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Development of a method for the determination of polyamines including *N*-acetylated forms in human saliva via benzoylation and gas chromatography-mass spectrometry

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ABSTRACT

A simple method for the determination of polyamines and their *N*-acetylated forms was developed using benzoyl chloride as derivatization reagent, and 1,6-diaminohexane as internal standard, followed by liquid-liquid extraction with ethyl acetate. The organic extract was injected in a gas chromatograph using a programmed temperature vaporizer and the determination and quantification was performed with a quadrupole mass spectrometer. There was no matrix effect with the proposed method, so internal calibration was used to quantify the corresponding derivatives. Good linear responses were obtained in the range from the limits of detection to 500 μg L^{-1} (50 μg L^{-1} for spermidine), with correlation coefficients varying from 0.9591 to 0.9968. The limits of quantification ($S/N = 10$) ranged 1.0 – 8.3 μg L^{-1}. Recoveries were found between 82 – 117%, showing the good accuracy of the proposed method. Intra- and inter-day precision assays, expressed as relative standard deviation (RSD) were evaluated at two different concentration levels (low and high), showing values in the range of 2.4 – 6.1% and 5.2 – 9.0% for repeatability and reproducibility, respectively (6.9 – 9.7% and 14.1 – 14.6% for spermidine). Successful determination of the studied polyamines and their *N*-acetylated forms was performed on the saliva of 17 volunteers.

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1. Introduction

Polyamines (putrescine, cadaverine, spermidine, spermine) represent a class of biogenic amines (BAs) naturally occurring in animals, plants and different microorganisms [1], coming from the amino acid metabolism. It is well known that abnormal values of polyamine concentrations are closely related to different diseases [2-8] and hence, their determination in different matrices has become an important challenge in the last years. Since these BAs can be formed through microbial activity [9], different food and drinks have been analysed to determine their concentration to verify quality and freshness [10-12], being not equally accumulated within foods [13]. Regarding human samples, polyamines and related compounds have been determined in matrices such as cancer cells [14], urine [15,16], serum and saliva [4,17-20]. The use of urine or saliva represent interesting non-invasive ways to determine these biomarkers avoiding the stress of sample collection to the patients. Different strategies have been followed to determine polyamines in saliva, usually derivatizing prior to analysis. Most commonly used derivatization reagents are *o*-phthaldialdehyde [21], 4-(*N*,*N*-dimethylaminosulfonyl)−7-fluoro-2,1,3-benzoxadiazole (DBD-F) [3,4], or fluorescein isothiocyanate [22] and most of the works use liquid chromatography [3,4,19,21] or capillary electrophoresis [17] as separation techniques prior to determination, mainly using mass spectrometry or fluorescence detection systems. When using gas chromatography, derivatization is also needed to overcome the main issues of high polarity and low volatility that polyamines present. Derivatization with benzoyl chloride (BzCl) has proved to be a very effective methodology for the determination of polyamines in different matrices such as beer [10], wine [11,23,24], plant foods [12] or cancer cells [14], and these derivatives have proved to be very stable in time [23], which facilitates handling and analysis of derivatized samples. Benzoyl chloride has also been applied to the analysis of human urine [25] but not to saliva. This is an easy-to-collect useful matrix [26] and, in our opinion, the development of new reliable methodologies that can take advantage of such non-invasive samples is of outmost value to avoid stress to patients during sample collection.

To the best of our knowledge, there are only two examples in literature where different polyamines were analysed in saliva using gas chromatography. In the first one, published by K.R. Kim et al. in 1997 [27], the authors claimed that their protocol, consisting of two consecutive derivatization reactions (isobutyloxycarbonyla-

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tion and *tert*–butyldimethylsilylation) using solid phase extraction, was applied to saliva samples, although they did not present any data to quantify the concentrations found. Recently, we have published the determination of putrescine, cadaverine, spermidine, L-ornithine and gamma-aminobutyric acid (GABA), in saliva *via* in situ derivatization with ethyl chloroformate and microextraction by packed sorbents using gas chromatography and mass spectrometry [18].

In our opinion, the development of new reliable methods for the determination of this class of biomarkers using non-invasive samples are of outmost importance, avoiding further disturbance to patients during sample collection.

Here, we describe the development and validation of a methodology based on derivatization with benzoyl chloride followed by liquid-liquid extraction (LLE) and further injection into a gas chromatograph with a programmed temperature vaporizer (PTV). A single quadrupole mass spectrometer was used for the determination and quantification of putrescine, cadaverine, *N*-acetyl-putrescine, *N*-acetyl-cadaverine and spermidine in saliva samples from 17 subjects, showing the applicability of this methodology.

2. Experimental

2.1. Reagents and standards

N-Acetyl-putrescine hydrochloride (98%, Ac-PUT), cadaverine dihydrochloride (98%, CAD), putrescine dihydrochloride (98%, PUT), spermidine (99%, SPD), 1,6 diaminohexane (98%, DAH), benzoyl chloride (99%, BzCl) and ethyl acetate (HPLC grade) were supplied by Sigma-Aldrich (Steinheim, Germany). *N*-Acetyl-cadaverine (*N*-5-aminopentyl acetamide, Ac-CAD, 97%) was purchased from ABCR (Karlsruhe, Germany). Sodium hydroxide (reagent grade) and sodium chloride (99.5%) were purchased from Scharlab (Barcelona, Spain). Stock solutions (1000 mg L^{-1}) of each single compound, were prepared in ultra-high-quality water (UHQ, obtained with a Wasserlab Ultramatic water purification system, Noain, Spain). The internal standard solution of DAH (1 mg L^{-1}), used to spike the different solutions studied, was prepared by further dilution of the previous 1000 mg L^{-1} stock solution. All solutions were stored at 8 °C. Before use, these solutions were left to warm up to room temperature and subsequently diluted to prepare the working solutions for spiking samples. Unless otherwise stated, all the optimisation studies were done with UHQ-water spiked with 500 μg L^{-1} of each analyte.

2.2. Saliva samples

Unstimulated saliva samples were obtained from 17 subjects (9 females and 8 males) and collected in 10 mL headspace vials. All the samples were collected after at least 1 hour without eating, drinking, smoking or brushing their teeth. The containers were seal-closed and kept at −20 °C until analysis (typically 24–72 h) [28]. After thawing at room temperature, samples were vortexed at maximum speed during 1 min for homogenization prior to use.

2.3. Derivatization, extraction procedure

Saliva samples were treated as follows: in a 10 mL headspace vial, 100 µL of saliva was added followed by 900 µL of UHQ-water and 100 µL of internal standard (1 mg L^{-1} DAH solution). Then, 1000 µL of NaOH (5 M) and 5.0 µL of benzoyl chloride neat were added. The vial was sealed, and the mixture was stirred with a magnetic bar for 60 s. Next, 1.0 mL of ethyl acetate was added for extraction, stirring for further 30 s. The vial was centrifuged at 3000 rpm for 5 min. One hundred microliters of the organic layer were then placed in a 1.0 mL GC vial with a 300 µL-insert and septum cap for injection.

Table 1
Retention times, selected ions for quantification (in bold) and identification of the polyamine derivatives, including internal standard, and SIM groups used.

Compound	t_R/min	**Quantifier** and qualifier ions	SIM group
Ac-PUT	5.05	**105**, 77, 134	**1**
Ac-CAD	5.34	**105**, 77, 134	
SPD	6.58	**105**, 77, 134	
PUT	7.31	**105**, 77, 174	**2**
CAD	7.99	**105**, 77, 134	**3**
DAH	8.78	**105**, 77, 134	

2.4. Instrumental conditions

All steps were automatically performed with a MPS2 Multi-Purpose Sampler (Gerstel, Mülheim an der Ruhr, Germany). A 20 μL volume (100 µL syringe from Gerstel GmbH & Co. KG) of the ethyl acetate extract was injected into the programmed temperature vaporizer filled with an empty deactivated baffled glass liner (71 mm x 2 mm I.D., Gerstel CIS-4). The inlet was operated in the solvent vent mode and the conditions were as follows: the initial temperature of the injector was set at 90 °C for 0.55 min with a vent flow of 100 mL min^{-1} (2 psi). After venting, the liner was rapidly heated (720 °C min^{-1}) up to 275 °C (injection time was 2 min). Then, the temperature was maintained during 5 min with split valve opened for cleaning, using a split vent purge flow of 100 mL min^{-1}. Finally, liquid CO_2 (Air Liquide) was used to reach initial conditions again.

All analyses were performed on a GC–MS instrument (Agilent Technologies, Santa Clara, CA, USA) consisting of an Agilent 7890A series gas chromatograph interfaced to an Agilent 5975C inert XL MSD. The chromatographic column was a 30 $m \times$ 0.25 mm, 0.25 μm HP-5MS UI capillary column (J&W Scientific, Folsom, CA, USA) and He (99.999% pure; Air Liquide) was used as carrier gas (flow rate of 1.5 mL min^{-1}). GC oven temperature program: 100 °C for 0.6 min to 115 °C (held for 0 min) at 95 °C min^{-1}. Then at 65 °C min^{-1} to 175 °C (held for 0 min), and finally at 45 °C min^{-1} to 300 °C (held for 5 min). Total chromatographic run time was 9.46 min. The total time needed for analysis per sample was 16 min: 1.0 min for derivatization reaction, 0.5 min for organic extraction, 5.0 min for centrifugation and 9.5 min for PTV-GC/MS run.

The MS was performed using a quadrupole mass spectrometer detector in EI mode (70 eV). The ion source and transfer line temperatures were 230 °C and 300 °C, respectively. Synchronous scan/SIM mode was used for collection of both types of data in each run (solvent delay: 4.50 min). The scan *m/z* range was set to 50–400 amu with a sampling rate of 8.01 scan s^{-1}. In the selected ion monitoring (SIM) mode, one quantification and two qualifier ions were monitored for quantification purposes. Three SIM groups with a dwell time value of 1 ms each (except ion 105 of SPD, set at 10 ms), and different *m/z* were employed (Table 1). Data were acquired using MSD ChemStation, Ver. E.02.00.493 software from Agilent Technologies. NIST_98 (NIST/EPA/NIH Mass Spectral Library, version 2.0) database was used for identification.

3. Results and discussion

For the optimisation studies, unless otherwise stated (changes corresponding to the optimised variable in each of the following sections), the instrumental conditions used were the ones aforementioned in Section 2.3 and using 1.0 mL of UHQ-water spiked with 500 μg L^{-1} of each analyte.

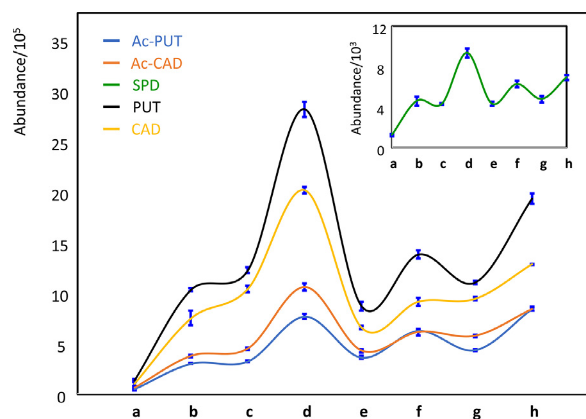

Fig. 1. Combined effects of concentrations of benzoyl chloride, sodium hydroxide and sodium chloride on 1.0 mL of UHQ-water spiked with 500 μg L^{-1} of each analyte: 500 µL NaOH (5 M) + 2.5 µL BzCl without NaCl (**a**), 500 µL NaOH (5 M) + 5.0 µL BzCl without NaCl (**b**), 1000 µL NaOH (5 M) + 2.5 µL BzCl without NaCl (**c**), 1000 µL NaOH (5 M) + 5.0 µL BzCl without NaCl (**d**), 500 µL NaOH (5 M) + 2.5 µL BzCl with NaCl (**e**), 500 µL NaOH (5 M) + 5.0 µL BzCl with NaCl (**f**), 1000 µL NaOH (5 M) + 2.5 µL BzCl with NaCl (**g**), 1000 µL NaOH (5 M) + 5.0 µL BzCl with NaCl (**h**).

3.1. Optimization of the derivatization reaction

As stated in the Introduction, derivatization with benzoyl chloride has been used previously for the determination of polyamines but never in saliva samples. The main variables optimised were the volume of BzCl and NaOH (5 M), ionic effect by addition of NaCl and reaction time.

Typical volumes used for this derivatization are around 5 µL of pure benzoyl chloride [14,29]. Results showed that the higher the volume of reagent used, the better the signal obtained. Considering the significant differences found, especially for putrescine and cadaverine, we decided to use 5.0 µL as standard derivatization reagent volume. Higher amounts would only produce more by-products [30], and reduce the lifetime of all the equipment parts involved. Volumes from 62.5 to 1000 µL of a 5 M NaOH solution were tested obtaining the best result with 1000 µL. It is well known that acyl chlorides are very reactive reagents, and they can react with water and other solvents to produce benzoic acid, benzoic anhydride and alkyl benzoates [30]. Both the optimisation of BzCl and NaOH (5 M) volumes were tested without and with the presence of NaCl (see Fig. 1). Reaction times were studied in the range of 1.0 to 30 min. The best results were already obtained with only 1.0 min of time.

3.2. Optimization of the extraction conditions

Different organic solvents were tested for the extraction step, such as: chloroform [14,29], diethyl ether [30,31], dichloromethane, carbon tetrachloride, ethyl acetate or toluene [32]. The best results were obtained with ethyl acetate. Organic solvent volume (1.0 and 2.0 mL) and extraction time (0.5, 1.0 and 2.0 min) were finally optimised to 0.5 min and 1.0 mL respectively.

3.3. Optimization of the instrumental conditions

3.3.1. Programmed temperature vaporizer conditions

Different liner packing materials were studied to compare with a baffled glass empty liner: glass wool and Tenax TA®. A baffled glass empty liner was the selected option since the other two were not compatible with all the polyamines studied.

Initial (60 – 120 °C, using 275 °C as final temperature) and final (200 – 275 °C, using 90 °C as initial temperature) PTV temperatures were evaluated selecting the solvent vent injection mode (see Fig. S1-a and S1-b, supplementary material). As the optimum initial temperature, we chose 90 °C where we observed the best initial retention and least loss of material during venting. For final temperature, 275 °C was chosen where signal improvements of 2.5, 3.1 and 2.4 folds for Ac-PUT, Ac-CAD and SPD derivatives, respectively. In all cases, blanks analysed after injections of samples containing polyamines provided no signals above detection limits, showing that desorption was complete.

Vent flow was evaluated with values ranging from 50 to 150 mL min^{-1}.(see Fig. S1-c, supplementary material). As working vent flow, we chose 100 mL min^{-1} since we observed an improvement from 50 mL min^{-1} but not with further increase to 150 mL min^{-1}. Injection time was also studied (1.0 – 2.0 min) obtaining 2.0 min as optimum (see Fig. S1-d, supplementary material).

Injection volume and speed were also optimised to 20 µL and 25 µL s^{-1} respectively.

3.3.2. Gas chromatography-mass spectrometry

Initial (50–150 °C, keeping final temperature at 300 °C) and final (250 °C - 325 °C, using 100 °C as starting temperature) oven temperatures were evaluated selecting always the maximum ramps permitted by the gas chromatograph configuration to achieve a fast separation (see Fig. S2-a and S2-b in supplementary material). Optimal temperatures were observed to be 100 °C and 300 °C respectively.

Mass spectrometer worked on simultaneous SIM/Scan mode for identification and quantification. Dwell times of the SIM mode were optimised, selecting 1 ms as the optimum value for all the ions except for *m/z* 105 of spermidine which gave better results using 10 ms. Selected ions for quantification and identification of the polyamine derivatives, including internal standard, and SIM groups used are shown in table 1.

3.4. Matrix effect and saliva sample volume

In some cases, analytes can be more present in the mucous part of saliva than in the aqueous part. To check this, we tested to centrifuge the saliva sample before reaction and then perform the derivatization and extraction process over the supernatant (including final extra centrifugation for separation of aqueous and organic layers). For this, 1.0 mL of pure saliva spiked with 420 µg L^{-1} for Ac-PUT, Ac-CAD, PUT and CAD and 42 µg L^{-1} for SPD was submitted to centrifugation at 3000 rpm for 5.0 min. The concentrations used here were optimised considering the expected values for saliva, as reported in literature. The supernatant was transferred to a different vial and then submitted to the standard process from here. The result was compared with the same saliva, diluted 1:10, and spiked with the same final concentration, which was submitted to the standard process as described in Section 2.3. Smaller signals (13 – 85% less) were obtained after using the supernatant of centrifuged saliva, proving that the analytes seem to be retained in the mucous part. Hence we decided to not centrifuge the samples.

Matrix effect was first evaluated by comparing the signals obtained from UHQ-water and a 1.0 mL of pure saliva sample, both spiked at the same concentration, that is, 420 µg L^{-1} for Ac-PUT, Ac-CAD, Put and CAD and 42 µg L^{-1} for SPD. DAH at 100 µg L^{-1} was added as internal standard in all solutions. After the extraction step with ethyl acetate, it was not possible to separate the organic from the aqueous layer. Then, we decided to dilute the saliva sample with UHQ-water (1:2, 1:5 and 1:10), keeping the same total concentration as before and final volume of 1.0 mL. Once the saliva sample was diluted, the issue of separating both layers was resolved and the analysis could be run. In addition, there was no significant difference between dilutions, showing that the presence

of saliva matrix did not affect the result and, hence, that there was no matrix effect. Considering the amount of saliva that a person can provide at once, we decided to use the lesser amount of pure saliva, i.e., 100 µL in this case, and dilute it to a final volume of 1.0 mL with UHQ-water, so we could be able to analyse more replicates using the same saliva sample.

The absence of matrix effect was further investigated by the analysis of the slopes of calibration curves from UHQ-water and 4 diluted saliva samples, obtained from 4 apparently healthy subjects, spiked in the range from 0.0 µg L^{-1} (unspiked sample) to 500 µg L^{-1} for all analytes and 50 µg L^{-1} for spermidine. DAH at 100 µg L^{-1} was added as internal standard in all solutions. All the calibration models presented good linear behaviour and no lack of fit was observed after ANOVA analysis. As shown in Table 2, there were not significant differences amongst the slopes of the different matrices and UHQ-water, indicating the absence of matrix effect. Thus, quantification of saliva samples was performed using internal standard calibration.

3.5. Method validation

Solutions of the studied polyamines in UHQ-water were used for the obtention of the calibration curves, as well as the determination of the limits of detection (LODs) and quantification (LOQs). Seven concentration levels were measured in triplicate, using the peak areas of the chromatograms obtained with the quantification ion extracted in the SIM mode for each analyte. Internal standard was always used to correct signals. As shown in Table 3, all calibration models presented good linear behaviour ($R^2 > 0.95$) and did not show any lack of fit after ANOVA analysis. LODs and LOQs were calculated following the ISO 11,843–1 and other authors' recommendation ($S/N = 2•H/h$, where H is the height of the neat peak corresponding to the considered analyte and h is the range of the blank noise measured around the place where the analyte elutes) [33] [34]. Values of $S/N = 3$ and $S/N = 10$ were used for the determination of LOD and LOQ, respectively. The detection limits ranged 0.3 – 2.5 µg L^{-1}, and quantification limits ranged 1.0 – 8.3 µg L^{-1}. Precision of intra- and inter-day assays were determined at two concentration levels, low level (Ac-PUT, Ac-CAD, PUT and CAD: 100 µg L^{-1}; SPD: 10 µg L^{-1}) and high level (Ac-PUT, Ac-CAD, PUT and CAD: 1000 µg L^{-1}; SPD: 100 µg L^{-1}). Repeatability (intra-day precision) was determined by analysing ten replicates of each concentration level on the same day. Reproducibility (inter-day precision) was determined by measuring five times each concentration level during five different days. The relative standard deviation (RSD, as percentage) obtained was equal or less than 6.1% and 9.0% for repeatability and reproducibility, respectively (9.7% and 14.6% for SPD). The accuracy of the method was calculated as the ratio of the found concentration to the added concentration (i.e., apparent recoveries, expressed as percentage) for four different spiked saliva samples, subtracting the signals obtained from the unspiked samples in every case. Saliva samples were 1:10 diluted and spiked with the following concentrations: 420 µg L^{-1} for Ac-PUT, Ac-CAD Put and CAD, and 42 µg L^{-1} for SPD. Good mean values were found between 82 and 117% (Table 4), highlighting the applicability of this method to quantify polyamines in saliva samples.

3.6. Analysis of saliva samples

To prove the applicability of the method proposed in this work, it was used to analyse 17 saliva samples (obtained from 9 females and 8 males). Results are shown in Fig. 2 (see table S1 in supplementary material). All concentrations found agreed with those reported in literature [3,35] and with results previously obtained by our group [18,19]. It is especially noteworthy the concentration

Table 2
Comparison of the slopes found for the linear equations obtained with UHQ-water and four diluted saliva samples from subjects S1, S2, S5 and S6.

Compound	UHQ-water		S1		S2		S5		S6	
	R^2	Slope	R^2	Slope	R^2	Slope	R^2	Slope	R^2	Slope
Ac-PUT	0.9700	0.0022 ± 0.0002	0.9936	0.0023 ± 0.0001	0.9225	0.0023 ± 0.0004	0.9433	0.0019 ± 0.0002	0.9252	0.0024 ± 0.0005
Ac-CAD	0.9707	0.0036 ± 0.0003	0.9697	0.0035 ± 0.0003	0.9559	0.0032 ± 0.0004	0.9295	0.0029 ± 0.0004	0.9756	0.0031 ± 0.0006
SPD	0.9591	0.007 ± 0.001	0.9211	0.009 ± 0.002	0.9606	0.006 ± 0.001	0.9263	0.008 ± 0.001	0.9214	0.005 ± 0.002
PUT	0.9968	0.020 ± 0.001	0.9887	0.022 ± 0.002	0.9547	0.019 ± 0.002	0.9539	0.022 ± 0.003	0.9548	0.021 ± 0.005
CAD	0.9949	0.019 ± 0.002	0.9802	0.020 ± 0.002	0.9861	0.018 ± 0.001	0.9957	0.019 ± 0.001	0.9423	0.024 ± 0.006

Table 3
Analytical characteristics of the proposed method for polyamine derivatives[a,b].

Compound	Calibration range (μg L^{-1})	Linear equation	R^2	LOD (μg L^{-1})	LOQ (μg L^{-1})	Repeatability (RSD,%)		Reproducibility (RSD,%)	
						Low level	High level	Low level	High level
Ac-PUT	LOD – 1000	$y = (0.0022 \pm 0.0002)$ x - (0.05 ± 0.06)	0.9700	2.0	6.6	6.1	4.6	8.2	8.0
Ac-CAD	LOD – 1000	$y = (0.0036 \pm 0.0003)$ x - (0.05 ± 0.09)	0.9707	2.5	8.3	5.8	4.6	7.5	6.6
SPD	LOD – 100	$y = (0.007 \pm 0.001)\,x + (0.01 \pm 0.02)$	0.9591	0.4	1.3	9.7	6.9	14.6	14.1
PUT	LOD – 1000	$y = (0.020 \pm 0.001)\,x + (0.2 \pm 0.4)$	0.9968	0.3	1.0	5.9	2.4	8.3	7.5
CAD	LOD – 1000	$y = (0.019 \pm 0.002)$ x - (0.4 ± 0.4)	0.9949	1.0	3.3	3.7	2.6	9.0	5.2

[a] Concentrations in level LOW: Ac-PUT, Ac-CAD, PUT and CAD: 100 μg L^{-1}; SPD: 10 μg L^{-1}.
[b] Concentrations in level HIGH: Ac-PUT, Ac-CAD, PUT and CAD: 1000 μg L^{-1}; SPD: 100 μg L^{-1}.

Table 4
Mean recovery values (%) obtained for 4 different spiked saliva samples.

Compound	Added concentration (mg L^{-1})	Found concentration (mg L^{-1})	Recovery (%)
Ac-PUT	0.420	0.49 ± 0.05	117
Ac-CAD	0.420	0.41 ± 0.03	97
SPD	0.042	0.045 ± 0.005	107
PUT	0.420	0.48 ± 0.03	113
CAD	0.420	0.34 ± 0.03	82

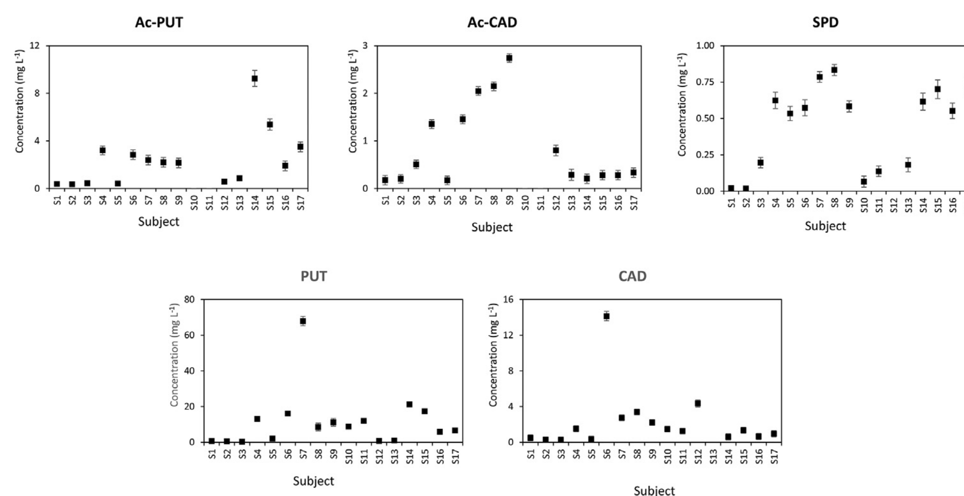

Fig. 2. Values of concentration of Ac-PUT, Ac-CAD, SPD, PUT and CAD (for numerical values, please see table S1 in supplementary material). Prediction intervals represented as uncertainty bars. See Experimental section for more details.

of putrescine found in subject S7, which belongs to a person diagnosed with rheumatoid arthritis. Although only one sample is not enough to make a significant conclusion, the results observed for this sample are in agreement with the already well known fact that high levels of putrescine are related to this disease [8,36-38]. For those samples where a high level of PUT was observed, two separate analyses were performed: one following the standard procedure, and one additional, diluting more the sample, taking 10 μL of saliva and adding 990 μL of UHQ-water, in order to correctly determine the concentration of PUT. High concentrations of cadaverine can be found in certain food like fish and its derivatives [39], which might explain the high levels found in subject S6.

As an example, Fig. 3 shows the chromatograms of two of the saliva samples analysed (S2, and S14) compared to the lowest level of calibration in UHQ-water (spiked with 100 μg L^{-1} of Ac-PUT, Ac-CAD, PUT and CAD and with 10 μg L^{-1} of SPD). Note that the peak at 8.13 min corresponded to a hydrolysis product of benzoyl chloride [30].

3.7. Comparison with other works

Although benzoylation is a well-known reaction to derivatize this type of compounds, it has not been applied yet for the analysis of saliva, but of other matrices such as beer [10], wine [11,23,24], plant foods [12] or cancer cells [14]. Other derivatization methods have been used to determine polyamines in saliva, but either using liquid chromatography or capillary electrophoresis for separation. Regarding the use of GC–MS, there are only two examples in literature where derivatization was used to quantify polyamines in saliva. In 1997, Kim et al. described a rather long protocol which included a first derivatization, followed by a solid phase extraction and then a second derivatization [27], but no LODs or LOQs were reported. More recently, our group published [18] the use of ethyl chloroformate as derivatization reagent and microextraction by packed sorbents. As shown in table 5, the protocol we propose here is faster than any of the available in literature at the moment and still allows the determination of these polyamines with LOQs in the range of μg L^{-1}.

4. Conclusions

Here we report the first application of derivatization with benzoyl chloride of putrescine, cadaverine, spermidine, acetylputrescine and acetyl-cadaverine and the analysis of the corresponding derivatives in saliva samples by PTV-GC/MS. All parts of this methodology have been optimised, from the derivatization reaction conditions and extraction process, to the PTV-injector, gas chromatograph and mass spectrometer parameters. We have suc-

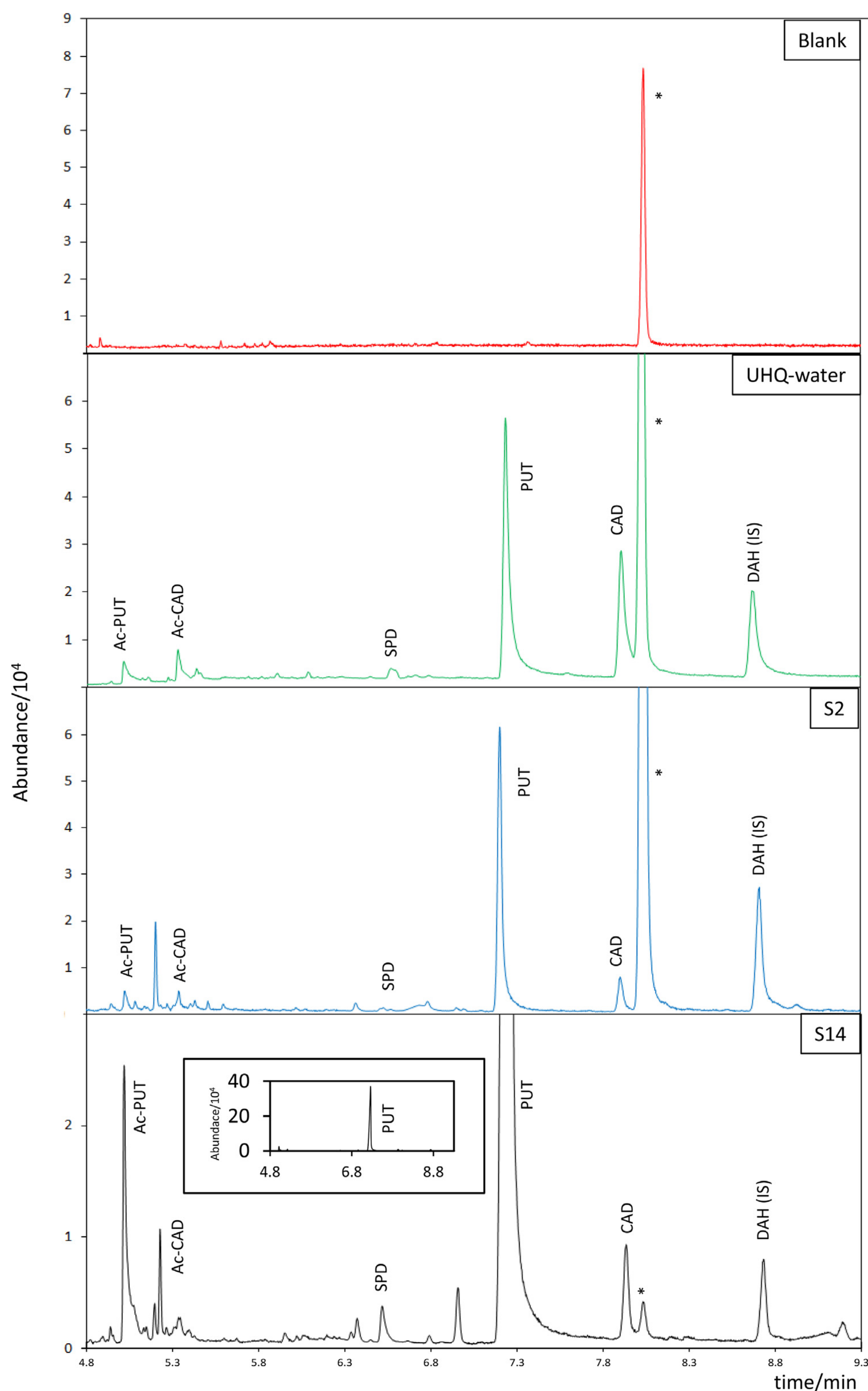

Fig. 3. Chromatograms ($m/z = 105$) obtained for the derivatization reagent blank, UHQ-water spiked at the lowest level of calibration (Ac-PUT, Ac-CAD, PUT and CAD: 100 μg L^{-1}; SPD: 10 μg L^{-1}), and saliva samples S2 and S14. See Experimental section for more details.

cessfully overcome matrix effect allowing to analyse multiple samples using internal calibration in UHQ-water. In this way, good LODs and LOQs have been reached (low μg L^{-1} range), as well as good precision in both, intra- and inter-day assays and accuracy (82–117%). The methodology has been applied to the determination of the studied compounds in 17 saliva samples, finding values in agreement with those reported in literature. The results observed in the analysis of the saliva samples show the applicability of the proposed methodology for the determination of these biomarkers in this type of matrix, and they are in agreement with values previously reported in literature.

Table 5
Comparison with other works.

Method	Derivatization	Polyamines[a]	LOQ[b] (µg L^{-1})	Analysis time per sample (min)[c]	Ref.
GC–MS	*iso*BOC + TBDMS	SPD, PUT, CAD(57)	ND	197	[27]
HPLC-FL	OPA	SPD, PUT (3)	5.2 – 7.2	59	[21]
UPLC-MS/MS	DBD-F	Ac-PUT, SPD, PUT, CAD (11)	0.3 – 3.6[d]	65	[4]
CE-LIF	FITC	SPD, PUT, CAD (6)	0.0024 – 0.062	1005	[22]
UPLC-MS/MS	DBD-F	Ac-PUT, SPD, PUT, CAD (12)	$< 2 \bullet 10^{-11}$ (e)	65	[3]
LC-MS/MS	none	PUT, CAD (8)	6.39 – 9.52	26.5	[19]
GC–MS	ECF	SPD, PUT, CAD (5)	8.76 – 14.3[f]	47	[18]
GC–MS	**BzCl**	**Ac-PUT, Ac-CAD, SPD, PUT, CAD**	**1.0 – 8.3**	**16**	**This work**

[a] only polyamines in common with present work; Total number of compounds studied in brackets.
[b] limits of quantification for the selected polyamines in this table.
[c] including sample treatment, derivatization, extraction and separation steps.
[d] ng L^{-1}.
[e] mol L^{-1}.
[f] determined with two saliva samples.ND: no data available; *iso*BOC: isobutyloxycarbonylation; SPE: solid phase extraction; TBDMS: *tert*–butyldimethylsilylation; CE-LIF: Capillary electrophoresis with laser-induced fluorescence detection; OPA: *o*-phthaldialdehyde; DBD-F: 4-(N,N-dimethylaminosulfonyl)−7-fluoro-2,1,3-benzoxadiazole; FITC: fluorescein isothiocyanate; ECF: ethyl chloroformate.

Credit author statement

Javier Peña: Methodology, Validation, Formal analysis, Investigation, Writing original and revised manuscript. **Mª Esther Fernández Laespada:** Methodology, Validation, Writing original and revised manuscript. **Carmelo García Pinto:** Methodology, Validation, Writing original and revised manuscript. **José Luis Pérez Pavón:** Term, Conceptualization, Supervision, Project administration, and Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462278.

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