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# Determination of polyamines and related compounds in saliva *via in situ* derivatization and microextraction by packed sorbents coupled to GC-MS

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ABSTRACT

Here we show the determination of different polyamines (putrescine, cadaverine, spermidine) and related compounds (gamma-aminobutyric acid and L-ornithine) in saliva samples. These compounds are known to be biomarkers for several diseases. We have optimised an *in situ* derivatization process using ethyl chloroformate, an automated microextraction by packed sorbent and the determination of the corresponding products using a programmed temperature vaporizer coupled to a gas chromatograph – mass spectrometer. After finding that saliva matrix has an effect on the analysis, quantitation was performed using the one-point standard additions method and normalization to IS. This allows the detection of the analytes in the range of μg/L within a matrix obtained by a non-invasive procedure. The method has been successfully validated and it has been used in the determination of these compounds in six saliva samples finding that putrescine and cadaverine present the highest concentrations in the subject diagnosed with rheumatoid arthritis. For ornithine and spermidine, the highest concentrations were found for male subjects, especially heavy smokers. All concentrations found for the compounds were in good agreement with data found in bibliography.

## 1. Introduction

Nowadays, there is an increasing interest on using samples involving non-invasive methods of collection for biomarker discovery and analysis. Among matrices, urine has been the most used [1,2], although saliva samples also represent an interesting option for analysis [3–5]. Saliva is a clear, slightly acidic mucoserous exocrine fluid secretion [6] formed by approximately 99% water, with a variety of electrolytes and proteins, along with glucose and nitrogenous metabolic products [7]. Saliva samples have been successfully used for the determination of several kind of compounds, such as drugs [7–9], organic contaminants [10], heavy metals [11,12] or biomarkers of exposure [13,14] and disease [15–19].

The selection of the aforementioned biological matrices implied the use of different sample preparation techniques, which is a critical step of any bioanalytical method development. Extraction procedures mainly include liquid-liquid extraction (LLE) or solid-phase extraction (SPE) [20]. However, in recent years, attention has been drawn to the development of microextraction methods due to the fact that they require lower volume of sample, together with easier handling and simpler equipment, as well as less consumption of reagents [21]. Among techniques, microextraction by packed sorbents (MEPS), a miniaturized version of SPE, represents an interesting option. The technique, discovered in 2004 by Mohamed Abdel-Rehim [22], has gained a broad attention and several reviews have already been published where the principles and main applications of the technique have been described [23–25]. MEPS has been applied to the determination of several kind of compounds in saliva samples, such as drugs [26–29] and steroids [30], but only coupled to liquid chromatography (LC). Although MEPS coupled to GC has been successfully applied for the determination of polyamines and amino acids in urine samples [31,32], to the best of our knowledge, there is no publication in bibliography where this methodology has been applied to saliva sample analysis.

Polyamines are organic compounds derived from amino acids metabolism, which have long been associated with cell growth, hypertrophy and tissue growth and with several diseases, such as cancer [33,34]. The determination of these compounds in saliva samples has become a potential non-invasive tool as markers of physiological conditions, as shown in several metabolomic studies [18,19,35]. LC [17,36–39], capillary electrophoresis (CE) [40,41] and fluorometric determination [42] have mainly been employed for the determination of these compounds in saliva. Due to the high polarity, low volatility,

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lack of fluorescence and weak UV absorption of these compounds, different derivatization reagents have been proposed for their determination, such as *o*-phthaldialdehyde [36], 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole [17,37] or fluorescein isothiocyanate [41]. With regard to gas chromatographic methods, to the best of our knowledge, until now it has not yet been reported any method about the determination of polyamines and related compounds in human saliva using this separation technique.

In this work, we propose for the first time a method based on an *in situ* derivatization reaction and MEPS extraction coupled to GC-MS for the determination of polyamines (putrescine, cadaverine and spermidine) in saliva samples. L-ornithine and gamma-aminobutyric acid have also been included since they have been described as the main precursor of polyamines [33] and as a catabolism product of putrescine, respectively. In order to reduce the polarity and increase the volatility of the compounds, ethyl chloroformate is proposed as derivatizating reagent. Chloroformates have been widely used for treating amino groups, as well as esterification reagents [43], and present important advantages, such as a very rapid reaction with no need for heat or water exclusion, negligible reagent cost and simple after reaction workup. In our opinion, the development of reliable methods for the determination of biomarkers of disease in samples involving non-invasive methods of sample collection are of outmost importance since patients are not exposed to disturbance during sample collection. To the best of our knowledge, this is the first time that this derivatization procedure, followed by MEPS extraction, injection using a programmed temperature vaporizer and GC-MS analysis, has been applied to the determination of polyamines and amino acids in saliva. We have previously applied this strategy in urine [31]. However, due to the different components and physicochemical properties of saliva in comparison to urine, many analysis parameters need to be re-optimized and new issues related with sample preparation need to be addressed.

## 2. Materials and methods

### 2.1. Chemicals, reagents and standard solutions

Putrescine (Put), cadaverine (Cad), spermidine (Spd), L-ornithine (Orn), gamma-aminobutyric acid (GABA), 1,6-diaminohexane (DAH, used as internal standard), pyridine, ethyl chloroformate (ECF) and methanol were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium hydroxide and ethanol were obtained from Scharlab (Barcelona, Spain). Each standard stock solution was made up in ultra-high quality (UHQ) water at a concentration of 1000 mg/L and stored in darkness at 6 °C until use. A Wasserlab Ultramatic water purification system (Noain, Spain) was chosen to obtain the water used throughout the study.

### 2.2. Saliva samples, collection and preparation

Unstimulated saliva samples for method optimization were taken from six different subjects (4 females and 2 males). Five samples corresponded to non-diagnosed individuals (one of them corresponded to a heavy smoker) and one corresponded to a subject diagnosed with rheumatoid arthritis. The subjects fasted and did not brush their teeth 1 h before collecting the samples. These, were directly collected into a 10-mL vial and stored at −20 °C until analysis. After thawing at room temperature, the saliva was centrifuged at 1815*g* during 10 min at room temperature to separate the denatured mucins. 715 µL of the supernatant was added to a 10-mL glass vial and diluted up to 5.0 mL with UHQ water.

### 2.3. Derivatization reaction

The evaluation of the optimum derivatization conditions was performed with an unspiked saliva sample (endogenous concentrations of compounds). 715 µL of saliva supernatant was mixed with 4285 µL of UHQ water in a 10-mL vial. Then, 66.5 µL of NaOH (5.0 M), followed by 165 µL of pyridine, 200 µL of ethanol and 200 µL of ECF were added successively. The vial was then hermetically sealed and vortexed for 1 min at room temperature. In order to eliminate the $CO_2$ generated during the reaction, vials were sonicated during 1 min at 35 KHz with a needle inserted in the cap septum of the vial. After sonication, samples were filtered (Nylon, 0.45 µm pore size attached to a 5 mL glass syringe) and analysed.

### 2.4. MEPS procedure

Automatic extraction of the analytes was performed using the Gerstel MPS2 Multi-Purpose Sampler (for more details please see reference [31]) using a C18 cartridge. Optimal conditions for MEPS (determined using an unspiked diluted saliva sample) were found to be first, conditioning of the sorbent with 100 µL of EtOH; then, passing 100 µL of UHQ water (flow rate, 25 µL/s). Next, extracting the sample (by drawing and discarding, 5 cycles of 100 µL at a flow rate of 5 µL/s). Then, washing the sorbent with a UHQ water:methanol mixture (80:20, v/v) to eliminate interferences and drying the cartridge by pumping air through it (10 × 100 µL) at a flow rate of 25 µL/s. The compounds were eluted with 10 µL of EtOH and injected into the programmed temperature vaporizer (PTV) at a flow rate of 5 µL/s.

### 2.5. PTV-GC-MS analysis

All experiments were performed using a liquid $CO_2$-cooled Gerstel PTV inlet (CIS-4, Gerstel, Baltimore, MD, USA) with an empty baffled liner (Gerstel) and using the solvent vent injection mode. Septum purge flow rate was fixed at 4 mL/min. The initial injector temperature was set at 90 °C (vent flow: 50 mL/min; vent pressure: 5.00 psi; vent time 0.5 min; the initial temperature of the liner was maintained for 0.55 min). After venting, the PTV was rapidly heated up to 300 °C (12 °C/s) with the split valve closed. Then, the analytes were injected during 2 min into the capillary column. Next, keeping the split valve open, the PTV temperature was maintained at 300 °C for 12.5 min.

GC analysis was carried out on an Agilent 7890A GC equipped with a HP-5MS Ultra Inert capillary column (30 m × 0.25 mm × 0.25 µm), from J&W Scientific (Folsom, CA, USA). Helium N50 (He, 99.9999% pure, from Air Liquide) was used as carrier gas at a flow rate of 1.8 mL/min with constant flow mode. Optimum conditions for GC oven were: initial temperature of 50 °C for 2.5 min, heat up to 70 °C (60 °C/min), then 115 °C (40 °C/min), then 175 °C (30 °C/min) and finally 300 °C (20 °C/min, 3 min). The total chromatographic run time was 15.21 min.

The GC was interfaced to an Agilent 5975C inert XL MSD detector (70 eV, electron ionization mode). The GC-MS interface temperature was set at 300 °C. The temperature of the ion source and quadrupole were 230 °C and 150 °C, respectively. Analysis was performed in synchronous SIM/Scan mode. Full scan data (*m/z* 50–300 amu) was used for identification and SIM data was used for quantification. Sampling of 2 ($2^N$, N = 1) and dwell time of 10 ms were used for acquisition. The number of SIM/Scan cycles/sec were 6.2. A solvent delay of 6 min was established. Data acquisition was performed with MSD ChemStation (v. E.02.00.493) software from Agilent Technologies. Identification of the compounds was performed based on previous published results [31]. Table 1 presents the retention times and the *m/z* ions used for quantitation and identification.

Fig. 1 shows a diagram of the whole analytical procedure.

## 3. Results and discussion

### 3.1. Optimization of the derivatization reaction

Among the several methods described in literature for the determination of amines and amino acids in biological samples, one of the

**Table 1**
Retention times and *m/z* ions used for quantification and identification of the compounds with the optimized method.

| Compound | $t_R$ (min) | *m/z* | |
|---|---|---|---|
| | | Quantitation ion | Qualifier ions |
| GABA | 7.14 | 116 | 86, 102 |
| Put | 8.78 | 102 | 142, 70 |
| Cad | 9.27 | 102 | 74, 128 |
| DAH (IS) | 9.76 | 102 | 130, 74 |
| Orn | 10.00 | 142 | 70, 102 |
| Spd | 12.14 | 142 | 116, 56 |

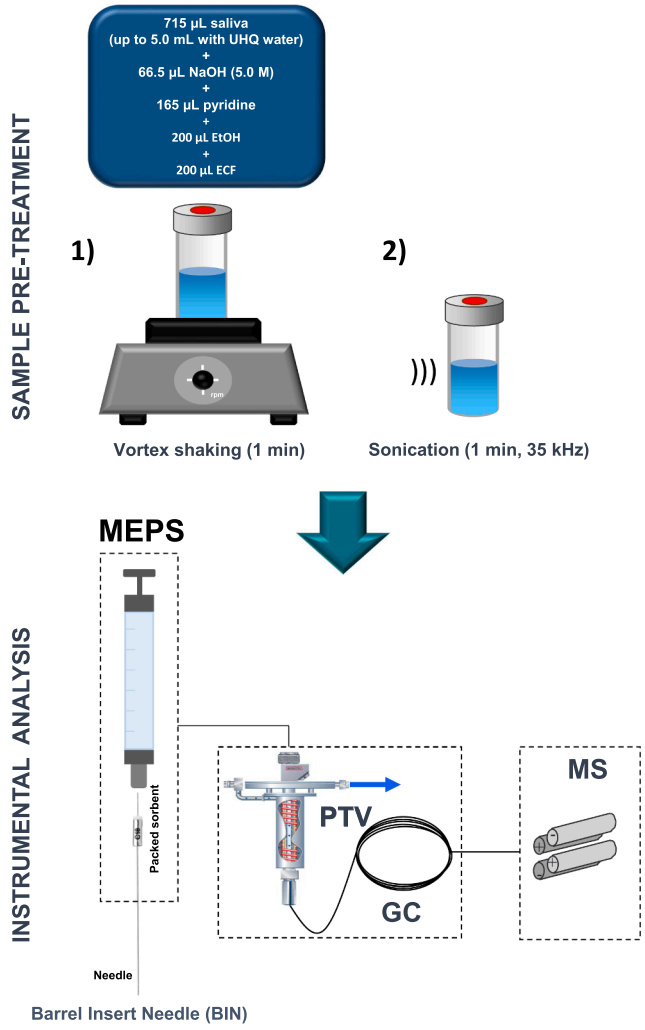


**Fig. 1.** Diagram of the whole analytical procedure.

most commonly used have been alkyl chloroformates [31,43,44], since it allows to perform the reaction directly in aqueous alkaline solution in just seconds. While amines react to form the corresponding carbamate, in the case of carboxylic acids, an ester is formed when pyridine is used after quick decarboxylation of the corresponding anhydride. Since two different products can be formed when the reaction is carried in the presence of an alcohol, it is necessary to choose the same alkyl group in both the alcohol and the alkyl chloroformate in order to have just one single product.

Based on previous results [31], an evaluation of the derivatization conditions in saliva samples was performed. In order to optimize the reaction, 715 µL of an unspiked sample was diluted up to 5.0 mL with UHQ water. Different volumes of the NaOH 5.0 M solution (22–66.5 µL), pyridine (55–165 µL), ethanol (66.5–200 µL) and ECF (66.5–200 µL) were evaluated, maintaining the same ratio of the reagents. Diminishing the concentration of the derivatization reagents implied an increment on the analytical signal for putrescine and cadaverine. However, worse results were obtained for ornithine and spermidine. No analytical signal was obtained for GABA, probably because the concentration of this analyte in the saliva used for optimization was below the limit of the detection (LOD) of the technique. Considering that the chromatographic signal obtained for spermidine was lower than the signals obtained for the rest of the analytes (due to the lower endogenous concentration of the compound in saliva), the highest volumes of the reagents were selected for further experiments, i.e. 66.5 µL of the NaOH solution 5.0 M, 165 µL of pyridine, 200 µL of EtOH and 200 µL of ECF.

During the reaction, $CO_2$ was generated as a by-product making the MEPS procedure less reproducible due to gas aspiring into the syringe during the sampling step. Sonication of samples (35 KHz, 1 min) was performed in order to minimize this effect. In addition, another issue was observed, since some small suspended particles appeared in the matrix after the reaction had been performed. This effect was observed at any concentration of the reagents and even after filtration of the sample before the reaction occurred. Thus, a post-reaction filtration step (Nylon filter, 0.45 µm pore size) was included in the protocol in order to avoid the blockage of the syringe and the MEPS cartridge.

### 3.2. Optimization of the MEPS procedure

Regarding the microextraction procedure, different parameters were evaluated, such as sample loading and washing and elution conditions. Furthermore, it is well known that MEPS extraction can be affected by the nature of the sample when biological fluids are analysed, with the need, on many occasions, to dilute the sample before analysis. Thus, different dilution ratios were also evaluated.

In order to optimize sample loading, it should be noted that the compounds evaluated in the present study are expected at different concentration levels in the saliva samples [3,19]. This should be taken into consideration when evaluating dilutions and number of extraction cycles. Sample loading was optimized using different saliva dilutions in UHQ water (1:50, 1:20, 1:10 and 1:7, v/v) in conjunction with different number of extraction cycles, from one extraction cycle (1 × 100 µL, 100 µL total sampling volume) up to ten cycles (10 × 100 µL, 1000 µL total sampling volume). Lower dilutions were not evaluated in order to avoid high sample consumption and possible cartridge blockage. In all cases, an unspiked saliva sample was used for optimization and final volume was set at 5.0 mL. Conditioning of the cartridge was performed with 100 µL of EtOH followed by 100 µL of UHQ water and sample loading was performed in the drawing-discarding mode. Fig. 2 shows the obtained results. In general, for a fixed number of extraction cycles, a decrease on the dilution ratio implied an increment on the analytical signal of the compounds. In the same way, for a fixed dilution ratio, an increment on the number of extraction cycles implied an increment on the analytical signal. Differences were observed for Spd and GABA. No analytical signal was obtained for Spd in the saliva used for optimization and GABA was only quantifiable with 1:10 and 1:7 dilutions and a minimum number of extraction cycles of 5 and 3, respectively. As a compromise situation, a 1:7 v/v dilution ratio (i.e. 715 µL of saliva and 4285 µL of UHQ water) and five extraction cycles were chosen as optimum. For most of the compounds, higher reproducibility was observed at these conditions. Moreover, sampling flow rate (aspiring and discarding) was also evaluated in the 5 to 25 µL/s range. A value of 5 µL/s was selected as optimum due to the higher signals obtained.

Washing step is a critical parameter during the MEPS procedure. At this point, unwanted weakly retained interferences can be eliminated. UHQ water with different amounts of methanol (0, 10, 20 and 30%, v/v) were evaluated as washing media (100 µL). Optimum results were obtained when 20% of methanol was added to the UHQ water. Fig. 3

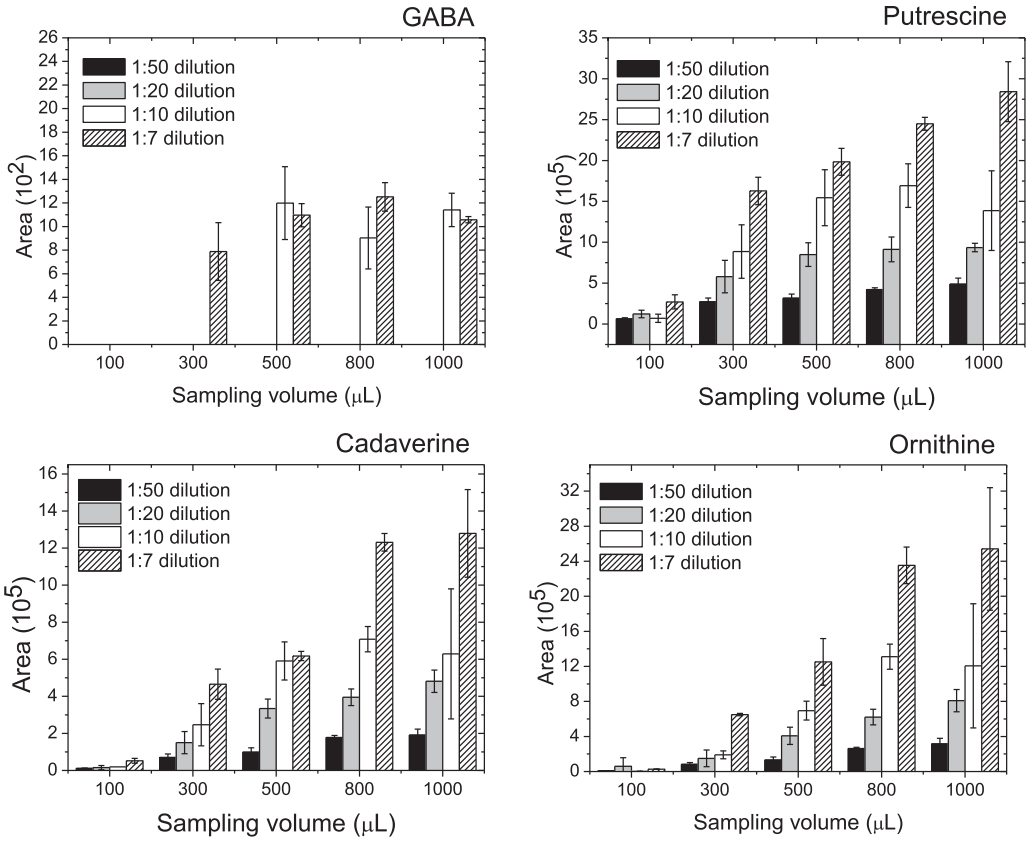


**Fig. 2.** Evaluation of extraction cycles (sample loading) and dilution of saliva.

shows a chromatogram of a saliva sample with two different washings, UHQ water and UHQ water:methanol 80:20, v/v. Cleaner chromatograms were obtained in the second case, mainly in the initial zone of the chromatogram (6.0–8.0 min). Higher organic content implied partial loss of the compounds.

Based on previous studies [31], EtOH was considered an adequate solvent for the elution of the compounds from the C18 cartridge. Hence, different elution volumes (10, 20, 30 and 50 μL) were evaluated with an elution flow rate of 5 μL/s. Similar results were obtained for 10 and 20 μL, indicating that 10 μL was enough volume to release the analytes from the sorbent. Higher volumes implied a decrease on the analytical signal for all of the compounds due to dilution. Thus, an elution volume of 10 μL (injection volume) was selected as optimum.

Carry-over is a common problem when pre-concentration techniques are used. In MEPS, the small amount of sorbent used can be easily washed between injections to reduce carry-over effects. Eight wash-discard cycles (4 with 100 μL EtOH and 4 with 100 μL UHQ water) were programmed after the elution of the compounds from the cartridge. With these conditions, carry-over was evaluated showing values below 0.08% proving the possibility of MEPS cartridge to be reused. In this work, cartridge reusability was evaluated. Under the optimized conditions, it was possible to perform around 110 extractions with the same cartridge.

### 3.3. PTV-GC-MS conditions

After the extraction of the compounds, injection of the eluent was automatically performed into a PTV inlet, using the solvent vent injection mode and an empty baffled liner. In order to optimize the injection, an evaluation of the initial temperature setting on the PTV was evaluated (50, 70, 90 and 110 °C). Previous studies have shown that vent flow and vent time had little influence on the process, so previous optimized values were maintained, i.e. 20 mL/min and 0.5 min, respectively. Similar results were obtained with all the temperatures

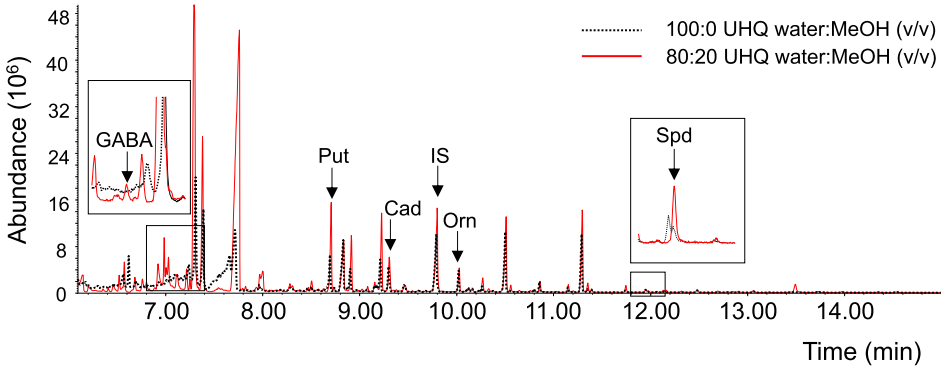


**Fig. 3.** Chromatograms obtained for a saliva sample using (A) only water or (B) a mixture of $H_2O$/MeOH 80:20 (v/v) for washing.

evaluated, so 90 °C was selected as optimum.

For GC-MS measurements, an injection time of 2 min was set, since it was the minimum time required for the analytes to reach the chromatographic column. Regarding the chromatographic separation, the temperature ramps chosen were the maximum ones permitted by the oven of the chromatograph, maintaining an initial column temperature of 50 °C. Synchronous SIM/Scan mode was selected for detection. Different samplings (N = 0, 1, 2, 4) and dwell time values (10, 30, 50, 70 and 100 ms) were evaluated. Optimum conditions were found to be N = 1 and a dwell time of 10 ms. Under these conditions, the number of SIM/Scan cycles/sec was 6.2.

### 3.4. Method validation

Evaluation of matrix effect is a crucial step of the method validation in order to verify if there is an influence of the nature of the matrix on the analytical procedure. In order to check this possibility, calibration curves obtained in two saliva samples (A and B) obtained from two apparent healthy subjects were constructed. The concentrations added to the saliva samples varied from 0.0 (unspiked sample) to 80 μg/L for GABA and Spd, 7.5 mg/L for Put and 4.5 mg/L for Cad and Orn. Signals obtained from the unspiked samples were subtracted in every case. All the models presented linear behaviour and ANOVA analysis was performed to check their validity, observing no lack of fit. The value of the determination coefficient was higher than 0.95 in all cases (Table 2). The slopes of the calibration curves were compared using a Student's *t*-test (significance level, 0.05). As shown in Table 2, *p*-values were found to be below 0.05 in all cases. Thus, there were significant differences in the slope of the two matrices indicating the existence of matrix effect. Quantitation was subsequently performed using the one-point standard additions method and normalization to IS.

The LODs and limits of quantification (LOQs) were also evaluated. As stated before, the nature of the sample influenced the analytical signals of the compounds. Thus, LODs and LOQs were matrix dependant. Due to the impossibility of finding analyte-free matrices, LODs and LOQs were calculated for the two salivas previously analysed. The LOD and the LOQ were determined as the analyte concentration giving a signal equal to the blank signal plus three and ten times the standard deviation of the blank, respectively [45]. Table 3 shows the values obtained for the two salivas, expressed as concentrations in the saliva sample (dilution factor 1:7 v/v applied).

The accuracy of the method (expressed as percentages) was calculated as the ratio of the measured concentration to the spiked concentration, i.e. apparent recoveries. The study was performed with a saliva sample spiked with the analytes at different concentrations: 20 μg/L for GABA and Spd, 250 μg/L for Put, 60 μg/L for Cad and 150 μg/L for Orn and values were calculated using the one-point standard addition method. Acceptable recovery values were found: 89% for GABA, 94% for Put, 103% for Cad, 131% for Orn and 130% for Spd. Due to the higher concentrations expected for Put, Cad and Orn in saliva, accuracy was evaluated again at higher concentration levels (2500 μg/L for Put, 1000 μg/L for Cad and 800 μg/L for Orn).

**Table 2**
Comparison of the slopes obtained for the calibration curves obtained in two saliva samples from different subjects with the proposed method.

| Compound | Saliva A | | Saliva B | | *p*-value |
|---|---|---|---|---|---|
| | $R^2$ | Slope | $R^2$ | Slope | |
| GABA | 0.9877 | 0.32 ± 0.02 | 0.9585 | 0.8 ± 0.1 | $3.3 \times 10^{-15}$ |
| Put | 0.9868 | 5.7 ± 0.5 | 0.9741 | 12 ± 1 | $4.5 \times 10^{-16}$ |
| Cad | 0.9905 | 8.9 ± 0.5 | 0.9961 | 14.1 ± 0.8 | $5.0 \times 10^{-29}$ |
| Orn | 0.9952 | 3.6 ± 0.5 | 0.9943 | 4.3 ± 0.2 | $2.8 \times 10^{-8}$ |
| Spd | 0.9852 | 3.3 ± 0.2 | 0.9913 | 2.0 ± 0.6 | $1.4 \times 10^{-16}$ |

**Table 3**
LODs and LOQs obtained for the two analysed salivas.

| Compound | Saliva A | | Saliva B | |
|---|---|---|---|---|
| | LOD (μg/L) | LOQ (μg/L) | LOD (μg/L) | LOQ (μg/L) |
| GABA | 3.12 | 10.4 | 4.10 | 13.7 |
| Put | 1.84 | 14.3 | 7.13 | 23.8 |
| Cad | 2.63 | 8.76 | 5.29 | 17.6 |
| Orn | 5.15 | 17.2 | 5.07 | 16.9 |
| Spd | 2.60 | 8.68 | 33.8 | 112.5 |

Acceptable values were obtained for all of the compounds, with values of 109% for Put, 74% for Cad and 97% for Orn.

Precision of the method was also evaluated in terms of the agreement between different replicates from the same reaction (repeatability, 4 replicates of a single vial) and of the closeness of agreement between independent results of seven different reactions (reproducibility, 4 replicates per vial). Values were found to be between 2.9 and 15.2% (Orn and Spd) and between 1.5 and 26.5% (Cad and GABA). Although the precision value among reactions obtained for GABA was slightly high, it could be considered acceptable taking into account that it covered variation of the derivatization reaction, extraction procedure, separation and detection.

Finally, saliva samples from six subjects, 4 females (S1-S4) and 2 males (S5 and S6) were analysed with the proposed methodology (five from non-diagnosed individuals and one (S2) corresponding to a subject diagnosed with rheumatoid arthritis). Sample 5 corresponded to a heavy smoker. Results are shown in Fig. 4. For Put and Cad, the highest concentrations found corresponded to subject 2 (diagnosed with rheumatoid arthritis). For Orn and Spd, the highest concentrations found corresponded to males subjects, especially to subject 5 (heavy smoker). The concentrations found for all of the compounds were in good agreement with previously published results [3,19].

## 4. Conclusions

In the present work, we have successfully applied for the first time an *in situ* derivatization process of putrescine, cadaverine, spermidine, L-ornithine and gamma-aminobutyric acid with ethyl chloroformate and MEPS-GC-MS for their determination in saliva samples. This new matrix presents different physicochemical characteristics in comparison to others previously analysed, such as urine [31]. The issues found have been studied, addressed and re-evaluated for every step of the analysis. After optimizing the reaction conditions, the microextraction step (MEPS) and the analysis procedure using a PTV coupled to GC-MS, we have been able to reach LODs in the range of μg/L. Matrix effect has been checked and quantitation of samples performed using the one-point standard addition method and normalization to IS. Accuracy (89–131%) and precision values in terms of repeatability (2.9–15.2%) and reproducibility (1.5–26.5%) have also been successfully proven to be within acceptable values. Finally, the method has been applied in the diagnosis of real saliva samples, finding results in accordance to those described in literature. Samples from diagnosed subject or heavy smoker presented higher concentrations than those corresponding to non-diagnosed ones.

## Declaration of Competing Interest

The authors declare that there is not conflict of interest.

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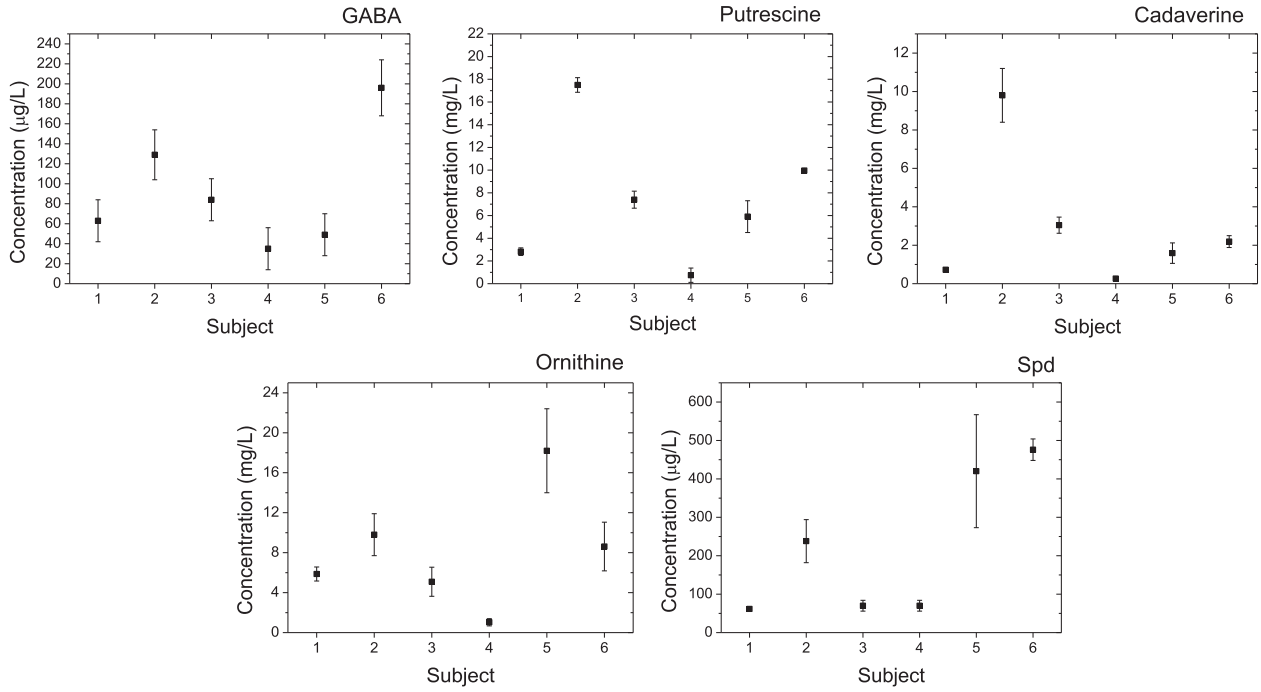


**Fig. 4.** Values of concentration of GABA, Put, Cad, Orn and Spd for different subjects. Note subject 2 was known to be affected of rheumatoid arthritis. Uncertainty bars represent the prediction intervals.

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