IsoF and 1a,1b-dihomo-PGF_{2α}. As a possible cause, sample treatment effect was evaluated and significant signals increase was observed for these analytes. Therefore, the use of calibration equations constructed from standard solutions subjected to UA-LLSME sample treatment to quantify these analytes, provided satisfactory recoveries (Table 4). Then, matrix effect was evaluated for each analyte. In all the cases, the slopes corresponding to the linear regressions of added concentrations (X) vs found concentrations (Y) were statistically comparable to 1 (at a confidence level of 95%), so the matrix effect was considered negligible.

In addition, four total parameters were determined as relative contents of salivary isoprostanes (IsoPs), isofurans (IsoFs), neuroprostanes (NeuroPs) and neurofurans (NeuroFs), they included the sum of isomers from each fatty acid oxidation family. Similarly, these parameters were determined in previous studies carried out in plasma [27] and urine samples [29].

Table 4

Accuracy and precision of the analytical method for standard solutions and saliva samples spiked at different concentration levels.

Analytes	Standard Solutions			Spiked saliva samples ^a		
	Standard concentration (nmol L ⁻¹)	[Recovery ± s (%)]		Sample added concentration (nmol L ⁻¹)	[Recovery ± s (%)]	
		Intra-day(n=3)	Inter-day(n=3)		Intra-day(n=3)	Inter-day(n=3)
2,3-dinor-15- <i>epi</i> -15- <i>F</i> _{2t} -IsoP	18.7	84 ± 2	110 ± 6	10	72 ± 2	80 ± 6
	75	99 ± 2	104 ± 6	50	104 ± 2	90 ± 9
	150	93 ± 2	104 ± 2	100	78 ± 3	85 ± 4
	18.7	89 ± 3	92 ± 8	10	92 ± 9	90 ± 10
15-keto-15- <i>F</i> _{2t} -IsoP	75	108 ± 7	83 ± 6	50	111 ± 9	107 ± 9
	150	96 ± 6	115 ± 10	100	100 ± 10	107 ± 8
	18.7	80 ± 2	117 ± 10	10	114 ± 9	81 ± 9
	75	84 ± 3	83 ± 5	50	97 ± 9	80 ± 10
15-keto-15- <i>E</i> _{2t} -IsoP	150	116 ± 3	108 ± 8	100	120 ± 10	104 ± 9
	18.7	107 ± 8	106 ± 5	10	79 ± 7	105 ± 17
	75	110 ± 4	94 ± 7	50	101 ± 12	105 ± 19
	150	104 ± 3	92 ± 5	100	92 ± 10	85 ± 8
15(<i>R</i>)-15- <i>F</i> _{2t} -IsoP	18.7	85 ± 5	112 ± 10	10	102 ± 9	93 ± 6
	75	105 ± 5	90 ± 7	50	112 ± 3	98 ± 7
	150	106 ± 3	100 ± 8	100	105 ± 5	104 ± 17
	18.7	81 ± 4	100 ± 10	10	89 ± 5	95 ± 10
15- <i>E</i> _{2t} -IsoP	75	94 ± 7	100 ± 9	50	87 ± 4	94 ± 8
	150	99 ± 6	111 ± 7	100	101 ± 6	72 ± 9
	18.7	100 ± 5	110 ± 5	10	84 ± 5	87 ± 9
	75	105 ± 5	92 ± 5	50	102 ± 10	100 ± 7
5- <i>F</i> _{2t} -IsoP	150	97 ± 5	90 ± 9	100	86 ± 8	93 ± 3
	18.7	110 ± 6	84 ± 9	10	98 ± 10	100 ± 20
	75	113 ± 7	96 ± 9	50	120 ± 10	99 ± 19
	150	94 ± 4	120 ± 6	100	95 ± 8	92 ± 8
15- <i>F</i> _{2t} -IsoP	18.7	100 ± 10	111 ± 11	10	98 ± 10	99 ± 17
	75	112 ± 6	96 ± 5	50	110 ± 5	112 ± 14
	150	96 ± 4	101 ± 7	100	104 ± 7	105 ± 16
	18.7	81 ± 8	80 ± 15	10	94 ± 13	78 ± 13
1a,1b-dihomo-PGF _{2α} ^b	75	83 ± 9	93 ± 11	50	95 ± 7	72 ± 9
	150	120 ± 7	120 ± 7	100	120 ± 9	109 ± 9
	16.9	102 ± 4	84 ± 10	8.6	88 ± 14	107 ± 15
	67.5	94 ± 9	101 ± 10	45	104 ± 20	90 ± 20
7(<i>RS</i>)-ST-Δ ⁸ -11-dihomo-IsoF	135	110 ± 6	96 ± 11	90	105 ± 5	112 ± 10
	17.7	91 ± 8	97 ± 8	9	120 ± 3	90 ± 10
	70.8	98 ± 3	96 ± 10	47.2	81 ± 3	94 ± 8
	142	102 ± 9	102 ± 7	94.4	93 ± 12	102 ± 9
4(<i>RS</i>)- <i>F</i> _{4t} -NeuroP	17.7	89 ± 8	102 ± 10	9	104 ± 14	84 ± 15
	70.8	99 ± 4	94 ± 7	47.2	95 ± 10	80 ± 11
	142	112 ± 6	99 ± 8	94.4	95 ± 6	80 ± 9
	17.5	107 ± 6	80 ± 6	8.9	116 ± 6	81 ± 8
17- <i>epi</i> -17- <i>F</i> _{2t} -dihomo-IsoP	70.2	87 ± 6	87 ± 7	46.8	80 ± 10	88 ± 6
	140	105 ± 8	105 ± 9	93.6	113 ± 10	116 ± 7
	17.5	75 ± 8	111 ± 10	8.9	70 ± 9	95 ± 7
	70.0	98 ± 10	98 ± 12	46.6	106 ± 16	106 ± 19
17- <i>F</i> _{2t} -dihomo-IsoP ^b	140	109 ± 6	108 ± 10	93.2	108 ± 16	108 ± 19
	17	82 ± 10	84 ± 11	8.6	105 ± 15	80 ± 20
	67.5	81 ± 2	84 ± 8	45	100 ± 9	81 ± 12
	135	114 ± 5	92 ± 3	90	114 ± 12	106 ± 15
17(<i>RS</i>)-10- <i>epi</i> -SC-Δ ¹⁵ -11-dihomo-IsoF ^b	17.5	107 ± 2	118 ± 15	8.9	117 ± 12	107 ± 10
	70.2	107 ± 3	106 ± 6	46.8	90 ± 8	109 ± 20
	140	114 ± 9	119 ± 10	93.6	118 ± 4	100 ± 10
	17.7	90 ± 10	80 ± 10	9	97 ± 9	100 ± 20
14(<i>RS</i>)-14- <i>F</i> _{4t} -NeuroP	70.8	100 ± 4	105 ± 6	47.2	120 ± 9	108 ± 19
	142	100 ± 9	110 ± 9	94.4	94 ± 10	93 ± 18

^a Saliva samples subjected to UA-LLSME as sample treatment.^b Quantification in saliva samples using standards subjected to UA-LLSME.

3.4. Analyte stability study

The analytes' stability assessment in saliva samples spiked at medium concentration level was carried out after several freeze-thaw cycles (long-term stability), and after 24 h at 4 °C (short-term stability). These samples were analyzed by triplicate, and the found concentrations were compared with results from freshly prepared solutions. In general, the recoveries were between 80 and 125%. Therefore, the analytes did not suffer from any significant deterioration.

3.5. Application to samples

The applicability of the new developed analytical method was assayed analyzing 30 saliva samples from elderly people with neurodegenerative diseases. Some analytes (5-*F*_{2t}-IsoP, 15-keto-15-*F*_{2t}-IsoP, 15-keto-15-*E*_{2t}-IsoP, PGE₂, 15-*E*_{2t}-IsoP, PGF_{2α}, 4(*RS*)-*F*_{4t}-NeuroP, 7(*RS*)-ST-Δ⁸-11-dihomo-IsoF, 10-*epi*-10-*F*_{4t}-NeuroP and 14(*RS*)-14-*F*_{4t}-NeuroP) were detected in all the saliva samples (Table 5), indicating the suitable analytical method sensitivity. In addition, signals from total parameters (IsoPs, NeuroPs, IsoFs, NeuroFs) were detected in saliva samples. These results are expressed

Table 5
Concentrations found for each compound in saliva samples from participants.

Analyte	Saliva samples (n = 30) (ng mg ⁻¹ protein)	
	Median	IQR ^a (Q1-Q3)
15(R)-15-F _{2t} -IsoP	0.04	0.03-0.2
1a,1b-dihomo-PGF _{2α}	0.56	0.35-1.16
2,3-dinor-15-epi-15-F _{2t} -IsoP	<LOD	-
15-keto-15-F _{2t} -IsoP	0.04	0.02-0.13
15-keto-15-E _{2t} -IsoP	0.09	0.02-0.18
PGE ₂	0.25	0.14-0.42
15-E _{2t} -IsoP	0.09	0.04-0.15
15-F _{2t} -IsoP	<LOD	<LOD -0.01
PGF _{2α}	0.24	0.17-0.37
4(RS)-F _{4t} -NeuroP	0.12	0-0.66
7(RS)-ST-Δ ⁸ -11-dihomo-IsoF	0.3	0.12-0.42
5-F _{2t} -IsoP	0.11	0.05-0.24
10-epi-10-F _{4t} -NeuroP	0.1	0.06-0.3
17-epi-17-F _{2t} -dihomo-IsoP	0.17	0.04-0.3
17-F _{2t} -dihomo-IsoP	0.25	0.09-0.54
17(RS)-10-epi-SC-Δ ¹⁵ -11-dihomo-IsoF	0.2	0.09-0.49
Ent-7(RS)-7-F _{2t} -dihomo-IsoP	0.05	0.01-0.49
14(RS)-14-F _{4t} -NeuroP	0.62	0.28-3.96
NeuroPs ^b	0.23	<LOD-0.037
IsoPs ^b	0.17	0.1-0.28
NeuroFs ^b	0.22	0.05-0.86
IsoFs ^b	0.16	0.1-0.44

^a IQR: interquartile range.

^b Total parameters results are expressed as (analyte signal intensity / (internal standard signal intensity)) mg⁻¹ of protein in saliva samples.

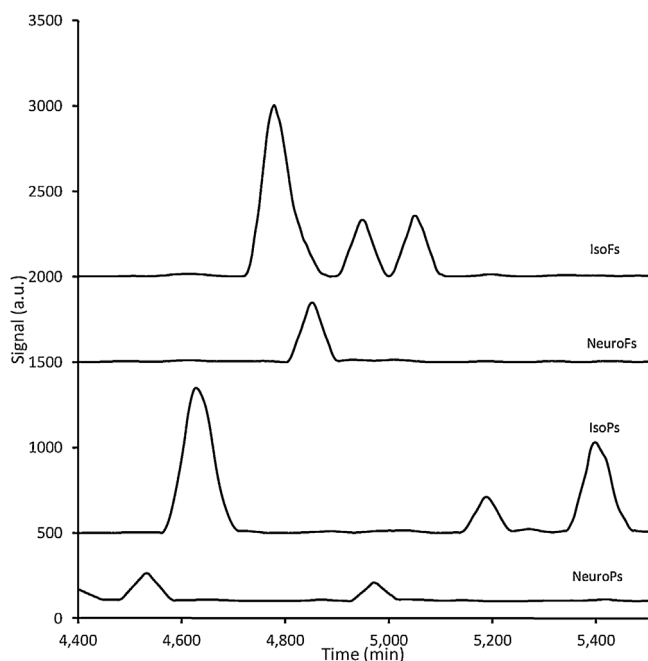


Fig. 2. UPLC-MS/MS chromatograms obtained with the validated method for total parameters (IsoFs, NeuroFs, IsoPs, NeuroPs) found in saliva samples from patients with neurodegenerative disease.

as (analyte signal intensity / (internal standard signal intensity)) mg⁻¹ of protein. As an illustrative example, Fig. 2 shows the chromatograms corresponding to total parameters found in patients' saliva samples.

4. Conclusions

A new and simple analytical method based on liquid-liquid extraction followed by UPLC-MS/MS has been developed to determine simultaneously 18 analytes (F₂-IsoPs, prostaglandins,

F₄-NeuroPs, F₂-dihomo-IsoPs, dihom-IsoFs) and 4 total parameters (IsoPs, NeuroPs, IsoFs, NeuroFs) in limited volume saliva samples. This method showed suitable sensitivity, as well as satisfactory precision and accuracy to be applied to saliva samples from patients with different neurodegenerative pathologies, in which lipid peroxidation could play an important role. This new set of lipid peroxidation biomarkers has not been previously measured in saliva samples, and the lack of validated analytical methods adds relevance to this work.

Nevertheless, further work analysing a higher number of saliva samples from well-defined groups of participants, and using the developed analytical method is required, in order to evaluate these analytes as potential salivary biomarkers in a specific neurodegenerative disease.

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Conflicts of interest

None of the authors of this manuscript declares having conflicts of interest.

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