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Development of a screening and confirmatory method for the analysis of polar endogenous compounds in saliva based on a liquid chromatographic-tandem mass spectrometric system

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ABSTRACT

In this paper, a high-throughput approach is proposed for the sensitive screening and the confirmatory analysis of polar compounds in saliva using a two-step approach based on a liquid chromatographic system coupled to a triple quadrupole mass spectrometer. A reversed-phase chromatographic column was used in both steps and changes in the composition of the mobile phase allowed the screening and the confirmatory analyses to be performed with the same instrumental configuration. The proposed strategy has been tested for the determination of a multiclass group of polar endogenous compounds (creatinine, polyamines and amino acids) in saliva samples. The validation of the entire procedure showed consistent results for all the compounds in both steps. Repeatability and reproducibility were evaluated for both procedures, with values below 8% in the case of repeatability and 17% in the case of reproducibility. The instrumental limits of detection were found to be between 1.22×10^{-3} and 46.1×10^{-3} mg/L for creatinine and lysine, respectively, and accuracy of the method was evaluated in terms of apparent recoveries and values were found to be between 80 and 127%. Matrix effects were evaluated and it was found that the analytical outcome was influenced by the matrix of the sample. Thus, a one-point standard addition method was used for quantification. The optimized two-step procedure was applied to saliva samples from apparent healthy volunteers. Overall, satisfactory results were obtained in both steps, demonstrating its applicability for quantitative analysis of polar endogenous compounds in this kind of matrices.

1. Introduction

In recent years, the development of high-throughput approaches for the simultaneous determination of a large number of compounds in complex samples is increasingly being demanded for research and for routine analytical laboratories application. Along with an effective reduction on the time of analysis, improvements in selectivity and sensitivity are also required to develop reliable methodologies capable of addressing the quantitative analysis of compounds of different chemical classes.

The use of non-separative methods is a very favourable option due to the reduction in the time needed to carry out the analysis, especially when a large number of samples are involved [1–3]. In some cases, the information obtained can be sufficient to solve the analytical problem at hand [4,5], but in other situations, it is necessary to validate the results previously obtained by using an additional separative step [6,7]. Usually, the non-separative and the separative methods are based on very different analytical techniques [8–12].

An interesting approach based on the use of mass spectrometry has been reported in which screening and confirmatory analyses were performed using the same instrumental configuration. This approach has been fully evaluated for the analysis of volatile organic compounds by using, on one hand, direct headspace sampling coupled to mass spectrometry (HS-MS) for screening purposes and, on the other, a gas chromatographic separation step (HS-GC-MS) for confirmatory purposes [1,13,14]. The same instrumental configuration is used for both screening and confirmatory purposes by setting the oven temperature high enough to eliminate the separative capacity of the GC column (non-separative method) or by using a temperature gradient when the separation is required for confirmation. Recently, improvements to this

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approach have been proposed such as the use of a programmed temperature vaporizer (PTV) as the injector device [15,16] or the direct coupling of microextraction by packed sorbents (MEPS) to the mass spectrometer [17].

High-throughput analytical methodologies involving liquid chromatographic approaches have also been proposed for non-volatile compounds [18–20]. Most of them propose a separative step and the use of high-resolution mass spectrometers. As an alternative to these configurations, different non-separative methods have been proposed, such as membrane extraction coupled to mass spectrometry [21] or direct infusion mass spectrometry [5,22,23]. However, none of them offers the possibility to use the same instrumental configuration for both non-separative and separative analyses.

This paper describes the development and validation of a high-throughput screening and confirmatory method that involves a two-step approach with the same instrumental configuration. It is based on a liquid chromatographic system coupled to a triple quadrupole mass spectrometer. A reverse-phase chromatographic column is included in the instrumental configuration. The two-step operation mode is simple: when the screening analysis is performed, the mobile phase has an eluting strength high enough to avoid the retention of the analytes in the chromatographic column; when confirmatory analysis is required, an elution gradient is proposed so the target compounds are separated and detected by the typical LC–MS/MS configuration. The main advantage of the proposed approach is that the instrumental configuration does not need to be modified in order to switch from the screening to the confirmatory analysis, unlike other reported methodologies in which the chromatographic column should be removed from the instrumental configuration and replaced by a polyether ether ketone (PEEK) tube to connect the automatic injector and the mass spectrometer [4,24–27]. It should be noted that although some LC systems allow injection to be performed using a multi-column setup, the methodology proposed here is an interesting alternative for those instruments that do not have this option.

In order to evaluate the possible use of the proposed approach, it was applied to the determination of a multiclass group of polar compounds (creatinine, polyamines and amino acids) in saliva samples from apparent healthy volunteers. These particular compounds have been associated with several diseases, such as chronic kidney disease [28], cancer [29,30] or inborn errors of metabolism [31]. Saliva can be quickly and easily collected through stress-free and non-invasive means and it has been shown that the concentration of many analytes in saliva and plasma are significantly related [32]. The possibility to trace the progression of certain diseases through non-invasive methods could be considered as one of the primary objectives in the field of Healthcare Research [32].

Different alternatives have been proposed for the determination of the aforementioned compounds in saliva samples, mainly based on separative methods. Underivatized salivary free amino acids have been determined by ultra-performance liquid chromatography mass spectrometry using hydrophilic interaction chromatography [33] or capillary electrophoresis with laser-induced fluorescence detection [34]. Reverse phase liquid chromatography with fluorescence detection [35] or mass spectrometric detection [28,29,36,37] has been proposed for the determination of underivatized creatinine and derivatized polyamines in saliva samples. As derivatization reagents, different alternatives have been proposed, such as *o*-phthaldialdehyde [35], 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole [29] and phenyl-isothiocyanate [37]. Flow injection analysis-mass spectrometry has also been proposed for, as an example, the determination of derivatized polyamines, using phenyl-isothiocyanate as derivatization reagent [37] or the determination of creatinine in urine samples [38]. To the best of our knowledge, this is the first time that a two-step screening-confirmatory procedure based on the same instrumental configuration is proposed for these type of compounds.

2. Experimental section

2.1. Chemicals

Putrescine (Put), cadaverine (Cad), creatinine (Cre), valine (Val), lysine (Lys), histidine (His), arginine (Arg) and tryptophan (Tryp) were supplied by Sigma-Aldrich (Steinheim, Germany). All standards were of analytical grade. Methanol, as well as heptafluorobutyric acid (HFBA), were also supplied by Sigma-Aldrich. The ultra-high quality (UHQ) water used throughout the study was obtained with a Wasserlab Ultramatic water purification system (Noain, Spain).

2.2. Standard solutions and samples

Stock solutions of the target analytes were prepared in UHQ water at a concentration of 2500 mg/L and stored in darkness at 4 °C. These solutions were used to spike the water and saliva samples at the different concentrations analysed.

Unstimulated saliva was obtained from six apparent healthy adults of both sexes and was directly collected into a 10-mL glass vial and stored at -20 °C until use. The subjects ingested no food or beverages and did not brush their teeth within 1 h before sample collection. After thawing, the saliva was centrifuged at 1811 ×*g* during 10 min to precipitate the denatured mucins. The supernatant (500 μL) was added to a vial and mixed with the same volume of UHQ water (0.1% HFBA, v/v) and an aliquot (10 μL) was injected into the system.

2.3. Instrumental configuration

Screening and confirmatory analyses were performed in a LC–MS/MS system consisting of a 1200 series HPLC system with a binary pump, a membrane degasser, an autosampler, a six-port valve and a 6410 LC/MS triple quadrupole (QqQ) mass spectrometer equipped with an electrospray (ESI) ionization source, all from Agilent Technologies (Waldbronn, Germany). The chromatographic column used in both steps was a Kinetex XB-C18 column (50 mm × 3.0 mm) from Phenomenex (Torrance, CA, USA) packed with 2.6 μm core-shell particles. The MS was operated in positive ion mode. The ESI source nebulizer pressure and voltage were set at 50 psi and +4000 V, respectively. Nitrogen was used as the drying (12 L/min, 350 °C) and collision gas. Analyte quantification was carried out under Multiple Reaction Monitoring (MRM) mode. All the parameters related to the instrumental configuration are listed in Table 1.

For screening purposes, the retention capacity of the column was eliminated using an isocratic mobile phase with a high eluting power (100% methanol, 0.1% HFBA, v/v) at a flow rate of 1 mL/min. The column was thermostatted at 25 °C. Under these conditions, the sample reaches the MS/MS detector directly being analyzed in less than 1.5 min.

For confirmatory purposes, the LC–MS/MS system worked in separative mode by using a mobile phase based on UHQ water (Solvent A) and methanol (Solvent B) mixtures. In both solvents, 0.1% HFBA (v/v) was added. The flow rate was set to 0.5 mL/min and the column was thermostatted at 25 °C. The solvent gradient used was as follows: 0% B for 0.5 min, then 0% to 70% B from 0.5 to 8.5 min, hold at 70% B during 2 min, and then returning to 0% B from 10.5 to 11.5 min and holding conditions during 5 min in order to re-equilibrate the column. The total chromatographic run time was 16.5 min.

Table 1
LC and MS/MS parameters of the target analytes.

Compound	t_R (min)	Precursor ion (m/z)	Product ions (m/z)	Fragmentor (V)	Collision energy (eV)
Creatinine	2.1	114.1	44.1/86.1	81	17/9
Lysine	2.5	147.1	84.1/130.1	41	13/5
Histidine	2.5	156.1	110.1/93.0	81	13/21
Putrescine	3.5	89.1	72.1	56	5
Valine	4.1	118.1	72.1/55.1	41	5/21
Arginine	4.5	175.1	70.1/60.1	81	21/13
Cadaverine	4.6	103.1	86.1	56	5
Tryptophan	8.0	205.1	188.1/146.1	81	1/13

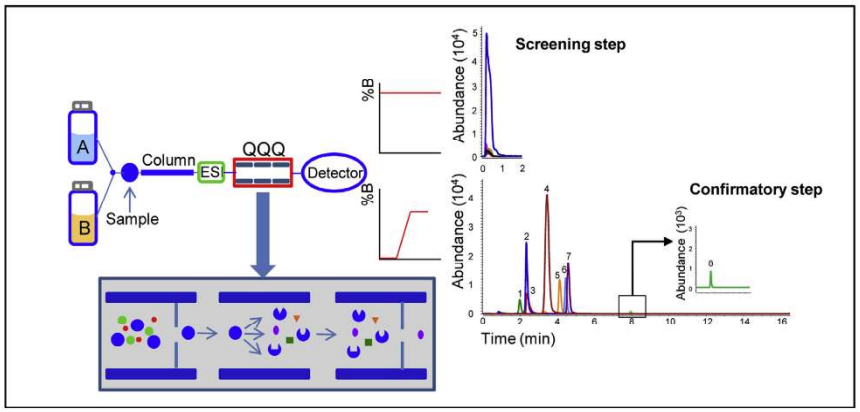

Fig. 1. Schematic diagram of the screening and confirmatory steps. 1, creatinine; 2, lysine; 3, histidine; 4, putrescine; 5, valine; 6, arginine; 7, cadaverine; 8, tryptophan.

Fig. 1 shows a general scheme of the proposed methods.

3. Results and discussion

The strategy described here is based on a LC–MS/MS system equipped with a reverse-phase column. To study the possibilities of the approach, several parameters were optimized, taking into account that, to perform the screening and the confirmatory analyses using the same instrumental configuration, the composition of the mobile phase is a critical parameter.

3.1. Optimization of the experimental LC–MS/MS conditions

For reversed-phase liquid-chromatographic analysis of underivatized amino acids and polyamines, the use of perfluorinated acids as ion-pair reagents have been described to improve the separation of these compounds on C18 columns [39,40]. Here, heptafluorobutyric acid (HFBA) was selected for such purpose and was added to the mobile phase at a concentration of 0.1% v/v.

The chromatographic separation of the target compounds was accomplished by using a solvent gradient based on UHQ water with 0.1% HFBA, v/v (solvent A) and methanol with 0.1% HFBA, v/v (solvent B). Four different elution conditions were evaluated. Best results were achieved with the following gradient: 0% B for 0.5 min, then 0% to 70% B from 0.5 to 8.5 min, hold at 70% B during 2 min, and then returning to 0% B from 10.5 to 11.5 min and holding conditions during 5 min. The flow rate was set at 0.5 mL/min. Under these conditions, a suitable separation was achieved in less than 10 min and another 6 min were required to reequilibrate the column. The total chromatographic run time was 16.5 min.

Multiple reaction monitoring (MRM) was used as the MS data acquisition mode. Two transitions were selected per analyte (Table 1) except for putrescine and cadaverine, where only one MRM transition was possible. Dwell time was evaluated in the range of 10–100 ms and, finally, a value of 10 ms was chosen, resulting in a rate of 5.29 cycles per second.

For saliva analysis, samples were first centrifuged and then a simple dilution step of the supernatant was assayed. Different dilutions in UHQ water (0.1% HFBA, v/v) were evaluated: 1:1, 1:5 and 1:10, v/v, using an unspiked saliva sample, in order to obtain the best analytical response without carryover effects. A 1:1 (v/v) dilution of the saliva sample showed the best analytical results. In order to evaluate carryover effects, quantification of the analytes in an UHQ water sample injected after the analysis of the 1:1 diluted saliva sample was performed. No signals of the target analytes were observed, so carryover effects were considered negligible. Therefore, this dilution was selected for further experiments.

3.2. Screening analysis

The screening analysis was accomplished in the same instrumental LC–MS/MS configuration including the chromatographic column. In this case, elution was carried out under isocratic conditions, maintaining the concentration of mobile phase at 100% methanol (with 0.1% HFBA, v/v). Thus, the separation ability of the column was eliminated and all the analytes eluted in a single peak that is directed toward the mass spectrometer.

Unlike other methods of analysis reported in the literature based on flow injection mass spectrometry [24,26,27] where the connection of the autosampler to the mass spectrometer is always done using a polyether ether ketone (PEEK) capillary (around 0.13 mm inner diameter), in this work the chromatographic column (3.00 mm inner diameter) was maintained during the screening step. This could imply the widening of the injection band.

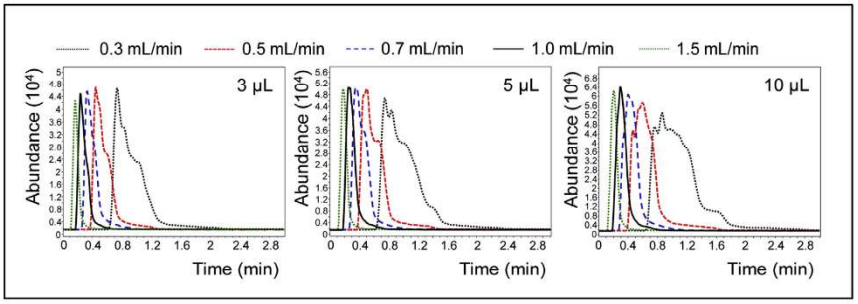

Fig. 2. Total ion current (TIC) corresponding to different injection volumes (3, 5 and 10 µL) and carrier flows (0.3, 0.5, 0.7, 1, 1.5 mL/min).

In order to optimize this screening step, different injection volumes (3–10 µL), as well as different flow rates (0.3–1.5 mL/min) were simultaneously evaluated (Fig. 2). Experiments were performed injecting a diluted saliva sample. As expected, higher injection volumes provided higher signal intensities but also wider peaks. Regarding the flow rate of the mobile phase, low values involved peak broadening; but at the highest flow rate assayed, lower signals were obtained due to the increased difficulty to eliminate the solvent during the electrospray ionization process [41]. Finally, 10 µL and 1 mL/min were chosen as the optimum value for injection volume and flow rate, respectively.

Under these conditions, it was possible to analyse each sample in less than 1.5 min. In addition, no time is required to reequilibrate the column since there is no chromatographic separation. The screening step described allowed us to perform around 30 injections per hour. These results confirm the high-throughput capacity of the proposed strategy.

A common problem encountered in all methods based on flow injection analysis mass spectrometry is the presence of interfering compounds, i.e., isobaric molecules, due to the elimination of the previous separation process. These interfering compounds can yield parent ions of the same mass-to-charge ratio, and if they exhibit similar gas-phase ion chemistry, they can also produce fragment ions of the same mass-to-charge ratio, causing quantification interferences when MRM is used [24]. This is even more significant when only one transition is available, which is the case for putrescine and cadaverine. Fig. 3 shows a chromatogram of an unspiked saliva sample. In this case, for each transition, no additional peaks were found for the majority of the compounds, except for lysine, arginine and tryptophan. However, other saliva samples could present different profile signals.

In the methodology described here, we propose to establish a cut-off value (i.e. a disease biomarker concentration level used to confirm or refute the presence of a disease in a subject) so that only samples that exceed it are analysed chromatographically for confirmation, without the need for any instrumental modifications. Therefore, only the suspicious samples would be analysed by the complete LC–MS/MS methodology to avoid false positives. In order to apply this method to a specific disease, it would be necessary to carry out an exhaustive evaluation of the existing literature to select the most significant analytes and the required cut-off values. Several publications have already shown elevated concentrations of several of the compounds evaluated here, such as putrescine, cadaverine and tryptophan, in saliva samples from breast, oral and pancreatic cancer patients [36,42], demonstrating the huge potential of the proposed methodology.

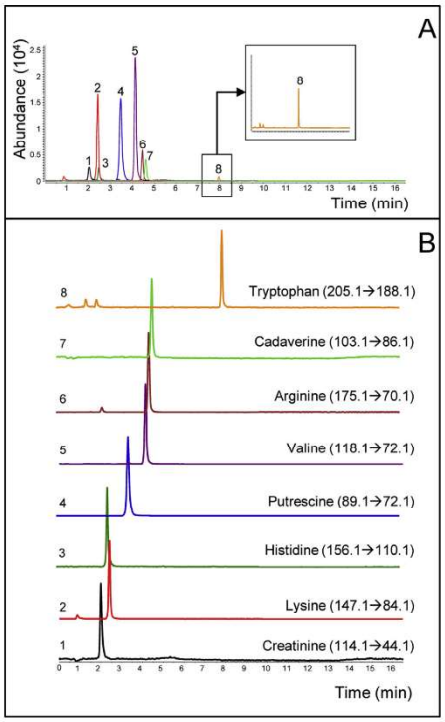

Fig. 3. A, MRM chromatogram of an unspiked saliva sample; B, individual MRM transitions of the target analytes. 1, creatinine; 2, lysine; 3, histidine; 4, putrescine; 5, valine; 6, arginine; 7, cadaverine; 8, tryptophan.

3.3. Evaluation of the screening and confirmatory approaches based on LC–MS/MS

The analytical characteristics of the screening and confirmatory approaches were evaluated for a mixture of the eight target compounds in water (Table 2). Standard calibration curves were obtained by analysing aqueous standard solutions of the analytes at six concentration levels. For putrescine, cadaverine and creatinine the concentration range was from 0.01 to 0.10 mg/L and for amino acids, the concentration range was from 0.02 to 0.20 mg/L.

Table 2
Analytical characteristics for the screening and confirmatory methods.

	Range (μg/L)	LC-MS/MS confirmatory method				MS/MS screening method			
		Slope	R^2	LOD (10^{-3} × mg/L)	LOQ (10^{-3} × mg/L)	Slope	R^2	LOD (10^{-3} × mg/L)	LOQ (10^{-3} × mg/L)
Creatinine	LOQ -100	244 ± 3	0.9993	1.22	4.06	113 ± 3	0.9973	2.17	7.23
Lysine	LOQ -200	16.4 ± 0.4	0.9981	10.7	35.6	6.2 ± 0.4	0.9930	46.1	153.5
Histidine	LOQ -200	13.3 ± 0.5	0.9946	18.2	60.6	4.1 ± 0.3	0.9863	33.3	110.9
Putrescine	LOQ -100	117 ± 4	0.9958	2.86	9.52	165 ± 3	0.9986	1.97	6.56
Valine	LOQ -200	40.7 ± 0.8	0.9987	11.5	38.3	18.9 ± 0.5	0.9995	31.6	105.2
Arginine	LOQ -200	10.9 ± 0.7	0.9854	2.92	9.72	8.5 ± 0.4	0.9963	11.5	38.3
Cadaverine	LOQ -100	80 ± 2	0.9974	1.91	6.39	79 ± 2	0.9998	2.24	7.46
Tryptophan	LOQ -200	7.8 ± 0.5	0.9856	6.50	21.6	5.7 ± 0.4	0.9890	9.52	31.7

Table 3
Comparison of the slopes obtained for the calibration curves obtained in UHQ water and in two saliva samples from different subjects with the screening and confirmatory methods.

Compound	LC-MS/MS confirmatory method			MS/MS screening method		
	UHQ water	Saliva 1	Saliva 2	UHQ water	Saliva 1	Saliva 2
Creatinine	227.8 ± 0.6	172 ± 3	123 ± 4	133.3 ± 0.7	70 ± 1	34 ± 3
Lysine	86 ± 2	35 ± 1	81 ± 2	24.4 ± 0.1	5.4 ± 0.2	18 ± 2
Histidine	77.6 ± 0.9	24.3 ± 0.9	41 ± 2	22.1 ± 0.3	6.3 ± 0.2	7.9 ± 0.3
Putrescine	71 ± 2	47 ± 2	51.4 ± 0.7	58 ± 3	38 ± 5	41 ± 1
Valine	162 ± 7	130 ± 4	152 ± 6	96.6 ± 0.6	13.3 ± 0.4	18.0 ± 0.8
Arginine	69 ± 1	52.7 ± 0.7	44.1 ± 0.9	50.3 ± 0.8	17.5 ± 0.7	11.8 ± 0.9
Cadaverine	84.4 ± 0.3	67 ± 1	68.2 ± 0.8	64 ± 1	20 ± 1	12.2 ± 0.3
Tryptophan	37.6 ± 0.3	36.7 ± 0.3	39.2 ± 0.4	29.2 ± 0.1	4.3 ± 0.1	6.1 ± 0.2

Each level was analysed in triplicate. All the calibration lines displayed linear behaviour. Their validity was checked using ANOVA analysis and it was observed that they did not exhibit any lack of fit. The values for the determination coefficient (R^2) were higher than 0.98 in all cases, as shown in Table 2.

The slopes obtained with the screening and confirmatory methods were compared using a Student's *t*-test to determine whether the slopes were significantly different (significance level, 0.05). The calculated value of *t* for most of the compounds was higher than the critical value. Thus, there were significant differences in the slopes for the two methods. These results showed that when the screening method was used, signal suppression/enhancement occurred due to the differences in the elution conditions of the analytes compared to the confirmatory method (mobile phase composition) [43] and due to the presence of co-eluting species. This was because the chromatographic separation process had not been carried out before MS/MS detection.

The instrumental limits of detection (LODs) and the limits of quantification (LOQs) for both screening and confirmatory methods were also evaluated using a UHQ water sample spiked with the compounds at different concentrations: 10 μg/L for Put, Cad, Creat and 20 μg/L for amino acids. For non-separative analysis, the concentrations used for Lys, His and Val were increased to 40 μg/L. LODs and LOQs 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 [44]. For the confirmatory method, LODs values between 1.22×10^{-3} and 18.2×10^{-3} mg/L and LOQs values in the 4.06×10^{-3}-60.6×10^{-3} mg/L range were obtained. For the screening method, higher values were obtained, with LODs between 2.17×10^{-3} and 46.1×10^{-3} mg/L and LOQs between 7.23×10^{-3} and 153.3×10^{-3} mg/L. The accuracy of the method was also evaluated in terms of apparent recoveries, calculated as the ratio of the measured concentration to the spiked concentration (expressed in percentages). The concentrations used for the calculations were those corresponding to the middle point of the calibration curve (i.e. 0.06 mg/L for creatinine, putrescine and cadaverine and 0.12 mg/L for amino acids). Values were found to be in the range of 88–111 % (for valine and putrescine, respectively).

3.4. Analysis of saliva samples

In order to evaluate matrix effects, calibration curves were obtained with both approaches for five calibration levels in three different matrices: UHQ water and two saliva samples from different subjects. Concentration intervals were selected based on the expected concentration of the compounds in saliva. These were from 10 to 30 mg/L for putrescine, from 0.4 to 1.6 mg/L for cadaverine, from 0.2 to 0.8 mg/L for creatinine and from 2.0 to 8.0 mg/L for amino acids. Each calibration level was analysed in triplicate. All these curves exhibited linear behaviour and did not exhibit any lack of fit.

The slopes of the calibration curves are shown in Table 3. They were compared by a Student's *t*-test (significance level, 0.05) [44,45]. Again, the calculated value of *t* for most of the compounds was higher than the critical value. Thus, there were significant differences in the slope of the different matrices evaluated. Similar results were obtained using both methods of analysis. In order to overcome the matrix effects, a one-point standard addition method was proposed for quantification. This is an alternative to the addition of isotopically labelled internal standards. Although the use of these compounds is the most powerful strategy for correcting suppression effects and improving quantitation accuracy, their use could be rather expensive, especially in a multi-class analysis and, in some situations, there are no commercial available standards [41,46].

Method reliability was evaluated in terms of the repeatability and reproducibility values, expressed as relative standard deviations (RSD, %), using an unspiked saliva sample (endogenous concentrations of the compounds). The sample was analysed ten times on the same day for repeatability and on three different days (three replicates per day) for reproducibility. Results are shown in Table 4. RSD values were found highly satisfactory, with values below 8% in the case of repeatability and 17% in the case of reproducibility. The results were found to be similar for both approaches, the screening and confirmatory methods. The accuracy of the method was also evaluated in terms of apparent recoveries. A saliva sample was spiked with the analytes at different concentration levels, 10 mg/L for putrescine, 3.0 mg/L for cadaverine, 0.2 mg/L

Table 4
Recovery, repeatability and reproducibility values (%) obtained for a saliva sample.

Compound	LC-MS/MS confirmatory method			MS/MS screening method		
	Recovery	Repeatability	Reproducibility	Recovery	Repeatability	Reproducibility
Creatinine	106	2.8	7.8	98	3.7	8.8
Lysine	90	7.7	10.0	99	3.5	9.7
Histidine	80	3.7	5.9	89	4.6	5.3
Putrescine	110	3.6	6.2	127	3.6	9.6
Valine	120	2.9	8.5	85	2.3	8.8
Arginine	100	1.8	11.8	95	4.6	16.7
Cadaverine	98	3.2	5.7	97	3.4	7.2
Tryptophan	100	1.9	5.1	85	3.6	6.1

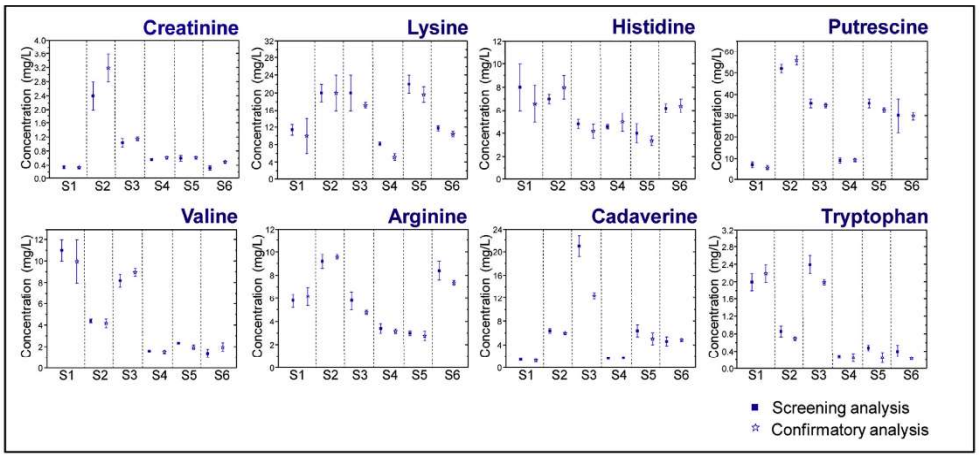

Fig. 4. Concentrations of the target compounds in different saliva samples (S1-S6). Uncertainty bars represent the prediction intervals. Screening results correspond to the filled squares and confirmatory results correspond to the empty stars.

for creatinine and 2.0 mg/L for the amino acids. The one-point standard addition protocol was used for the accurate determination of the compounds. The saliva sample was analysed before being spiked, and the area of the signals obtained for each compound were subtracted to the signals of the standard addition. Results are shown in Table 2. Values were found to be between 85 and 127% for the screening analysis (values found for valine and putrescine, respectively) and between 80 and 120% for the confirmatory analysis (values found for histidine and valine, respectively).

Due to the elevated concentrations of the compounds in saliva samples and the need to use the standard addition method, carryover effects were evaluated after the injection of the spiked level of the saliva sample. It was shown that carryover existed at these elevated concentrations, although the magnitude did not reach 0.4% (with values of 0% for creatinine, valine and tryptophan). A second injection of an UHQ water sample was also evaluated. Carryover was observed but only for lysine and putrescine, with values below 0.1%. Although the magnitude of the effect was very small, it could be recommended to inject an UHQ water sample after the analysis of the spiked level of the saliva sample.

Finally, saliva samples from six subjects were analysed with the proposed methods in order to estimate the concentration of these endogenous compounds. All samples were obtained from apparent healthy individuals of both sexes and were analysed in triplicate. The one-point standard addition protocol was applied; two vials were prepared per sample (three replicates each). The first vial comprised the unspiked saliva sample (1:1 v/v, saliva:UHQ water, 0.1% HFBA). The second vial comprised the spiked saliva sample (1:1 v/v, saliva:UHQ water, 0.1% HFBA). The concentrations added in each case were calculated as 2.5 to 3 times the expected concentration of the analyte in the saliva sample. Final ratios were found to be between 1.8 and 4 times the real concentration.

As shown in Fig. 4, all compounds were detected and quantified in all of the samples. For each subject, results corresponding to the screening method correspond to the filled square and the empty star corresponds to the confirmatory method. The concentrations found for all of the analytes are in good agreement with previous published research [37,47,48]. Similar results were obtained using both methods of analysis. However, differences were observed for several compounds as, for example, tryptophan in sample 3. In this case, interfering compounds were observed in the chromatograms of this sample. Fig. 5 represents the workflow of the proposed LC–MS/MS strategy. First, samples are analysed using the screening method. As can be seen, samples from subject 1 and 3 are suspicious because the target analyte concentration is very close or higher than the concentration level stablished to confirm or refute the presence of the disease in the subject. Thus, only those samples were analysed using the chromatographic approach for confirmatory purposes. After chromatographic analysis, subject 1 is still suspicious and further evaluation of the subject would be needed (i.e. medical diagnosis). However, subject 3 could be discarded as suspicious because the concentration found using the confirmation step was below the cut-off value, avoiding a false positive result.

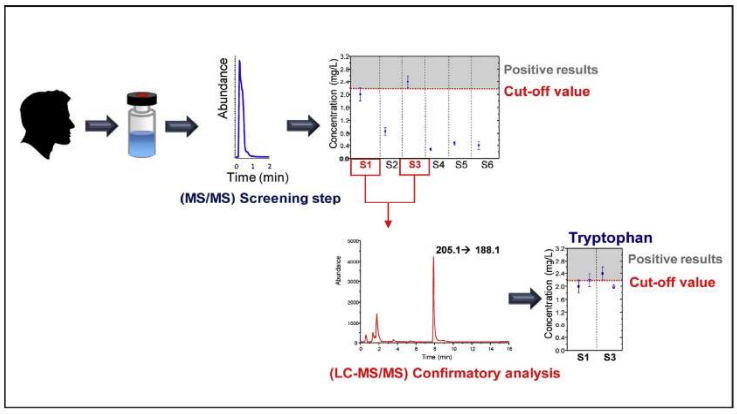

Fig. 5. Workflow of the proposed methodology. Uncertainty bars represent the prediction intervals. Screening results correspond to the filled squares and confirmatory results correspond to the empty stars.

4. Conclusions

In the present study, a rapid and reliable screening and confirmatory methodology for the analysis of polar compounds is described. The instrumental configuration is based on a liquid chromatographic system coupled to a triple quadrupole mass spectrometer. A two-step procedure has been developed for screening and confirmatory purposes without any instrumental modification. When the screening step is performed, a mobile phase with a strong eluting power was used in order to prevent the analyte retention; when the confirmation step was required, a solvent gradient is proposed in order to obtain the appropriate chromatographic separation of the target compounds. In addition, a cut-off value (i.e. a disease biomarker concentration level used to confirm or refute the presence of a disease in a subject) for the target compounds could be established and only those samples that exceed that value in the screening step can be easily analysed using the confirmatory procedure to validate the results obtained. In order to check the proposed approach, it was applied to determine a multiclass group of polar compounds (creatinine, polyamines and amino acids) in saliva samples. The quantitative results obtained by the screening and confirmatory steps were in good agreement, demonstrating the validity of the proposed strategy.

According to the obtained results, the screening step offers the important advantage of an effective reduction in time analysis, as around 30 injections per hour can be analysed. On the contrary, it presents some demerits, as possible matrix affects, since no separation takes place. The main advantage of the strategy proposed here is that the screening analysis step can be easily switched to the confirmatory step involving chromatographic separation without any modification in the configuration of the LC–MS/MS instrument.

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