ORIGINAL ARTICLE

HPLC method with post-column derivatization for the analysis of endogenous histidine in human saliva validated using the total-error concept

Anna-Maria Gkantiri[1] · Apostolia Tsiasioti[1] · Constantinos K. Zacharis[2] · Paraskevas D. Tzanavaras[1]

Abstract

Histidine (His) is an essential amino acid that plays an important biological role and associated with various pathological conditions. A simple and reliable method for the determination of endogenous histidine in human saliva was optimized and validated. The analyte was separated from the saliva matrix by cation exchange chromatography and detected fluorimetrically ($\lambda_{ex}/\lambda_{em} = 360/440$ nm) after online, specific post-column derivatization (PCD) reaction with o-phthalaldehyde. The chemical and instrumental variables of the post-column reaction were optimized using Box–Behnken experimental design to achieve maximum sensitivity. Method validation was carried out employing the total-error concept. Histidine could be analyzed reliably in the range of 0.5–5.0 µM, with an LOD ($S/N = 3$) of 50 nM. Monte Carlo simulations and capability analysis were used to investigate the ruggedness of the PCD reaction. The sampling strategy, sample preparation and stability were also investigated. Seventeen saliva samples were successfully analyzed with histidine levels being in the range of 2.7–19.5 µM.

Keywords Histidine · Human saliva · Cation exchange chromatography · Post-column derivatization · o-Phthalaldehyde

Abbreviations

HPLC	High-pressure liquid chromatography
PCD	Post-column derivatization
His	Histidine
OPA	o-Phthalaldehyde
BISM	Binary inlet static mixer
LOD/LOQ	Limits of detection/quantitation
β-ETI	β-Expectation tolerance intervals
ANOVA	Analysis of variance
BBD	Box–Behnken design
LoF	Lack of fit
LLOQ	Lower limit of quantitation

Handling editor: D. Tsikas.

✉ Paraskevas D. Tzanavaras
  ptzanava@chem.auth.gr

[1] Laboratory of Analytical Chemistry, School of Chemistry, Faculty of Sciences, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

[2] Laboratory of Pharmaceutical Analysis, Department of Pharmaceutical Technology, School of Pharmacy, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

Introduction

L-Histidine (His) is an essential amino acid with interesting biochemical properties, including proton buffering, chelation of metals and antioxidant activity. The rather unique properties of His are attributed to its imidazole ring and are not restricted solely to His, but expanded to His-containing peptides as well (Holeček 2020). His has proven to offer therapeutic solutions to a variety of pathological situations ranging from rheumatoid arthritis (Kim et al. 2018) to atopic dermatitis (Tan et al. 2017) and neurological disorders (Scalise et al. 2018). Recent research reports have proven strong relation of the levels of His and/or His-rich peptides in saliva with chronic stressful psychological conditions (Nakamura et al. 2010; Marvin et al. 2017). The potentially advantageous effects of His on human health have led to the commercialization of His-based nutritional supplements (Wang et al. 2020).

Saliva is secreted from the salivary glands and has multiple functions, including mouth cleaning and protection, antibacterial effects and digestion. Saliva composition includes a variety of organic (urea, uric acid, glucose, cholesterol, fatty acid, triglycerides, neutral lipid, etc.) and inorganic compounds (ammonia, Na^+, Cl^-, Ca^{2+}, K^+, HCO_3^-, $H_2PO_4^-$, etc.) (Zhang et al. 2016). During the last years, human saliva

has emerged as an attractive, alternative biological substrate for the diagnosis and prognosis of human health, ranging from the obvious oral diseases to systemic disorders such as cancers (Lee and Wong 2009; Melguizo-Rodríguez et al. 2020). Collection of human saliva is non-invasive and painless, while its composition is less complex and, as it is produced by exorcine glands, it offers real-time monitoring. Additionally, the stability of saliva samples has proven to be better compared to other biological materials (serum, plasma, urine, etc.) (Emekli-Alturfan et al. 2009; Elmongy and Abdel-Rehim 2016).

Due to the clinical significance of His, a variety of bioanalytical methods have been recently reported in the literature for its determination in biological material. Razavi et. al. have described a colorimetric, paper-based sensor for the determination of His in urine (Razavi and Khajehsharifi 2021). Despite the fact that the authors analyzed only one urine sample, they concluded that the method could be useful for clinical testing. In a similar way, another recently published paper-based method that combines a His-selective enzymatic reaction and the molybdenum blue chemistry claims potential clinical usefulness without presenting data from real sample analysis (Kugimiya et al. 2020). The enzymatic His assay proposed by Yamaguchi et al. (2019) was able to measure His in three human plasma samples with good agreement to a corroborative LC–MS method. The complexity of the procedure, including the design of a new L-histidine decarboxylase mutant, renders the assay rather unattractive for routine clinical applications. An indirect fluorimetric method based on the inhibitory effect of His on the oxidation of thiamine by cobalt-containing Prussian Blue nanocubes requires additional treatment steps to improve selectivity against biothiols and uric acid, while despite the fact that the authors analyzed human serum, they did not report endogenous levels of His (Yao et al. 2020). On the other hand, LC–MS/MS has been applied to the monitoring of His metabolism in mouse feces, including histamine and imidazole-4-acetate (Acuña et al. 2021). To overcome matrix interferences common in bioanalysis of mass spectrometry, the authors have utilized two different internal standards. Finally, in a recent report salivary His has been determined by HPLC, in a detailed study involving numerous samples from different stressful conditions (Wu et al. 2021). The method requires tedious off-line pre-column derivatization coupled to polystyrene nanofiber solid phase extraction prior to chromatographic analysis.

In the present study, we extend our recent research reports on the analysis of His in biological material (Stampina et al. 2021), by optimizing and validating a reliable method for the selective and sensitive determination of endogenous levels of His in human saliva. Due to the lack of intrinsic fluorescence, one of the most widely applied strategies for the sensitive quantification of His—and other amino acids—is chemical

derivatization using suitable reagents (Rigas 2012). Recently, we have exploited the analytical potentials of highly specific reactions of o-phthalaldehyde (OPA) with a group of analytes including histamine, glutathione, homocysteine and histidine (Alevridis et al. 2020; Tsiasioti et al. 2020a; Tsiasioti and Tzanavaras 2021). The combination of cation exchange separation with online post-column reaction with OPA in the absence of nucleophilic reagent offers an analytical scheme that enables the accurate analysis of His following minimal sample preparation (Håkanson et al. 1974; Yoshimura et al. 1990). The post-column derivatization (PCD) reaction has been optimized using experimental design, while validation was carried out by the total-error concept.

Materials and methods

Reagents and solutions

Analytical-grade reagents were employed throughout this study. Histidine (His, 99%) and acetonitrile were obtained from Sigma. The derivatizing reagent (o-phthalaldehyde, OPA) and concentrated HNO_3 were provided by Fluka, while KH_2PO_4 and NaOH were purchased from Merck. High-purity water (18 MΩ cm resistivity) was produced by a B30 water purification system (Adrona SIA).

All solutions (including standards and reagents) were prepared on a daily basis. The standard stock solution of His was prepared at 1000 μM by dissolving the required amount in the mobile phase (aqueous solution of $HNO_3 = 5$ mM). The diluted working standard solutions were also prepared in the same solvent. The post-column derivatizing reagent (OPA) was prepared at an amount concentration of 20 mmol L^{-1} in water and was typically consumed within a working day (50 or 100 mL). The PCD buffer (150 mM phosphate buffer, pH = 8.5) was also prepared daily (50 or 100 mL). The mobile phase was degassed ultrasonically and filtered under vacuum through 0.45 μm membrane filters (Whatman®).

The artificial saliva surrogate matrix that was used during validation studies was prepared as follows: NaCl (0.13 g L^{-1}), KCl (0.96 g L^{-1}), KH_2PO_4 (0.66 g L^{-1}), $NaHCO_3$ (0.63 g L^{-1}), KSCN (0.19 g L^{-1}), $CaCl_2$ (0.23 g L^{-1}), urea (0.2 g L^{-1}), Na_2SO_4 (0.76 g L^{-1}), NH_4Cl (0.18 g L^{-1}). The pH of the final mixture was adjusted to 6.8 (Gal et al. 2001). Following preparation, the artificial saliva was kept refrigerated in an amber glass bottle.

Instrumentation

HPLC setup: (i) LC-9A binary pump (Shimadzu); (ii) Elite™ vacuum degasser (Alltech); (iii) Rheodyne 7725i injection valve ($V = 10$ μL); (iv) column oven ($T = 60$ °C,

Jones Chromatography); (v) MetroSep C4 column (150 × 4.0 mm i.d., 5 μm, Metrohm).

PCD setup: (i) Minipuls™ 3 peristaltic pump (Gilson) equipped with Tygon tubing; (ii) 200-cm long PTFE reaction coil (0.5 mm i.d.); (iii) reaction coil thermostat (HiChrom Limited); (iv) RF-551 spectrofluorimetric detector operated at the high sensitivity mode ($\lambda_{ex}/\lambda_{em} = 360/440$ nm) (Shimadzu). Data acquisition was carried out via the Clarity® software (version 8.2.1.84, DataApex®).

## Samples collection and preparation

Human saliva samples were donated on a voluntary basis from faculty members and postgraduate students of the Department of Chemistry. All participants were informed about the nature of this study and provided their written consent. Unstimulated saliva samples were collected using Salivette units equipped with cylindrical-shaped cotton swabs according to the instructions of the manufacturer (Salivette); the volunteers were informed not to consume food or drinks for at least 2 h prior to sampling. In brief, the cylindrical cotton swabs were removed from the unit and gently chewed by the volunteers for ca. 60 s. After removal from the mouth, the saliva-soaked cotton swabs were placed back in the Salivette units and centrifuged at 1000*g* for 2 min to remove undissolved material and obtain a clear saliva solution. The centrifuged Salivette® units were kept at − 18 °C until analysis.

Accurately measured volumes of the collected saliva (500–1000 μL) were mixed with equal volumes of ice-cold acetonitrile and centrifuged again (4000*g* for 5 min). Ten microliters of the clear supernatants was injected directly to the HPLC–PCD setup without any other treatment.

HPLC–PCD method

A graphical depiction of the HPLC–PCD configuration is included in Fig. 1. The mobile phase (5 mmol L^{-1} HNO_3) was pumped through the cation exchange column at a flow rate of 1.0 mL min^{-1} ($T = 60$ °C) (Stampina et al. 2021). 10 μL of samples and/or standards were injected in all cases. Following separation from the sample matrix, His was merged downstream with a combined stream of the PCD reagents (OPA and phosphate buffer were mixed online at equal flow rates of 0.25 mL min^{-1} each through a binary inlet static mixer equipped with a 250 μL mixing cartridge (BISM, ASI-Analytical Scientific Instruments)). Chemical derivatization of the analyte was allowed to proceed online upon passage through a thermostated 200 cm long reaction coil (knitted tightly around a 4.6 mm i.d. stainless steel rod to facilitate thermostating at 50 °C). The formed derivative was
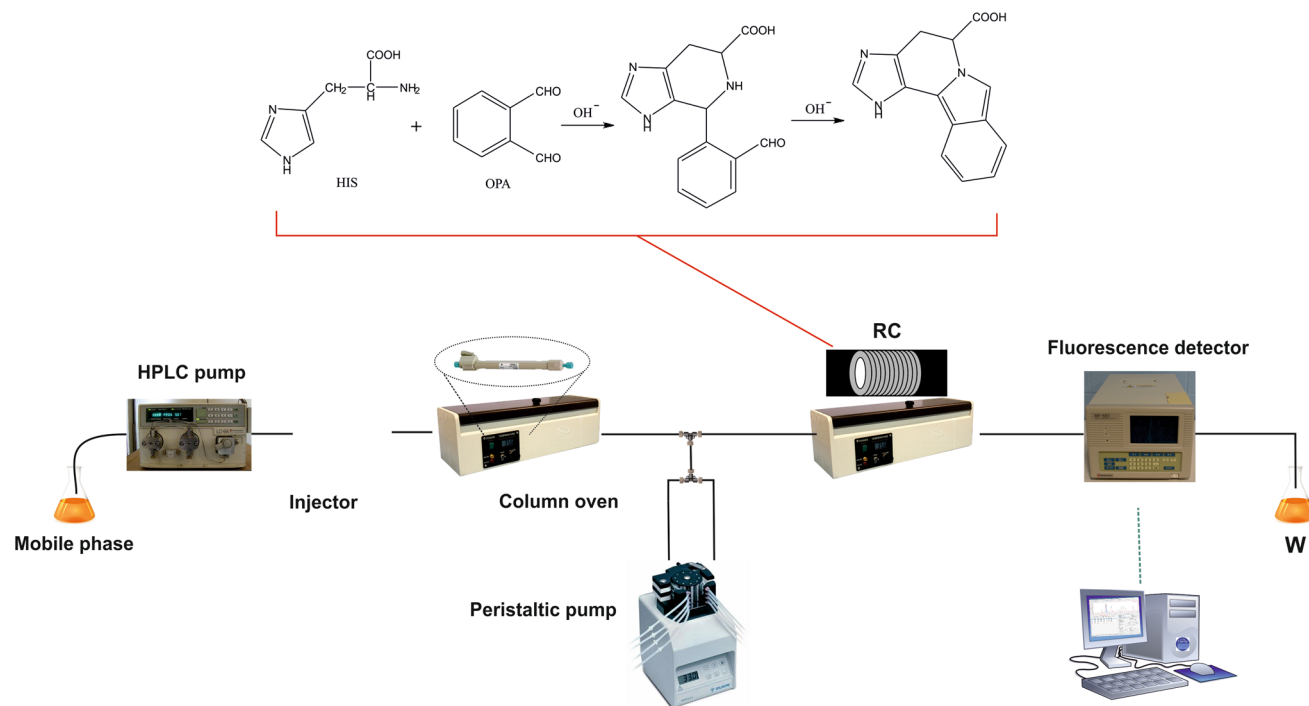
Fig. 1 Depiction of the optimized HPLC–PCD setup used for the determination of endogenous His in human saliva, including the structure of the His-OPA derivative (Håkanson et al. 1974; Yoshimura et al. 1990). Experimental details: Mobile Phase = 5 mM HNO₃; Q(mobile phase) = 1.0 mL min^{-1} ($T = 60$ °C); V(sample) = 10 μL; [OPA] = 20 mM; Phosphate Buffer = 150 mM/pH = 8.5; Q(PCD) = 0.25 mL min^{-1} each stream; Reaction coil = 200 cm ($T = 50$ °C); FL detection at $\lambda_{ex}/\lambda_{em} = 360/440$ nm

monitored spectrofluorimetrically ($\lambda_{ex}/\lambda_{em} = 360/440$ nm) and recorded as a single peak ($t_R = 2.8$ min).

Optimization of the PCD reaction using Box–Behnken design

The post-column derivatization reaction between His and OPA was optimized using response surface methodology through the Box-Behnken Design (Ferreira et al. 2007; Bezerra et al. 2008; Hibbert 2012). For simplicity and rapidity reasons, all optimization experiments were carried out in a flow injection analysis (FIA) configuration, by removing the ion exchange column and using the mobile phase as carrier (5 mM HNO_3). The selected range of each studied experimental parameter was $T = 30–70$ °C, pH = 7–10, $c(OPA) = 5–20$ mM, Q(PCD) = 0.15–0.35 mL min^{-1}, $c(buffer) = 50–150$ mM. The amount concentration of the analyte was $c(His) = 5$ μM in all cases.

Method validation using accuracy profiles

The validation of the analytical method was implemented by constructing the accuracy profiles by taking into consideration the total error (systematic and random errors) (Hubert et al. 2003, 2004, 2007). The experimental implementation of this validation procedure involved the organization of replicate measurements of both calibration and validation standards of the analyte at five concentration levels ($m = 5$) ranging from 0.5 to 5.0 μM. Each calibration (aqueous) and validation (artificial saliva surrogate matrix) level was independently prepared in triplicate ($n = 3$) for three consecutive days ($k = 3$). One injection of calibration/validation standard was performed resulting in a total of 90 injections (for more details on the concept of accuracy profiles are included in the supplementary section).

Monte Carlo simulation and capability analysis for the robustness study

High sensitivity of the His response in relation to PCD variables can influence the robustness of the method. Therefore, it is essential to find out the maximum variation of each factor that can be allowed to demonstrate its robustness. The robustness of the PCD method was examined using Monte Carlo simulations and capability analysis (Mandur and Budman 2014; Reuven and Rubinstein 2017). On this basis, 100,000 iterations were conducted using Monte Carlo simulations experiments and the simulated data were utilized to estimate the Cpk values. The acceptance criteria of the His peak area was established to ±5% of the predicted value obtained from the optimization step. To generate the simulated data, the response function (derived from the Box-Behnken Design) was used with the aid of a statistical software (more details on the principles of the procedure are presented in the supplementary section).

Matrix effect and samples stability

The matrix effect (ME) was evaluated in a pooled human saliva matrix ($n = 12$). Following sampling, collection and centrifugation, 500 μL of each individual sample was mixed and vortexed to prepare the pooled matrix (total volume of 6 mL). 450 μL fractions were spiked with 50 μL of His standards, vortexed and subsequently treated with 500 μL of acetonitrile (see sample preparation above). An aqueous calibration curve was prepared in an analogous way, by replacing the 450 μL of saliva with equal volumes of water. Two independent solutions were prepared in all cases and concentration levels. The amount concentration of His ranged between 1 and 10 μM ($n = 6$). The potential matrix effect was validated by comparing the slopes of the matrix matched calibration curve versus the aqueous one.

The stability of the samples was investigated in both treated (protein precipitation with acetonitrile) and untreated saliva. For this purpose, a pooled saliva mixture ($n = 6$) was prepared as described in the previous paragraph and a fraction was treated and analyzed immediately ($t = 0$) to determine the levels of endogenous His. This fraction was also used to evaluate the "autosampler stability" (RT at 0, 24, 72 h). Untreated pooled saliva was stored at RT, 4 °C and – 18 °C and was analyzed at 24 and 72 h. In all cases the stability was evaluated based on the endogenous content of His in the human saliva matrix.

Results and discussion

Preliminary experiments

Preliminary experiments were focused on the selection of the most appropriate buffer species prior to optimization. On this basis we compared borate and phosphate buffers (50 mM, pH = 9 for both cases). The LC conditions were kept fixed at 5 mM HNO_3 as mobile phase, T(column) = 60 °C and Q(MP) = 1 mL min^{-1} (Stampina et al. 2021). Although borate is the buffer of choice in most OPA applications that are based on the generic mechanism for primary amines (Zuman 2004), the experimental results showed ca. threefold lower sensitivity compared to analogous phosphate buffer (see Fig. 1S in supplementary material). Due to the significantly lower expected values of His in human saliva compared to urine and serum, the PCD step was re-optimized using experimental design to achieve the maximum possible sensitivity.

## Optimization of PCD parameters using Box–Behnken design

Response surface methodology was employed for the optimization of the PCD parameters. On this basis, the effect of the reaction temperature (Factor A), the buffer pH (Factor B), the OPA amount concentration (C(OPA)) (Factor C), the flow rate of each PCD reagent (Factor D) and the buffer concentration (Factor E) were investigated and optimized using a Box–Behnken design (BBD). The BBD is an efficient alternative statistical tool that can be utilized for the optimization of chromatographic methods (Ferreira et al. 2007). Generally, it involves $2\,k(k\text{-}1) + n_c$ experiments where k represents the number of factors and n_c the center points. In our case, a total of 46 runs were performed and each selected factor was studied at three levels (-1, 0, 1). The BBD and the experimental responses were analyzed using the Design-Expert® 13 software (Stat-Ease Inc, Minneapolis, MN, USA). The experiments were randomized to avoid systematic error. The factorial design points are shown in Table 1S (supplementary data). A quadratic polynomial model was considered:

$$y\left(X_{1\leq i\leq 5}\right) = \beta_o + \sum_{i=1}^{5}\beta_i X_i + \sum_{i=1}^{5}\beta_{ii}X_i^2 + \sum_{1}^{5}\sum_{j=1}^{i-1}\beta_{ij}X_iX_j + \varepsilon,$$

where $y(X_{1\leq i\leq 5})$ represents the experimental response, X_i the factor, β_o the constant, β_i the linear coefficients, β_{ii} the quadratic coefficients, β_{ij} the quadratic coefficients and ε the random error. The estimates of the coefficients for the models were calculated by least squares multi-linear regression and these models were validated by analysis of variance (ANOVA) (Table 2S, supplementary data). To conclude to a more "realistic" model, we excluded the non-significant factors ($p > 0.05$) using the "backward elimination" approach. The adjusted and the predicted R^2 values were 0.9365 and 0.9116, respectively, revealing that the described models have adequate reliability and predictability. The adequate precision value was 31.27 which is much higher than 4 indicating the significance of the model. Diagnostic plots such as the normal probability plot of residuals and the plot of residuals against the predicted values are illustrated in Fig. 2S (supplementary material). The data were randomly scattered around the line indicating that the model was appropriately fitting the data. The lack-of-fit (LoF) parameter ($p = 0.7677$) was obtained by the comparison of the variability of the actual model residuals with those of replicate settings and found to be non-significant ($p > 0.05$). As shown in 3D plots (Fig. 2A and B), there is a significant augmentation of the His peak area at elevated values of C(OPA), buffer concentration and pH, respectively.

To optimize the His response, Derringer's desirability function (D) was utilized to obtain the optimum set of conditions. This function was based on a scale of desirability ranging from 0 to 1 as a fully desirable response (Derringer and Suich 1980). It was calculated by combining single desirability functions, usually as the geometric mean (Ferreira et al. 2007). In our case, the goal of the optimization has been set to maximize the His peak area. A desirability function of 1.0 was achieved. All desirability surface
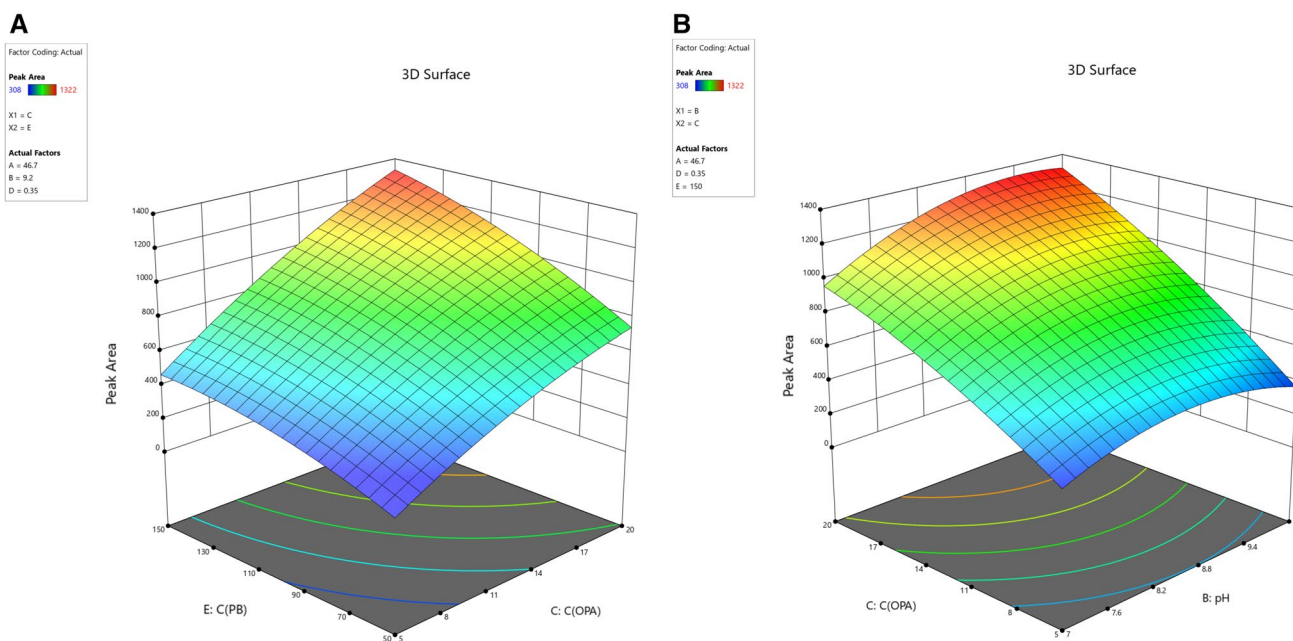
Fig. 2 Response surface (3D) plots indicating the effects of **A** C(PB) and C(OPA) and **B** C(OPA) and pH on the peak area of His

contour plots are illustrated in Fig. 3S. The optimization of D resulted to the following predicted values: reaction temperature 49.2 °C, buffer pH 8.5, C(OPA) 19.9 mM, flow rate 0.242 mL min^{-1} and buffer concentration 146 mM. For simplicity of operation, the temperature, the C(OPA), the flow rate and the buffer concentration were rounded off to 50 °C, 20 mM, 0.25 mL min^{-1} and 150 mM, respectively, and were adopted for further experiments. Finally, to confirm these optimum set of conditions, six replicate injections of the mixture were analyzed. The differences between the obtained experimental values with the predicted values were less than 4%.

Sample collection and preparation

Salivette® units for saliva collection are branded with two alternative swabs, namely cotton-based and synthetic (polystyrene). Ngamchuea et al. have reported data indicating random errors during sampling for analytes having chemical affinity to cotton-based swabs. They also reported that the extent of the errors depends on the sample volume and the time frame between sampling and centrifugation (Ngamchuea et al. 2017b).

A series of experiments was therefore carried out to validate the suitability of the Salivette® units for the specific application. Aqueous solutions of His (5 μM) were employed in all cases. In brief, 500 and 1000 μL of the His solution were slowly applied directly on the cotton and synthetic swabs and were absorbed by the materials. Centrifugation was carried out either as soon as possible ($t = 0$) and after equilibration for 10 min. The collected fractions were analyzed directly by the proposed HPLC–PCD method. The experimental results showed recovery of His between 94 and 97% when using the cotton swabs, indicating minimum binding of the analyte to the material. The phenomenon was also independent of the applied volume and the time between sampling and centrifugation. On the other hand, despite the fact that when using the synthetic swabs we recovered practically all of the applied solution in terms of volume, the percent recoveries of His were < 5%, indicating strong binding on the material. His could be recovered quantitatively from the synthetic swab using 500 μL acetonitrile. The Salivette® units with the cotton swabs were therefore selected for the reliable sampling of the saliva samples.

His is reported to be present in human saliva at the low micromolar level (Cheng et al. 2015). On an ideal basis sample treatment should be as simple as possible and additionally have minimum contribution to the dilution of the sample. Based on the robustness of the cation exchange column and the specificity of the PCD reaction we investigated two alternative treatment protocols that mainly targeted on the removal of salivary proteins; (i) 1: 1 dilution with 5% TFA and (ii) 1 + 1 dilution with ice-cold acetonitrile, followed

by centrifugation for 10 min at 4000g. As can be seen in Fig. 4S (supplementary section) the presence of TFA at a final concentration of 2.5% v/v affected the chromatographic behavior of His. Serial dilutions to lower the concentration of TFA improved the shape of the peak, but they also limit the applicability of the method in terms of the expected content of the analyte in the real samples.

The effect of acetonitrile on the chromatographic pattern of His and on the sensitivity of the determination was evaluated by analyzing standard solutions at the 5 μM level containing elevated fractions of the organic solvent (10–50%). As can be seen in the overlaid chromatograms of Fig. 5S (supplementary section), the effect of acetonitrile was negligible in both terms of the PCD reaction (sensitivity) and the chromatographic behavior of the analyte. Based on these findings, a very simple and rapid sample preparation protocol was established (see experimental section) that also offered minimum dilution of the sample matrix (1:1 with acetonitrile).

Method validation

The proposed method was validated using the total-error concept by constructing the accuracy profiles "HPLC–PCD method". Since His is an endogenous saliva compound, validation experiments were carried out using artificial saliva as surrogate matrix (Ngamchuea et al. 2017b). The accuracy profiles are represented in Fig. 3A. The red and the dashed blue lines symbolize the relative bias and the β-expectation tolerance limits at 95% probability level, respectively. The β-expectation tolerance intervals are calculated for each concentration level of the validation standards. In case of the dashed blue lines are contained within the black dotted lines, the (predefined acceptance limits of 15% for bioanalytical methods), method can be considered as valid. All validation results are presented in Table 1.

Linearity, LOD, and LOQ

The linearity of the results expresses the ability of the analytical method to provide results directly proportional to the concentrations. To simplify the future calculations, simple linear unweighted regression model was utilized for His determination. As it can be seen in Fig. 3A the relative errors (e_r %) for all concentrations gave results within the acceptance limits ($\pm$ 15%) apart from the lowest calibration level of 0.25 μM. The e_r values of this level were included in the range of $\pm$ 20% and therefore it could be considered as LOQ according to the bioanalytical method validation guidelines (FDA 2018). However, the obtained β-expectation profile overpassed the lower acceptance limit even of -20%. We therefore decided to exclude this level by taking into consideration the level

Fig. 3 **A** Accuracy profiles for the His determination in artificial saliva using a linear unweighted regression model. The red plain, blue dashed and blank dotted lines correspond to the relative error (%), the accuracy profile and the acceptance limits λ ($\pm 15\%$), respectively, **B** Linearity profile of His. The plain blank line corresponds to the identity line ($Y = X$), blue dashed line represents the accuracy profile (β-ETI) and the dotted curves illustrate the acceptance limits $\lambda \pm 15\%$ expressed in μM
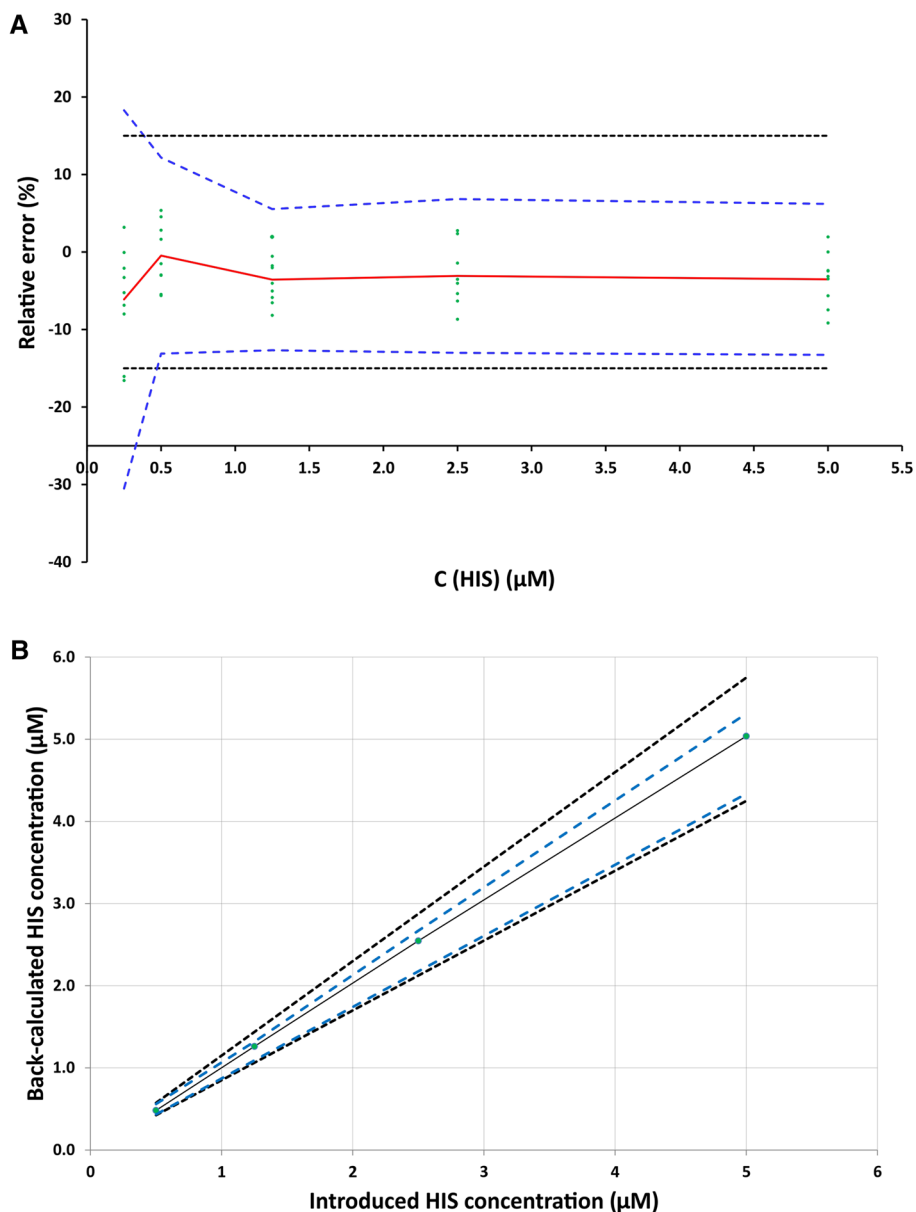
of 0.5 μM as the lower limit of quantitation (LLOQ), as it provides accurate and reproducible results and is adequately low for this kind of bioanalytical application. The limit of detection (LOD) was estimated by the S/N criterion and was calculated to be 50 nM.

The linearity of the method was assessed by fitting least squares regression lines on the back-calculated concentrations of the validation standards as a function of the introduced His concentration. Calibration parameters (slope, intercept, correlation coefficient, etc.) are shown in Table 1. The linearity of the method was also verified since the absolute β-expectation tolerance limits were within the absolute acceptance limits (Fig. 3B).

Trueness, accuracy and precision

Trueness is expressed as the mean bias (%) between the measurements and the target concentrations. As mentioned in Table 1, the relative biases ranged between − 0.5 and − 3.6% for the studied analyte showing the good trueness of the proposed LC–PCD method. Precision was expressed as the RSD of repeatability (s_r, %) and time-dependent intermediate precision (s_R, %) for each concentration level. In all cases, s_r and s_R values were lower than 4.1 and 3.8%, respectively demonstrating the adequate precision of the proposed method. The upper and lower β-ETIs for each level for His are entirely included inside the acceptance limits of $\pm 15\%$.

Table 1 Validation results for the quantitation of His in artificial saliva

Validation criteria			
Response function (linear unweighted)	Slope	Intercept	r
(k^a = 3; m = 5; n = 1) (0.50–5.0 µM)			
Day 1	105.21	5.036	0.9979
Day 2	120.37	4.193	0.9985
Day 3	118.57	13.50	0.9990
Precision (k = 3; n = 1)			
C (µM)	s_r (%)[b]	s_R (%)[c]	
0.50	3.4	2.8	
1.25	2.8	2.9	
2.50	4.1	3.8	
5.00	4.0	3.7	
Trueness (k = 3; n = 1)			
C (µM)	Relative bias (%)		
0.50	− 0.5		
1.25	− 3.6		
2.50	− 3.1		
5.00	− 3.5		
Accuracy (k = 3; n = 1)			
C (µM)	Relative β-ETI (%)		
0.50	[− 13.11, 12.18]		
1.25	[− 12.67, 5.55]		
2.50	[− 13.00, 6.83]		
5.00	[− 13.27, 6.21]		
Linearity (k = 3; m = 5; n = 1) (0.50 – 5.0 µM)			
Slope	1.010		
Intercept	− 0.0061		
r^2	0.9999		
LOD (µM)	0.05		
LLOQ (µM)	0.5		

[a]k, m and n correspond to the series of experiments, number of calibration levels and replicates, respectively

[b]s_r (%): relative standard deviation under repeatability conditions

[c]s_R (%): relative standard deviation under intermediate precision

Therefore, the proposed analytical scheme can be considered as accurate in the examined range.

Robustness using Monte Carlo simulations and capability analysis

A group of simulation experiments was performed considering the mean value of 50 °C (temperature), 8.5 (pH), 20 mM (C(OPA), 0.25 mL min^{-1} (flow rate), and 150 mM (buffer concentration) with standard deviation (SD) values of 1, 0.1, 0.5, 0.01, 1, respectively. The capability analysis revealed that the CpK value was 0.9516 (0.39% of the results will be out of specification), which is lower than the acceptable
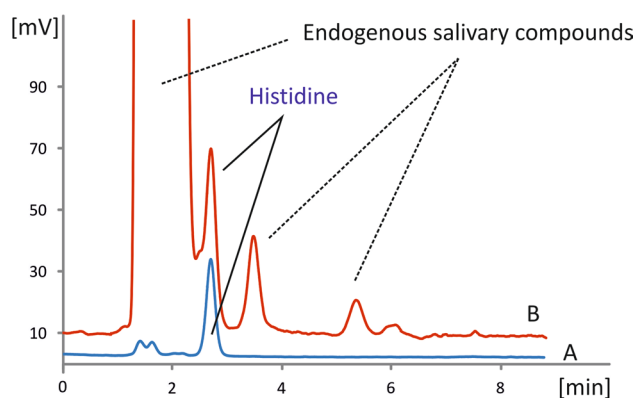
Fig. 4 Selectivity studies: representative chromatograms from the analysis of a pooled human saliva matrix (n = 6). Chromatogram A = analysis by the proposed HPLC–PCD method under the optimal conditions; Chromatogram B = analysis using the same HPLC conditions and a non-specific PCD reaction (R_A = 2 mM OPA and R_B = 5 mM N-acetylcysteine in 100 mM borate buffer (pH = 10), $\lambda_{ex}/\lambda_{em}$ = 340/460 nm)

value of 1.33 indicating minimization of the SD values. After readjustment of the SD value of the most robustness-sensitive parameter (i.e., C(OPA)) resulted in a satisfactory CpK value of 1.34 at SD value of 0.2. Figure 6S (supplementary data) illustrates the histogram from the capability analysis of the examined response.

Selectivity and carryover

Saliva is a rather complex biological matrix that—besides His—is known to contain a variety of amino acids (Balci et al. 2021). To evaluate the specificity of our method, we analyzed a pooled saliva sample (n = 6) using two different PCD reaction mechanisms; (i) with the current optimized reaction of His with OPA in the absence of nucleophilic reagent that is highly specific for His and (ii) with the generic OPA mechanism for primary amines that includes the use of N-acetyl-cysteine as nucleophilic reagent (López-Grío et al. 2000). In the second experiment, all salivary compounds with primary amino moieties are expected to react and yield fluorescent derivatives. The experimental results depicted in the chromatograms of Fig. 4 clearly demonstrate that although saliva contains a substantial amount of primary amino compounds (chromatogram B, generic OPA mechanism), only the peak of His is present when the same sample is analyzed by our method (chromatogram A, specific OPA mechanism).

Another series of selectivity experiments were carried out to investigate the potential interfering effect of glutathione (GSH). The latter biologically active tri-peptide is reported to be present in saliva (Ngamchuea et al. 2017a) and is also reported to react with OPA in the absence of nucleophilic compounds in a similar manner to His (with significantly

shifted excitation and emission wavelengths though) (Tsiasioti et al. 2020b; Tsiasioti and Tzanavaras 2021). As can be seen in Fig. 7S (supplementary section), GSH is not retained in the cation exchange column under the selected conditions, it is practically eluted at the void volume of the system and it is efficiently separated from the peak of the target analyte ($R_s > 4$).

The potential carryover was studied by subsequently analyzing a blank and a spiked saliva sample at the highest calibration level. No influence on the signal was recorded indicating that the "between-injection" washing procedures of the injection valve and the column were satisfactory.

Evaluation of the matrix effect

As mentioned above, validation experiments based on the accuracy profiles were carried out using a surrogate matrix (artificial saliva). It was therefore necessary to investigate the existence/absence of matrix effects in real saliva samples analysis. Due to the complexity of biological samples, the evaluation of the matrix effect is a critical analytical parameter that dictates the accuracy and design of the quantification procedure. As a general rule, methods that are based on post-column reactions are less prone to phenomena associated with the sample matrix compared for example to the pre-column derivatization mode (Zacharis and Tzanavaras 2013; Rigas 2013).

Following the procedure described in the Experimental section and due to the simple treatment of the saliva and the specificity of the PCD reaction, the matrix effect was calculated to be + 2.5%, enabling the use of the aqueous calibration curve for quantification. The analytical scheme is therefore simplified, avoiding the necessity of standard addition or matrix matched calibration.

Samples stability studies

Deproteinized saliva proved to be stable at RT for at least 72 h with percent recoveries ranging between 91 and 104% (autosampler stability). On the other hand, in untreated saliva stored at RT the levels of His increased almost fivefold between 0 and 72 h (Fig. 8S, supplementary data). This behavior can be attributed to the release of His from His-rich peptides and proteins that are reported to be present in human saliva (Khurshid et al. 2017; Soraya et al. 2019). For example, Histatins are salivary His-rich peptides (18–28 mol%) that are present in saliva at concentration ranges between 50 and 450 µM (Melino et al. 2014). Refrigeration at 4 °C improved the stability but it did not eliminate the phenomenon (twofold increase within 72 h). The experimental findings showed that untreated saliva is stable for His analysis only upon freezing at – 18 °C, with

percent recoveries in the range of 92–97%. Alternatively, saliva samples could be deproteinized upon collection.

Analytical application

Seventeen human saliva samples were collected and treated as described in the experimental section. The samples were analyzed either directly after treatment or following dilution with 1 + 1 water + acetonitrile when needed (elevated levels of His). The experimental results are presented in Table 2. His was found to be present in all samples at quantifiable concentrations ranging between 2.7 and 19.5 µM (mean value = 9.1 µM, $n = 17$).

The accuracy of the procedure was evaluated by spiking the samples at two levels of His, namely 2.5 and 5.0 µM. The percent recoveries ranged between 81.5 and 118.3% (mean value = 97.8 ± 13.9%) at 2.5 µM His and 85.0–107.3% (mean value = 94.6 ± 8.3%) at 5.0 µM His. Representative chromatograms of blank and spikes saliva samples are depicted in Fig. 5.

Conclusions

In the present study, a robust and reliable method for the determination of endogenous His in human saliva has been optimized and validated. The proposed analytical scheme offers some interesting features including: (i) optimization of the PCD conditions using experimental design and

Table 2 Endogenous levels of Histidine in saliva samples donated by male/female volunteers as determined by the proposed HPLC–PCD method

Sample	Gender/age	Histidine (µM)	SD (µM)
1	F/23	2.7	0.5
2	F/25	8.9	0.1
3	F/27	6.2	0.2
4	F/23	4.3	0.1
5	M/27	11.9	0.3
6	F/25	7.0	0.4
7	F/23	8.7	0.5
8	M/39	12.1	0.2
9	F/23	7.3	0.3
10	M/23	13.2	0.6
11	M/21	17.1	0.2
12	M/25	6.3	0.1
13	M/27	10.1	0.4
14	M/25	19.5	0.5
15	F/29	7.8	0.3
16	M/45	6.6	0.2
17	F/27	4.5	0.4
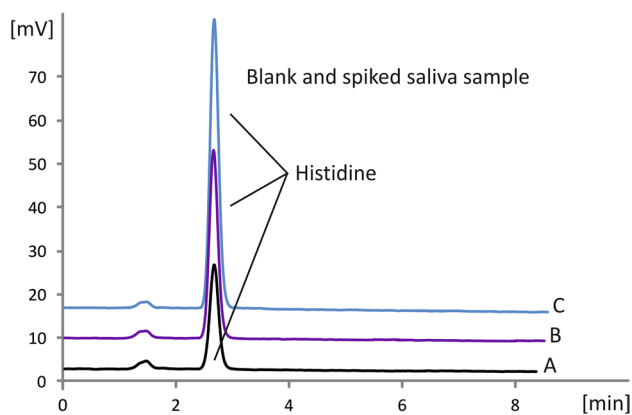
Fig. 5 Representative chromatograms from the analysis of a blank (**A**) and spiked (**B** = 2.5 µM and **C** = 5.0 µM) human saliva samples by the proposed HPLC–PCD method (for experimental details please see the relevant section)

validation based on the concept of total error; (ii) highly specific PCD chemistry versus amino acids; (iii) fast and simple sample treatment avoiding complicated extraction/purification steps; (iv) the method is free from matrix effects enabling convenient quantification using aqueous calibration curves; (v) endogenous His was successfully quantified in all human saliva samples. Ongoing research in cooperation with a state hospital involves the application of the method to an extensive study focusing on the correlation of salivary His levels with various pathological and psychological situations (e.g., stressful situations).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00726-022-03135-7.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

Research involving human participants, their data or biological material All human participants in this study were members of the Laboratory of Analytical Chemistry of the Department of Chemistry and donated saliva samples on a voluntary basis. They were informed in detail about the non-medical nature of the work and provided written consent.

Informed consent All human volunteers participating in this research were informed and provided written consent to participate in the study, including publication of the results.

References

Acuña I, Ruiz A, Cerdó T et al (2021) Rapid and simultaneous determination of histidine metabolism intermediates in human and mouse microbiota and biomatrices. BioFactors. https://doi.org/10.1002/BIOF.1766

Alevridis A, Tsiasioti A, Zacharis CK, Tzanavaras PD (2020) Fluorimetric method for the determination of histidine in random human urine based on zone fluidics. Molecules. https://doi.org/10.3390/MOLECULES25071665

Balci N, Kurgan Ş, Çekici A et al (2021) Free amino acid composition of saliva in patients with healthy periodontium and periodontitis. Clin Oral Investig 25:4175–4183. https://doi.org/10.1007/S00784-021-03977-7/TABLES/3

Bezerra MA, Santelli RE, Oliveira EP et al (2008) Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta 76:965–977. https://doi.org/10.1016/j.talanta.2008.05.019

Cheng F, Wang Z, Huang Y et al (2015) Investigation of salivary free amino acid profile for early diagnosis of breast cancer with ultra performance liquid chromatography-mass spectrometry. Clin Chim Acta 447:23–31. https://doi.org/10.1016/J.CCA.2015.05.008

Derringer G, Suich R (1980) Simultaneous optimization of several response variables. J Qual Technol 12:214–219. https://doi.org/10.1080/00224065.1980.11980968

Elmongy H, Abdel-Rehim M (2016) Saliva as an alternative specimen to plasma for drug bioanalysis: a review. TrAC Trends Anal Chem 83:70–79. https://doi.org/10.1016/J.TRAC.2016.07.010

Emekli-Alturfan E, Kasikci E, Alturfan AA et al (2009) Effect of sample storage on stability of salivary glutathione, lipid peroxidation levels, and tissue factor activity. J Clin Lab Anal 23:93–98. https://doi.org/10.1002/JCLA.20296

FDA (2018) Bioanalytical Method Validation Guidance. In: Food Drug Adm. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry. Accessed 11 Jan 2021

Ferreira SLC, Bruns RE, Ferreira HS et al (2007) Box-Behnken design: an alternative for the optimization of analytical methods. Anal Chim Acta 597:179–186. https://doi.org/10.1016/j.aca.2007.07.011

Gal JY, Fovet Y, Adib-Yadzi M (2001) About a synthetic saliva for in vitro studies. Talanta 53:1103–1115. https://doi.org/10.1016/S0039-9140(00)00618-4

Håkanson R, Rönnberg AL, Sjölund K (1974) Improved fluorometric assay of histidine and peptides having NH2-terminal histidine using o-Phthalaldehyde. Anal Biochem 59:98–109. https://doi.org/10.1016/0003-2697(74)90014-1

Hibbert DB (2012) Experimental design in chromatography: a tutorial review. J Chromatogr B 910:2–13. https://doi.org/10.1016/j.jchromb.2012.01.020

Holeček M (2020) Histidine in health and disease: metabolism, physiological importance, and use as a supplement. Nutr 12:848. https://doi.org/10.3390/NU12030848

Hubert P, Nguyen-Huu JJ, Boulanger B et al (2003) Validation of quantitative analytical procedure, harmonization of approaches. STP Pharma Prat 13:101–138

Hubert P, Nguyen-Huu JJ, Boulanger B et al (2004) Harmonization of strategies for the validation of quantitative analytical procedures: a SFSTP proposal—part I. J Pharm Biomed Anal 36:579–586

Hubert P, Nguyen-Huu J-J, Boulanger B et al (2007) Harmonization of strategies for the validation of quantitative analytical procedures: a SFSTP proposal—part III. J Pharm Biomed Anal 45:82–96

Khurshid Z, Najeeb S, Mali M et al (2017) Histatin peptides: pharmacological functions and their applications in dentistry. Saudi Pharm J 25:25–31. https://doi.org/10.1016/J.JSPS.2016.04.027

Kim D, Mun S, Lee J et al (2018) Proteomics analysis reveals differential pattern of widespread protein expression and novel role of histidine-rich glycoprotein and lipopolysaccharide-binding protein in rheumatoid arthritis. Int J Biol Macromol 109:704–710. https://doi.org/10.1016/J.IJBIOMAC.2017.12.075

Kugimiya A, Fujikawa A, Jiang X et al (2020) Microfluidic paper-based analytical device for histidine determination. Appl Biochem Biotechnol 192:812–821. https://doi.org/10.1007/S12010-020-03365-Z/FIGURES/7

Lee Y-H, Wong DT (2009) Saliva: an emerging biofluid for early detection of diseases. Am J Dent 22:241–248

López-Grío S, Torres-Lapasió JR, Baeza-Baeza JJ, García-Alvarez-Coque MC (2000) Micellar liquid chromatographic separation of amino acids using pre- and post-column o-phthalaldehyde/N-acetylcysteine derivatization. Anal Chim Acta 418:153–165. https://doi.org/10.1016/S0003-2670(00)00965-X

Mandur J, Budman H (2014) Robust optimization of chemical processes using Bayesian description of parametric uncertainty. J Process Control 24:422–430. https://doi.org/10.1016/j.jprocont.2013.10.004

Marvin RK, Saepoo MB, Ye S et al (2017) Salivary protein changes in response to acute stress in medical residents performing advanced clinical simulations: a pilot proteomics study. Biomarkers 22:372–382. https://doi.org/10.1080/1354750X.2017.1279215

Melguizo-Rodríguez L, Costela-Ruiz VJ, Manzano-Moreno FJ et al (2020) Salivary biomarkers and their application in the diagnosis and monitoring of the most common oral pathologies. Int J Mol Sci 21:1–17. https://doi.org/10.3390/IJMS21145173

Melino S, Santone C, Di Nardo P, Sarkar B (2014) Histatins: salivary peptides with copper(II)- and zinc(II)-binding motifs. FEBS J 281:657–672. https://doi.org/10.1111/FEBS.12612

Nakamura Y, Kodama H, Satoh T, Adachi K, Watanabe S, Yokote Y, Sakagami H (2010) Diurnal changes in salivary amino acid concentrations. In Vivo (brooklyn) 24:837–842

Ngamchuea K, Batchelor-McAuley C, Compton RG (2017a) Rapid method for the quantification of reduced and oxidized glutathione in human plasma and saliva. Anal Chem 89:2901–2908. https://doi.org/10.1021/ACS.ANALCHEM.6B04186/SUPPL_FILE/AC6B04186_SI_001.PDF

Ngamchuea K, Chaisiwamongkhol K, Batchelor-Mcauley C, Compton RG (2017b) Chemical analysis in saliva and the search for salivary biomarkers—a tutorial review. Analyst 143:81–99. https://doi.org/10.1039/C7AN01571B

Razavi F, Khajehsharifi H (2021) A colorimetric paper-based sensor with nanoporous SBA-15 for simultaneous determination of histidine and cysteine in urine samples. Chem Pap 757(75):3401–3410. https://doi.org/10.1007/S11696-021-01548-4

Reuven Y, Rubinstein DPK (2017) Simulation and the monte carlo method. Wiley

Rigas PG (2012) Review: liquid chromatography—post-column derivatization for amino acid analysis: strategies, instrumentation, and applications. Instrum Sci Technol 40:161–193. https://doi.org/10.1080/10739149.2011.651669

Rigas PG (2013) Post-column labeling techniques in amino acid analysis by liquid chromatography Amino Acid Analysis. Anal Bioanal Chem 405:7957–7992. https://doi.org/10.1007/S00216-013-7127-3/FIGURES/4

Salivette Salivette - Hygienic saliva collection for diagnosis and monitoring. In: sarstedt AG. https://www.sarstedt.com/fileadmin/user_upload/99_Broschueren/Englisch/156_Salivette_GB_0813.pdf

Scalise M, Galluccio M, Console L et al (2018) The Human SLC7A5 (LAT1): the intriguing histidine/large neutral amino acid transporter and its relevance to human health. Front Chem. https://doi.org/10.3389/FCHEM.2018.00243

Soraya GV, Abeyrathne CD, Buffet C et al (2019) (2019) Ultrasensitive and label-free biosensor for the detection of Plasmodium falciparum histidine-rich protein II in saliva. Sci Reports 91(9):1–10. https://doi.org/10.1038/s41598-019-53852-5

Stampina E, Tsiasioti A, Klimatsaki K et al (2021) Determination of histidine in human serum and urine by cation exchange chromatography coupled to selective on-line post column derivatization. J Chromatogr B Analyt Technol Biomed Life Sci. https://doi.org/10.1016/J.JCHROMB.2021.122697

Tan SP, Brown SB, Griffiths CEM et al (2017) Feeding filaggrin: effects of L-histidine supplementation in atopic dermatitis. Clin Cosmet Investig Dermatol 10:403–411. https://doi.org/10.2147/CCID.S146760

Tsiasioti A, Tzanavaras PD (2021) Determination of glutathione and glutathione disulfide using zone fluidics and fluorimetric detection. Talanta 222:121559. https://doi.org/10.1016/j.talanta.2020.121559

Tsiasioti A, Andreou A, Tzanavaras PD (2020a) Selective reaction of homocysteine with o-phthalaldehyde under flow conditions in highly alkaline medium: fluorimetric determination using zone fluidics. Luminescence 35:1402–1407. https://doi.org/10.1002/BIO.3904

Tsiasioti A, Zacharis CK, Tzanavaras PD, Zotou AS (2020b) Study of the oxidative forced degradation of glutathione in its nutraceutical formulations using zone fluidics and green liquid chromatography. Separations 7:16. https://doi.org/10.3390/SEPARATIONS7010016

Wang Y, Zhao Z, Qin J et al (2020) Rapid in situ analysis of l-histidine and α-lactose in dietary supplements by fingerprint peaks using terahertz frequency-domain spectroscopy. Talanta. https://doi.org/10.1016/J.TALANTA.2019.120469

Wu X, Huang J, Zhang C, Kang X (2021) Nanofibers comprising polystyrene and zinc acetate 2 for extraction of salivary histidine prior to its determination by HPLC-DAD. Anal Sci. https://doi.org/10.2116/analsci.21P134

Yamaguchi H, Nakata K, Tatsumi M et al (2019) Development of a novel l-histidine assay method using histamine dehydrogenase and a stable mutant of histidine decarboxylase. Anal Biochem 570:13–20. https://doi.org/10.1016/J.AB.2018.12.017

Yao Z, Liu H, Liu Y et al (2020) Fluorimetric determination of histidine by exploiting its inhibitory effect on the oxidation of thiamine by cobalt-containing Prussian Blue nanocubes. Microchim Acta 187:1–7. https://doi.org/10.1007/S00604-019-3930-7/TABLES/2

Yoshimura T, Kamataki T, Miura T (1990) Difference between histidine and histamine in the mechanistic pathway of the fluorescence reaction with ortho-phthalaldehyde. Anal Biochem 188:132–135. https://doi.org/10.1016/0003-2697(90)90540-P

Zacharis CK, Tzanavaras PD (2013) Liquid chromatography coupled to on-line post column derivatization for the determination of organic compounds: a review on instrumentation and chemistries. Anal Chim Acta 798:1–24. https://doi.org/10.1016/j.aca.2013.07.032

Zhang CZ, Cheng XQ, Li JY et al (2016) Saliva in the diagnosis of diseases. Int J Oral Sci 83(8):133–137. https://doi.org/10.1038/ijos.2016.38

Zuman P (2004) Reactions of orthophthalaldehyde with nucleophiles. Chem Rev 104:3217–3238. https://doi.org/10.1021/cr0304424