

The effect of sampling procedures on the urate and lactate concentration in oral fluid

T. Lomonaco^{a*}, S. Ghimenti^a, D. Biagini^a, E. Bramanti^b, M. Onor^b, F. G. Bellagambi^a,
R. Fuoco^a, F. Di Francesco^{a,c}

^a Department of Chemistry and Industrial Chemistry, University of Pisa, Pisa, Italy

^b Institute of Chemistry of Organometallic Compounds, CNR, Pisa, Italy

^c Institute of Clinical Physiology, CNR, Pisa, Italy

* Corresponding Author

E-mail address: tommaso.lomonaco@unipi.it (T. Lomonaco)

51 **Highlights**

- 52 ➤ Analytical methods for the determination of urate and lactate in oral fluid.
- 53 ➤ Sampling procedures influence urate and chloride concentrations, not lactate.
- 54 ➤ Methods suitable for the routine analysis of oral fluid.

55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101

102 **Abstract**

103 The study was aimed at evaluating the influence of sampling procedure on the determination
104 of uric acid and lactate in oral fluid. Samples of non-stimulated and stimulated oral fluid were
105 collected from 22 healthy volunteers. Different degrees of stimulation were obtained by moving a
106 polyester swab within the mouth at 50, 100 and 150 min⁻¹. Three oral fluid samples were
107 consecutively collected from a subgroup of 5 volunteers at a constant stimulation (70 min⁻¹) and at
108 a self-selected pace to evaluate reproducibility.

109 The urate concentration in oral fluid decreased with the increase of the stimulation and oral
110 fluid flow rate ($r = -0.98$, $p = 0.01$). Also, the lactate concentration was much ($p = 0.03$, two tailed)
111 lower in samples collected under a mild stimulation (50 min⁻¹) than in samples collected without
112 stimulation. Nevertheless, it progressively increased at higher stimulations (100 and 150 min⁻¹). A
113 transfer process mediated by membrane carriers (i.e. urate transporter and organic anion
114 transporters) was hypothesized to explain these results. Finally, a reduced variability (relative
115 standard deviation below 10%) of the urate concentration was obtained when oral fluid was
116 sampled at constant stimulation (70 min⁻¹), but it increased remarkably (20–50%) in case of
117 sampling at self-selected pace. Nevertheless, expressing the salivary excretion of urate as a function
118 of time (μgmin^{-1}), the variability of sampling procedure at self-selected pace was lower than 15%.

120
121 **Keywords**

122 Oral fluid, sampling procedures, uric acid, lactate, flow rate, pH
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151

152 1. Introduction

153 The correlation between drug concentration in oral fluid (OF) and the corresponding
154 concentration of the unbound and pharmacologically active fraction in blood [1] has recently made
155 OF analysis attractive for many researchers working in the field of pharmacokinetic [2] and
156 therapeutic drug monitoring (TDM) [3,4]. In addition, the minimally invasive sampling and the
157 simpler matrix compared to blood has made OF analysis popular for human biomonitoring [5].

158 In humans, OF mainly originates from three pairs of major salivary glands (parotid, sublingual
159 and submandibular) and from a large number of minor salivary glands [6]. Healthy adults normally
160 produce 500–1500 mL of OF per day at an approximate rate of 0.3–1.0 mLmin⁻¹, but several
161 physiological and pathological conditions can modify this secretion rate [7]. The non-invasive
162 sampling that does not require trained personnel is the main advantage of OF analysis compared to
163 the analysis of blood and its derivatives (e.g. plasma).

164 Several procedures for collecting non-stimulated and stimulated OF samples are currently
165 available [8]. Non-stimulated OF samples can be collected by draining, spitting, suction and/or
166 adsorption into swab, whereas secretion can be chemically stimulated with few drops of citric acid
167 (0.1–0.2 M) on the tongue or mechanically stimulated by asking the patient to chew paraffin wax,
168 parafilm, rubber bands or chewing gum. After stimulation, the OF can be spat out, suctioned or
169 absorbed [8]. Assuming a unit density for OF [9], flow rate (mLmin⁻¹) is estimated from the ratio of
170 sample weight (grams) to sampling time (min). Swab saturation and swallowing of saliva from the
171 patient should be avoided in order to have a reliable estimate of the flow rate. Stimulation allows to
172 collect large sample volumes (>1 mL) in a short time (30–60 s) and limits the variability of salivary
173 pH, as most samples' pH values lie in a narrow range centred at pH 7.5 [10].

174 Generally, the transfer of a compound from blood to OF occurs by passive transport through
175 the salivary membrane or active processes mediated by a protein carrier. The actual mechanism (or
176 combination of mechanisms) depends on the chemical-physical properties of the compound (i.e.
177 molecular size, pKa and lipid solubility), as well as on the OF pH and flow rate [11]. When passive
178 transport is involved, the concentrations of lipid-soluble acidic or basic compounds in OF depend
179 on the degree of ionization in plasma and OF. Since salivary membranes are not permeable to
180 charged “ionized” molecules, permeability is governed from the pH-partition hypothesis [11]. The
181 Rasmussen model allows to calculate the theoretical OF to plasma concentration ratio (OF/P) for a
182 compound crossing the salivary membrane by a rapid diffusion through the acinar cells. Fast
183 diffusion makes the concentration of such molecules in OF independent of flow rate [12]. On the
184 contrary, a hydrophilic compound is a poor membrane-permeant due to its limited solubility in
185 lipids. For this reason, it slowly enters OF only via the tight junctions of the acinar cells (the
186 paracellular route) making its concentration in such fluid significantly influenced by flow rate (an
187 increased salivation dilutes the compound and decreases concentration) [11]. In this case, the “tube”
188 model of capillary diffusion is needed to estimate the OF/P concentration ratio [10]. In the case of
189 an active process, a specialized membrane protein guarantees the movement of a compound across
190 the salivary membrane against the concentration gradient. In primary active transport, these proteins
191 require energy in the form of adenosine triphosphate, whereas in the secondary transport the
192 electrochemical gradient is responsible for the transfer of the compound [13].

193 The inter- and intra-subject variability of OF pH and flow rate affects the OF to plasma
194 concentration ratio and limits the use of OF analysis for therapeutic monitoring to a restricted set of
195 drugs (e.g. unconjugated steroids) characterized by a high permeability through the salivary
196 membranes [14]. Other drugs require ad hoc collection protocols, as recently discussed for warfarin
197 monitoring [15], to make sure that data are representative of the actual conditions of patients as well
198 as to compare data collected at different times.

199 Uric acid (AU) and lactic acid are two important metabolites produced from the xanthine
200 oxidase enzyme via the purine metabolism pathways [16] and from pyruvate via anaerobic
201 glycolysis respectively [17]. Several papers proposed to monitor the concentrations of these two
202 compounds because of correlations with several pathological conditions (e.g. chronic heart failure)

[18–22] or therapies (e.g. haemodialysis) [23]. Uric acid is a weak acid (pKa equal to 5.4 at 20 °C) [24] distributed throughout the extracellular fluid compartment by protein carriers, namely urate transporter (URAT1) and organic anion transporters (OATs). Lactic acid (pKa equal to 3.9 at 20 °C, [17]) is produced by lactate dehydrogenase in skeletal muscles, liver and red blood cells under anaerobic conditions [16]. At physiological pH (7.40 ± 0.02) [25], UA and lactic acid are mainly dissociated to urate (UR) and lactate (LAC) respectively.

In the kidney, URAT1 and OATs mediates the uptake of UR from the renal tube into the renal tubular cells in exchange of organic anions, such as lactate and nicotinate [26,27]. Recently, Ikarashi et al. found that URAT1 and OATs are expressed both in the ductal cells (i.e. OAT1, 2 and 4) and in the acinar cells (i.e. OAT2 and 3) of the salivary glands [28]. Sato et al. reported that the reabsorption mechanism of UR by URAT1 is influenced by different anions, among which hydroxyl and chloride [29]. Since stimulation increases the OF concentrations of both these anions up to ten times [30,31], it is reasonable to speculate that the transfer mechanism of UR from blood to OF may be affected.

Based on this information, the objective of this study was to i) compare the composition of non-stimulated and stimulated OF samples collected at different degrees of stimulation (50, 100 and 150 min⁻¹) and then ii) set up a reliable sampling protocol for the non-invasive monitoring of AU and LAC.

2. Materials and methods

2.1. Statement of ethics and study subjects

The study was approved by the Ethics Committee of the Azienda Ospedaliero-Universitaria Pisana (“A co-operative mHEALTH environment targeting adherence and management of patients suffering from Heart Failure”, protocol number: 643694). Twenty-two nominally healthy subjects (12 males and 10 females) volunteered to participate and signed a written informed consent.

2.2. Chemicals and materials

Uric acid, i.e. 7,9-Dihydro-1H-purine-2,6,8(3H)-trione (purity $\geq 99\%$), EHNA hydrochloride, i.e. erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride (purity $\geq 98\%$), dipyrindamole (purity $\geq 98\%$), sodium hydroxide (purity $\geq 98\%$, pellets anhydrous), lactate (TraceCERT, 1000 ± 2 mgL⁻¹), 9-chloromethyl-anthracene (purity $\geq 98\%$), tetra-n-butylammonium bromide (purity $\geq 98\%$) and triethanolamine (purity $\geq 99\%$), sodium chloride (purity $\geq 99\%$), sodium nitrate (purity $\geq 99\%$), phosphoric acid (purity $\geq 99\%$) and acetonitrile at HPLC grade were purchased from Sigma Aldrich (Italy). HPLC grade water was produced by a milli-Q reagent water system (Millipore, USA).

All the liquid solutions and OF samples were stored in sterile polypropylene containers from Eppendorf (Italy).

2.3. Oral fluid collection

Oral fluid samples were collected by roll-shaped polyester swabs (Salivette, Sarstedt, Germany) between 9 and 11 AM, in order to avoid that variations related to circadian rhythms could affect results [32]. Each volunteer was asked to refrain from eating, drinking, smoking, chewing gum, and oral hygiene practices for at least 1 h prior to OF collection. Subjects placed the swab in the mouth between the gum and cheek and kept it steady for 2 min (procedure A) to collect non-stimulated OF samples. On the contrary, different degrees of stimulation were obtained by moving the swab in the mouth at 50, 100 and 150 min⁻¹ (procedure B). Three stimulated OF samples were consecutively collected from a subgroup of 5 volunteers moving the swab for 1 min at 70 min⁻¹ (procedure C) and for 1 min at self-selected pace (procedure D) to evaluate the

repeatability of the sampling procedure. The beat of a digital metronome (Real Metronome, Australia) helped volunteers to move the swab at the selected frequency.

The OF flow rate (grams per minute) was calculated from the ratio of sample weight and collection time, assuming a unit density for OF. Immediately after sampling, two experimenters measured OF sample pH by narrow range ($5.2 < \text{pH} < 8.1$) pH paper strips (Pehanon, Macherey Nagel, Germany) with a resolution of 0.3 pH units.

The OF samples were then recovered by centrifuging the swabs at 3000 rpm for 5 min at room temperature (25 ± 1 °C). The sample was split into three aliquots and then frozen at -80 °C until assay.

2.4. Oral fluid preparation and analysis

2.4.1 Determination of uric acid

An aliquot of sample (20 μL) was diluted at 1 mL with a water solution containing 0.1% (v/v) formic acid and 3% acetonitrile. The resulting solution was then vortex-mixed for 30 s and injected (10 μL) into the HPLC system.

Separation of UA in diluted OF samples was carried out by high performance liquid chromatography in isocratic mode at 25 °C, with a mobile phase consisting of 3% acetonitrile and 97% of 0.1% (v/v) aqueous formic acid at a flow rate of 1 mL min^{-1} . Ultraviolet detection was performed at a wavelength of 290 nm.

2.4.2 Determination of lactate

The full details of the analytical procedures are reported elsewhere [33]. Briefly, an aliquot of sample (10 μL) was added to the amber reaction vial together with 20 μL of triethanolamine (5%), 90 μL of tetra-n-butylammonium bromide (90 mM) and 370 μL of 9-chloromethyl-anthracene (9-CMA) (10 mM) (final volume of 490 μL). The resulting solution was incubated at 70 ± 1 °C for 60 min in a thermostatic water-bath in the dark to facilitate the derivatization reaction. The solution was cooled for 1 min in ice and then diluted 10-fold with a water-acetonitrile mixture (80:20, v/v). This solution was injected (10 μL) into the HPLC system.

High performance liquid chromatography separation was performed as follow: isocratic conditions for 1 min (20% of acetonitrile), followed by an increase of acetonitrile at 40% in 15 min and isocratic for other 9 min at 40% of acetonitrile. Excitation and emission were set at wavelength of 365 and 410 nm, respectively.

2.4.3 Determination of chloride

An aliquot of sample (0.2 mL) was diluted with 0.04 mL of NaNO_3 (5 M) and 1.76 mL of milli-Q water (final volume 2 mL). The solution was vortex-mixed for 30 s and then transferred into a Pyrex-glass cell for the measurement, which was carried out at room temperature (25 ± 1 °C) under constant magnetic stirring. The electrode was rinsed before and after the analysis with milli-Q water. Blank samples were prepared in the same way by diluting 0.2 mL of milli-Q water and analysed every four OF samples.

2.5. Equipment

High performance liquid chromatography (HPLC) analysis was carried out using a Jasco HPLC system equipped with an AS 2055 autosampler, a PU 2089 quaternary low-pressure gradient pump, an UV 2070 ultraviolet detector and a FP 2020 fluorescence detector. The column temperature was controlled by a HT 3000 thermostat (ClinLab, USA). The HPLC system was controlled using ChromNAV™ software (v. 1.9, Jasco, Japan).

A ZX4 Advanced Vortex Mixer (VELP Scientifica, Italy) and a Centrifuge 5804 R equipped

with an A-4-44 swinging bucket rotor (Eppendorf, Italy) were used for sample vortex-mixing and centrifugation, respectively.

Chromatographic separation of UA was carried out with a Zorbax SB-Aq reversed-phase column (Agilent, 250 × 4.6 mm, 5 µm) connected to a Zorbax SB-Aq guard column (Agilent, 12.5 × 4.6 mm, 5 µm). The LAC-9-CMA adduct was separated using a Poroshell EC-C-18 reversed-phase column (Agilent, 100 × 4.6 mm, 2.7 µm) connected to a guard column TC-C-18 (Agilent, 12.5 × 4.6 mm, 5 µm).

Absorption spectra of UA were recorded by a Lambda 25 spectrophotometer (PerkinElmer, USA).

A chloride Ion Selective Electrodes (ISE) (Crison, Spain) was used to determine the concentration of chloride in OF samples. All the potential measurements were carried out by a PC2700 pH/mV/Conductivity/°C/°F meter (Eutech Instrument, USA) capable of reading to 0.01 mV.

All data were analysed using GraphPad Prism (v. 6.0, GraphPad Software Inc., USA).

2.6. Standard solutions and quality control samples

A mobile phase composed of 3% of acetonitrile and 97% of 0.1% (v/v) aqueous formic acid was prepared and filtered through a 0.45 µm cellulose membrane, then stored at 4 °C for 1 month in a sterile polypropylene container.

A stock solution of UA (970 µgmL⁻¹) was prepared by dissolving a weighed amount of the pure compound in milli-Q water and adding an aliquot of NaOH 10 M to help dissolution of UA, which has a poor solubility in pure water (0.06 mgmL⁻¹ at 20 °C) [34]. This solution was further diluted with the mobile phase to obtain standard solutions in the required range (0.5–20 µgmL⁻¹), which were protected from light and then stored at 4 °C up to 1 month.

A stop solution for the analysis of UA was prepared by mixing 150 µL of a dipyridamole solution (42 mg dissolved in 25 mL of ethanol), 850 µL of a solution of EHNA (24 mg dissolved in 25 mL of saline solution) and 30 mL of saline solution (0.9% w/v of NaCl). Once prepared, the stop solution was protected from light and stored at 4 °C up to 1 month.

Stock solutions of triethanolamine (5%), tetra-n-butylammonium bromide (90 mM) and 9-chloromethyl-anthracene (10 mM) were prepared by dissolving weighed amounts of the pure compounds in acetonitrile. Each solution was filtered through a 0.45 µm cellulose membrane and stored at 4 °C up to 1 month in an amber vial.

Working solutions of LAC were prepared in the range 47 and 498 µgmL⁻¹ by diluting the stock solution (1000 ± 2 mgL⁻¹) with milli-Q water and stored at 4 °C up to 1 month in an amber vial.

A stock solution of sodium nitrate (5 M) was prepared by dissolving a weighed amount of the pure compound in milli-Q water. Sodium chloride was kept at 120 °C for at least 2 hours and then stored in a desiccator. A stock solution of NaCl (0.1 M) was prepared by dissolving a weighed amount of the pure compound in milli-Q water. This solution was further diluted with milli-Q water to obtain working solutions in the required range (5–80 mM).

Pooled OF samples (POFSs) were obtained by pooling samples from 10 healthy volunteers. Aliquots of POFSs were spiked with known amounts of UA, LAC and NaCl to obtain standard OF samples at different concentration levels. All these samples were stored at -80 °C until analysis.

3. Results and discussion

3.1. Oral fluid collection

Three collection devices (cotton swab, cotton swab impregnated with citric acid and synthetic swab), commercially available from Sarstedt, were compared to assess recovery of analytes and the possible release of interfering compounds from the swab material.

For this purpose, two standard solutions containing UA ($50 \mu\text{g mL}^{-1}$) and LAC ($100 \mu\text{g mL}^{-1}$) were prepared and analysed according to the procedures described in section 2.4.1 and 2.4.2 respectively. Then, three aliquots (6 mL) were prepared from each solution and further split into three additional aliquots (2 mL) that were absorbed in the collection devices (the capacity of a device is about 2.2 mL). Overall, three replicates were used for each type of device and solution, for a total number of 18 samples. The devices were centrifuged for 5 min at 3000 rpm and room temperature, then contents were analysed. For each compound, the recovery was calculated from the ratio of the concentration measured in content of the swab to the concentration of the initial solution. The synthetic swab showed the lowest background and the highest recovery percentage ($>95\%$) for both UA and LAC (data not shown), and was so selected to be used for collecting OF in UA and LAC analysis. No specific tests were performed to assess the recovery of chloride, as we thought it unlikely for an ionic compound to be absorbed in the swab.

Further tests were carried out with the synthetic swab to verify the possible influence of sample pH on the recovery of the target compounds from the swab. A pooled OF sample, having a pH of 7.2, was spiked with 50 and $100 \mu\text{g mL}^{-1}$ of UA and LAC respectively and then split into three aliquots (7 mL). Two of them were acidified at pH 5 and 6 by adding 7 and 5 μL of H_3PO_4 (1 M) respectively, whereas the last one was alkalized to pH 8 by adding 8 μL of NaOH (1 M). These samples were directly analysed and then three aliquots (2 mL) of each were absorbed into polyester swabs, recovered by centrifugation and analysed as described before.

Table 1 shows that both compounds were quantitatively recovered independently of pH value, confirming that the synthetic swab is a reliable device for this kind of analyses.

Table 1

Recovery of uric acid ($50 \mu\text{g mL}^{-1}$) and lactate ($100 \mu\text{g mL}^{-1}$) in a pooled oral fluid sample from synthetic swabs at pH values ranging from 5 to 8.

Measured pH	Average recovery % (RSD ^a)	
	Uric acid	Lactate
5.3	98% (1%)	97% (6%)
6.0	99% (2%)	94% (5%)
7.2	101% (0.4%)	98% (7%)
8.1	99% (1%)	96% (8%)

^a Three replicates.

3.2. HPLC separation of uric acid and lactate-9-CMA adduct in oral fluid

Uric acid is a highly polar compound ($\log K_{ow}$ of -2.17 [35]) and therefore its retention on traditional C-18 and other alkylsilyl stationary phases under RP-HPLC conditions is problematic. Ion paring reagents are commonly used to enhance retention of highly polar compounds, but these additives increase the complexity of the mobile phase and can result in lengthy column equilibration time. The chromatographic behaviour of UA was evaluated by a Zorbax SB-Aq reversed-phase column, which allows the use a purely aqueous mobile phase. We found that an isocratic elution with a mobile phase consisting of 3% acetonitrile and 97% water with 0.1% (v/v) of formic acid, at a flow rate of 1 mL min^{-1} and 25°C , achieved a good retention ($k' = 1.5$) of UR with a run time of 9 min. Fig. 1 shows the HPLC-UV chromatograms of blank (0.1% (v/v) of formic acid), a standard solution of UA ($50 \mu\text{g mL}^{-1}$), a pooled OF ($35 \mu\text{g mL}^{-1}$) and pooled OF sample spiked with UA ($50 \mu\text{g mL}^{-1}$).

Unlike Pellegrini et al. [33], the chromatographic separation of the LAC-9-CMA adduct was carried out by a C-18 reversed-phase column with smaller particles (2.7 vs 5 μm), which guarantees a higher separation efficiency. Tests were performed to improve the resolution of the LC method. We found that the use of isocratic conditions for 1 min (20% acetonitrile), followed by an increase

of acetonitrile to 40% in 15 min and additional 9 min isocratic at 40% acetonitrile, represented an efficient way to separate the LAC-9-CMA adduct. Fig. 2 shows the HPLC-FP chromatograms of blank (milli-Q water), standard working solution of LAC ($100 \mu\text{g mL}^{-1}$), pooled OF ($47 \mu\text{g mL}^{-1}$) and pooled OF sample spiked with LAC ($50 \mu\text{g mL}^{-1}$).

3.3. Analytical figures of merits

3.3.1 Calibration curves, limit of detection (LOD) and quantification (LOQ)

The LOD and LOQ values for UA, LAC and chloride were calculated, in accordance with IUPAC guidelines [36], as three and ten times the standard deviation (sb) of the low level spiked blank or blank sample. The calibration curves ($a = m \times b$) for all the analytes were evaluated by the Deming regression analysis.

In the case of UA, a pooled OF sample spiked with $50 \mu\text{g mL}^{-1}$ of UA was split into five aliquots ($50 \mu\text{L}$) and then diluted (10000-fold) with 0.1% (v/v) of formic acid and 3% of acetonitrile in water. The LOD and LOQ values were 3 and 10 ng mL^{-1} , respectively.

Five aliquots ($10 \mu\text{L}$) of milli-Q water were analysed for the determination of LAC and LOD and LOQ values resulted 6 and $19 \mu\text{g mL}^{-1}$, respectively.

Similarly, five aliquots ($200 \mu\text{L}$) of milli-Q water were analysed for the determination of chloride and LOD and LOQ values resulted 0.2 and 0.6 mM, respectively.

The working range of $0.5\text{--}20 \mu\text{g mL}^{-1}$ was chosen for the determination of UA into OF sample. The best-fit model for the seven-point calibration curves ($n = 3$ at each concentration) of UA was: $a = (370 \pm 2) \times 10^3 b$, ($R^2 = 0.999$).

For the determination of LAC in OF, the FP signal increased linearly with the LAC concentration between 47 and $498 \mu\text{g mL}^{-1}$ and the best fit model for the five-point calibration curves ($n = 3$ at each concentration) was: $a = (50 \pm 5) \times 10^5 b$, ($R^2 = 0.999$).

The working range of 5–80 mM was chosen for the determination of chloride into OF sample and the five-point calibration curves ($n = 3$ at each concentration) resulted: $a = (-54 \pm 3) \times b$, ($R^2 = 0.997$) at 25°C , in agreement with the value reported in the electrode datasheet ($-55 \pm 4 \text{ mV/pCl}^-$ at $20 \pm 5^\circ\text{C}$).

3.3.2 Recovery and precision

The recovery and precision for UA and chloride were determined with validation experiments over 5 days, whereas data concerning LAC are available from Pellegrini et al. [33]. The recovery from spiked pooled OF samples was calculated as the percentage ratio of the difference between the analyte concentrations measured in the spiked and the non-spiked samples to the concentration in spiked samples. Pooled OF samples, spiked at 50, 100, 150 and $200 \mu\text{g mL}^{-1}$ of UA and 20, 50 and 80 mM of chloride, were prepared and each one was evaluated in triplicate within the same day and on three consecutive days. The recovery of both compounds and the corresponding intra- and inter-day precision, expressed as relative standard deviation (RSD), are reported in Table 2.

Table 2
Recovery, intra- and inter-day precision relevant to the measurement of uric acid and chloride concentration in spiked pooled oral fluid samples.

Uric acid concentration ($\mu\text{g mL}^{-1}$)			Recovery %	Intra-day ^a RSD	Recovery %	Inter-day ^b RSD
Non-spiked	Spiked	Total				
40	44	79	89	4%	88	5%
	86	116	88	6%	91	7%
	128	150	86	5%	85	8%
	183	201	88	7%	90	8%
Chloride concentration (mM)			Recovery %	Intra-day ^a RSD	Recovery %	Inter-day ^b RSD
Non-spiked	Spiked	Total				
17	20	35	90	6%	92	9%
	51	64	92	4%	91	8%
	84	95	93	5%	90	7%

^a Calculated from three replicates at each concentration value.

^b Calculated from three replicates at each concentration in three days.

3.3.3 Interference and matrix effect

The absence of interferences in the analytical procedures was evaluated by comparing the slopes [37], reported with the corresponding standard deviation, of the calibration curves for a set of standard working solutions (AU: $a (370 \pm 2) \times 10^3 b$, LAC: $a = (50 \pm 5) \times 10^5 b$ and chloride: $a = (-54 \pm 3) \times b$) and a set of treated POFS samples (AU: $a (360 \pm 7) \times 10^3 b + 160 \times 10^3$, LAC: $a = (51 \pm 4) \times 10^5 b + 5 \times 10^3$ and chloride: $a = (-53 \pm 4) \times b + 30$). For UA, LAC and chloride these slopes were not significantly different at a confidence level of 95%, highlighting the absence of interferences into OF.

Our chloride data were in good agreement with those reported by Narashimam et al. [38], who showed that the signal (mV) of a solution containing 120 ng mL^{-1} of chloride was not influenced by inorganic ions (i.e. bromide, sulphide, cyanide and iodide) at concentrations two times lower.

3.3.4 Stability

The stability of standard working solutions (UA, LAC and chloride) was assessed at room temperature ($25 \pm 1^\circ\text{C}$) and 4°C , whereas the stability in OF samples was also studied at -80°C . The initial concentrations of the analytes ($t = 0 \text{ h}$) were used as the reference values and samples' stability was evaluated by analysing the variance (ANOVA) at a confidence level of 95%.

Standard working solutions of UA ($0.5, 2$ and $10 \mu\text{g mL}^{-1}$) and LAC-9-CMA adduct ($47, 200$ and $498 \mu\text{g mL}^{-1}$) were stable throughout the duration of a typical sequence of chromatographic analyses (storage in the autosampler for about 24 h at room temperature) and up to one month at 4°C . The stability of chloride standard working solutions ($5, 20$ and 100 mM) at 4°C and room temperature ($25 \pm 1^\circ\text{C}$) showed no significant variations over six months.

Adenosine deaminase, involved in the metabolism of purine, catalyses the irreversible hydrolytic deamination of adenosine to inosine. This reaction is an important source of hypoxanthine and xanthine; therefore, an increase of deamination reaction produces in turn an increase of UA. Since this degradation may also occur in OF because of the presence of adenosine deaminase [39], the stability of UR in OF samples at room temperature ($25 \pm 1^\circ\text{C}$) was also evaluated. Immediately after collection, an OF sample was split into two aliquots: the first aliquot was added (1:1, v/v) with an appropriate stopping solution (composed of EHNA and dipyrindamole) and analysed after 1 h , whereas the second aliquot was kept at room temperature and analysed after 1 h . These two procedures produced similar results. In addition, no significant degradation was

observed in diluted samples (50-fold) during the typical sequence of chromatographic analyses (which includes a storage in the autosampler for about 24 h at room temperature) after a two-month storage at 4 °C. The concentration of UR in OF was stable after two thaw-freeze cycles, whereas a degradation (about 40%) was observed after the third cycle. After six months at -80 °C, the UR concentration in OF was stable at confidence level of 95%.

Several studies highlighted that lactate dehydrogenase (LDH), an enzyme able to catalyses the interconversion of pyruvate and LAC with concomitant interconversion of NADH and NAD⁺, is expressed in OF samples [40,41,42]. Thus, the stability of LAC in OF represents a key-point to obtain reliable data. For this reason, POFSs were split after collection in three aliquots and then analysed immediately (t_0), 3 hours (t_{0+3h}) and 6 hours (t_{0+6h}) later. Fig. 3 shows the trend over time of the LAC-9-CMA concentration normalized to the concentration corresponding to the first observation time.

As reported in Fig. 3, the concentration of LAC in OF showed a slight increase over time although the amount measured after 6 hours was not statistically different ($p = 0.22$, two tailed) in comparison to the value measured at t_0 . The concentration of LAC was also stable up to three thaw-freeze cycles and for six months at -80 °C.

In the case of chloride, the concentration of both standard working solution and OF was not affected by the different storage conditions (i.e. room temperature, 4 °C and -80 °C), confirming the results recently discussed by Puertos [43].

3.4. Influence of oral fluid flow rate and pH on the oral fluid concentrations of urate and lactate

The concentration of UR, LAC and chloride were determined in OF samples collected from 22 nominally healthy volunteers. Subjects were asked to follow the procedures A and B, whereas a subgroup of 5 subjects also provided samples according to the procedures C and D. Volunteers didn't suffer from gout or disturbance of UR metabolism, and their average age and body mass index (BMI) were 29 ± 9 years (range, 21–50) and 23 ± 3 Kg m⁻² (range, 19–28) respectively.

Fig. 4 shows the effect of different collection protocols (non-stimulated vs stimulated) and intensity of stimulation (50, 100 and 150 min⁻¹) on the OF flow rate (Fig. 4A), pH (Fig. 4B) and chloride (Fig. 4C), UR (Fig. 4D) and LAC concentration (Fig. 4E) for all the enrolled subjects (N = 22).

Fig. 4B and 4C show that both pH and chloride increased with the intensity of stimulation, which promotes higher OF flow rates. Good correlation between OF pH and flow rate ($r = 0.75$, $p < 0.0001$) as well as between chloride and flow rate ($r = 0.72$, $p < 0.0001$) were observed in the overall data set (N = 88). These results are in good agreement with the current two-stage theory of saliva formation [44] stating that the primary fluid secreted from salivary acinus is almost isotonic with plasma. As this initial fluid reaches the ductal system of the salivary gland, an energy-dependent transport processes reabsorbs chloride and secretes bicarbonate ions. Since the ductal membranes are not permeable to water, the resulting saliva becomes hypotonic with plasma. At high flow rates (2–4 mL min⁻¹), time is insufficient for an effective reabsorption and secretion to take place, thus increased flow rates lead to higher chloride (50 mM) [6] and bicarbonate concentrations (70 mM) [6]. Under stimulated conditions, the bicarbonate ion mainly contributes to the OF buffer system [45] and then, considering the physiological value of 70 mM, the OF pH is estimated to 7.5.

Figure 4D and 4E show the effect of stimulation on the OF concentration of UR and LAC. Urate concentration significantly decreased from 70 ± 20 µg mL⁻¹ to 30 ± 10 µg mL⁻¹ with flow rate. Concentrations relevant to sample collected without stimulation and the highest flow rate were significantly different ($p < 0.0001$, two tailed) at a confidence level of 95%. In the same conditions, the LAC concentration in OF decreased slightly from 40 ± 20 µg mL⁻¹ to 30 ± 10 µg mL⁻¹, but such difference was not statistically significant ($p = 0.17$, two tailed). However, the LAC concentration

showed a different trend in comparison with UR, in which LAC significantly decreased ($p = 0.03$, two tailed) from non-stimulated to mild stimulated (50 min^{-1}), followed by a progressively increase at higher flow rate values (100 and 150 min^{-1}). Generally, acidic and hydrophilic compounds such as UA (pK_a of 5.4 at 20°C [17] and $\log K_{ow}$ of -2.17 [35]) and lactic acid (pK_a of 3.9 at 20°C [17] and $\log K_{ow}$ of -0.72 [46]) are poor membrane-permeants, so that they principally cross the salivary membranes through the OF tight junctions of the acinar cells (the paracellular route). This process occurs at a low rate and for this reason the concentration in OF is expected to decrease with increasing flow rate [6,11]. As aforementioned, the concentration of LAC did not follow this general rule (Fig. 4D) and then additional phenomena should be considered. According to literature, the urate transporter (URAT1) and the organic anion transporters (OATs) mediates the uptake of UR from the renal tube into the renal tubular cells in exchange of organic anions, such as LAC and nicotinate [47]. Sato et al. indicated that the reabsorption mechanism of UR in blood by URAT1 is influenced by a variety of anions (e.g. chloride) and dicarboxylic acids, such as glutaric acid and 2-oxoglutaric acid [29]. In particular, they highlighted that the change of UR uptake was mainly observed in the presence of chloride gradients. If one considers that URAT1 and OAT1, 2, and 4 are present in ductal cells, and OAT2 and 3 are in acinar cells of the salivary glands [28], it possible to speculate that the both URAT1 and OATs transport UR and LAC in OF.

Since the inter-subject variability is generally higher than the intra-subject variability, trends can be better evaluated by focusing on a single volunteer. In fact, Fig. 5 shows the effect of different collection protocols (non-stimulated vs stimulated) and degrees of stimulation (50, 100 and 150 min^{-1}) on the normalized value of flow rate (black circle), pH (black square) and chloride (white up-pointing triangle), UR (black diamond) and LAC (white down-pointing triangle) concentration for a representative subject.

To assess the reliability of these results, the repeatability of the proposed stimulated sampling procedure was investigated by collecting three consecutive OF samples by procedures C and D from five volunteers (3 males and 2 females). Using the procedure C, we found an RSD lower than 10% for both UR and LAC, whereas a much larger variability (RSD 20–50%) of the UR concentration was observed when the synthetic swab was moved at self-selected pace (procedure D). However, the variability of the UR salivary excretion (μgmin^{-1}), calculated as the UR concentration ($\mu\text{g mL}^{-1}$) times the flow rate (mLmin^{-1}), was lower (RSD 10–15%).

4. Conclusions

In this work, we evaluated the influence of collection procedures on the concentrations of UR, LAC and chloride in OF. The chloride concentration significantly increased ($r = 0.67$, $p < 0.0001$) with the intensity of stimulation. The UR concentration monotonically decreased with the intensity of stimulation and negatively correlated with the OF flow rate ($r = -0.98$, $p = 0.01$). On the contrary, LAC showed a significant decrease ($p = 0.01$, two tailed) from non-stimulated to stimulated (50 min^{-1}) sample, followed by a progressively increase at higher flow rate values (100 and 150 min^{-1}). Since UR and LAC do not diffuse through the salivary membrane due to their low hydrophobicity, a transfer process probably mediated by the membrane carriers (i.e. URAT1 and OATs) was hypothesized.

When three OF samples were collected keeping constant the frequency of stimulation (70 min^{-1}), the variability of collection procedure was always lower than 10%. On the contrary, when the OF was collected without any control of stimulation the variability of collection procedure resulted much higher (20–50%). Nevertheless, expressing the salivary excretion of UR as a function of time (μgmin^{-1}), the variability was lower than 15%.

Acknowledgements

578 The author would like to thank Dr. Bernardo Melai from Department of Chemistry and
579 Industrial Chemistry of University of Pisa for his support and help in the analysis of chloride in oral
580 fluid samples.
581

582
583 **Funding**

584 This work was supported by the European Union [grant number 643694].
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626

References

- [1] S. Pichini, I. Altieri, P. Zuccaro, R. Pacifici, Drug monitoring in nonconventional biological fluids and matrices, *Clin. Pharmacokinet.* 30 (1996) 211–228.
- [2] W.A. Ritschel, G.A. Tompson, Monitoring of drug concentrations in saliva: a non-invasive pharmacokinetic procedure, *Methods Find. Exp. Clin. Pharmacol.* 5 (1983) 511–525.
- [3] H. Liu, M.R. Delgado, Therapeutic drug concentration monitoring using saliva samples. Focus on anticonvulsants, *Clin. Pharmacokinet.* 36 (1999) 453–470.
- [4] S. Ghimenti, T. Lomonaco, M. Onor, L. Murgia, A. Paolicchi, R. Fuoco, L. Ruocco, G. Pellegrini, M.G. Trivella, F. Di Francesco, Measurement of warfarin in the oral fluid of patients undergoing anticoagulant oral therapy, *PLoS One* 6 (2011) 1–7.
- [5] A.K. El-Naggar, L. Mao, G. Staerkel, M.M. Coombes, S.L. Tucker, M.A. Luna, G.L. Clayman, S. Lippman, H. Goepfert, Genetic heterogeneity in saliva from patients with oral squamous carcinomas: implication in molecular diagnosis and screening, *J. Mol. Diagn.* 3 (2001) 164–170.
- [6] J.K. Aps, L.C. Martens, Review: the physiology of saliva and transfer of drugs into saliva, *Forensic Sci. Int.* 150 (2005) 119–131.
- [7] S. Chiappin, G. Antonelli, R. Gatti, E.F. De Palo, Saliva specimen: a new laboratory tool for diagnostic and basic investigation, *Clin. Chim. Acta* 383 (2007) 30–40.
- [8] M. Navazesh, Methods for collecting saliva, *Ann. N. Y. Acad. Sci.* 694 (1993) 72–77.
- [9] J.L. Chicharro, A. Lucía, M. Pérez, A.F. Vaquero, R. Ureña, Saliva composition and exercise, *Sports Med.* 26 (1998) 17–27.
- [10] R. Haeckel, P. Hänecke, Application of saliva for drug monitoring. An in vivo model for transmembrane transport, *Eur. J. Clin. Chem. Clin. Biochem.* 34 (1996) 171–191.
- [11] W.J. Jusko, R.L. Milsap, Pharmacokinetic principles of drug distribution in saliva, *Ann. N. Y. Acad. Sci.* 694 (1993) 36–47.
- [12] F. Rasmussen, Salivary excretion of sulphonamides and barbiturates by cows and goats, *Acta Pharmacol. Toxicol.* 21 (1964) 11–19.
- [13] E. Roussa, Channels and transporters in salivary gland, *Cell Tissue Res.* 343 (2011) 263–287.
- [14] T. Higashi, Y. Shibayama, T. Ichikawa, K. Ito, T. Toyo'oka, K. Shimada, K. Mitamura, S. Ikegawa, H. Chiba, Salivary chenodeoxycholic acid and its glycine-conjugate: their determination method using LC–MS/MS and variation of their concentrations with increased saliva flow rate, *Steroids* 75 (2010) 338–345.
- [15] T. Lomonaco, S. Ghimenti, I. Piga, D. Biagini, M. Onor, R. Fuoco, F. Di Francesco, Influence of sampling on the determination of warfarin and warfarin alcohols in oral fluid, *Plos One* 9 (2014) 1–23.
- [16] W.S. Waring, D.J. Webb, S.R. Maxwell, Uric acid as a risk factor for cardiovascular disease, *Q. J. Med.* 93 (2004) 707–713.
- [17] B. Levy, Lactate and shock state: the metabolic view, *Curr. Opin. Crit. Care* 12 (2006) 315–321.
- [18] M.K. Kutzing, B.L. Firestein, Altered uric acid levels and disease states, *J. Pharmacol. Exp. Ther.* 324 (2008) 1–7.
- [19] K.C. Lin, H.Y. Lin, P. Chou, The interaction between uric acid level and other risk factors on the development of gout among asymptomatic hyperuricemic men in a prospective study, *J. Rheumatol.* 27 (2000) 1501–1505.
- [20] D.S. Freedman, D.F. Williamson, E.W. Gunter, T. Byers, Relation of serum uric acid to mortality and ischemic heart disease. The NHANES I Epidemiologic Follow-up Study, *Am. J. Epidemiol.* 141 (1995) 637–644.
- [21] T. Kawase, M. Toyofuku, T. Higashihara, Y. Okubo, L. Takahashi, Y. Kagawa, K. Yamane, S. Mito, H. Tamekiyo, M. Otsuka, T. Okimoto, Y. Muraoka, Y. Masaoka, N. Shiode, Y. Hayashi, Validation of lactate level as a predictor of early mortality in acute decompensated

- 678 heart failure patients who entered intensive care unit, *J. Cardiol.* 65 (2015) 164–170.
- 679 [22] P. Attanà, C. Lazzeri, C. Picariello, C.S. Dini, G. Gensini, S. Valente, Lactate and lactate
680 clearance in acute cardiac care patients, *Eur. Heart. J. Acute Cardiovasc. Care* 1 (2012) 115–
681 121.
- 682 [23] C. Donadio, D. Calia, S. Ghimenti, M. Onor, E. Colombini, R. Fuoco, F. Di Francesco, Uric
683 acid is the major determinant of absorbance in spent dialysate allowing spectrophotometric
684 evaluation of dialysis dose, *J. Nephrol.* 27 (2014) 331–337.
- 685 [24] G. Kortum, W. Vogel, K. Andrussov, Dissociation constants of organic acids in aqueous
686 solution, Intern. union of pure and applied chemistry, London, 1961.
- 687 [25] J.C. Atherton, Acid-base balance: maintenance of plasma pH, *Anaesth. Intens. Care*, 4
688 (2003) 419–422.
- 689 [26] M.A. Hediger, R.J. Johnson, H. Miyazaki, H. Endou, Molecular physiology of urate
690 transport, *Physiology (Bethesda)*. 20 (2005) 125–133.
- 691 [27] D.B. Mount, Molecular physiology and the four-component model of renal urate transport.
692 *Curr. Opin. Nephrol. Hypertens.* 14 (2005) 460–463.
- 693 [28] R. Ikarashi, K. Shibasaki, A. Yamaguchi, Immunohistochemical studies of organic anion
694 transporters and urate transporter 1 expression in human salivary gland, *Acta Odontol.*
695 *Scand.* 71 (2013) 312–316.
- 696 [29] M. Sato, T. Wakayama, H. Mamada, Y. Shirasaka, T. Nakanishi, I. Tamai, Identification
697 and functional characterization of uric acid transporter Urat1 (Slc22a12) in rats, *Biochem.*
698 *Biophys. Acta* 808 (2011) 1441–1447.
- 699 [30] K. M. Johan, L. C. Martens, Review: The physiology of saliva and transfer of drugs into
700 saliva, *Forensic. Sci. Int.* 150 (2005) 119–131.
- 701 [31] C. A. Schneyer, H. D. Hall, Effects of varying frequency of sympathetic stimulation on
702 chloride and amylase levels of saliva elicited from rat parotid gland with electrical
703 stimulation of both autonomic nerves, *Proc. Soc. Exp. Biol. Med.* 196 (1991) 333–337.
- 704 [32] C. Dawes, Circadian rhythms in human salivary flow rate and composition, *J. Physiol.* 22
705 (1972) 529–545.
- 706 [33] D. Pellegrini, M. Onor, I. Degano, E. Bramanti, Development and validation of a novel
707 derivatization method for the determination of lactate in urine and saliva by liquid
708 chromatography with UV and fluorescence detection, *Talanta* 130 (2014) 280–287.
- 709 [34] Z. Wang, E. Konigsberger, Solubility equilibria in the uric acid-sodium urate-water system,
710 *Thermochim.* 310 (1998) 237–242.
- 711 [35] A. Nahum, C. Horvath, Evaluation of octanol-water partition coefficients by using high-
712 performance liquid chromatography, *J. Chromatogr. A* 192 (1980) 315–322.
- 713 [36] L.A. Currie, Nomenclature in evaluation of analytical methods including detection and
714 quantification capabilities, *Pure Appl. Chem.* 67 (1995) 1699–1723.
- 715 [37] J.H. Zar, *Biostatistical Analysis*, fifth ed, Prentice Hall, 1998.
- 716 [38] S.V. Narasimhan, G. Visalakshi, Interference measurement in the potentiometric
717 determination of chloride at ppb level using an ion-selective electrode, *J. Electroanal. Chem.*
718 131 (1982) 325–330.
- 719 [39] B. Rai, J. Kaur, R. Jacobs, S.C. Anand, Adenosine deaminase in saliva as a diagnostic
720 marker of squamous cell carcinoma of tongue, *Clin. Oral. Investig.* 15 (2011) 347–349.
- 721 [40] S. Patel, R. Metgur, Estimation of salivary lactate dehydrogenase in oral leukoplakia and
722 oral squamous cell carcinoma: a biochemical study, *J. Cancer. Res. Ther.* 11 (2015) 119–
723 123.
- 724 [41] P.R. Joshi, S. Golgire, A study of salivary lactate dehydrogenase isoenzymes levels in
725 patients with oral leukoplakia and squamous cell carcinoma by gel electrophoresis method,
726 *J. Oral Maxillofac. Pathol.* 18 (2014) S39–S44.
- 727 [42] M. Sivaramakrishnan, B. Sivapathasundharam, M. Jananni, Evaluation of lactate
728 dehydrogenase enzyme activity in saliva and serum of oral submucous fibrosis patients, *J.*

Oral. Pathol. Med. 44 (2015) 449–452.

[43] E. Puertos, Extended stability of Intravenous 0.9% sodium chloride solution after prolonged heating or cooling, Hosp. Pharm. 49 (2014) 269–272.

[44] J. H. Thaysen, N. A. Thorn, I. L. Schwartz, Excretion of sodium, potassium, chloride and carbon dioxide in human parotid saliva, Americ. J Phys. 178 (1954) 155–159.

[45] J. Bray, Lectures notes on human physiology, Wiley, New York, 1999.

[46] C. Hansch, A. Leo, D. Hoekman, In exploring QSAR. Hydrophobic, electronic and steric constants, first ed., American Chemical Society, Washington DC, 1995.

[47] H.J. Shin, M. Takeda, A. Enomoto, H. Fujimura, H. Miyazaki, N. Anzai, H. Endou, Interactions of urate transporter URAT1 in human kidney with uricosuric drugs, Nephrology 16 (2011) 156–162.

780 **List of captions**

781

782 **Fig. 1.** HPLC-UV chromatograms of diluted samples: 0.1% (v/v) aqueous formic acid (A), standard
783 working solution of uric acid ($50 \mu\text{g mL}^{-1}$) (B), pooled control oral fluid sample ($35 \mu\text{g mL}^{-1}$) (C)
784 and pooled control oral fluid sample spiked with uric acid ($50 \mu\text{g mL}^{-1}$) (D). Retention time of uric
785 acid was 8.0 min.

786

787 **Fig. 2.** HPLC-FP chromatograms of derivatized samples: milli-Q water (A), standard working
788 solution of lactate ($100 \mu\text{g mL}^{-1}$) (B), pooled control oral fluid sample ($47 \mu\text{g mL}^{-1}$) (C) and pooled
789 control oral fluid sample spiked with lactate ($50 \mu\text{g mL}^{-1}$) (D). Retention time of lactate-9-
790 chloromethyl-anthracene adduct was 24.1 min.

791

792 **Fig. 3.** Average values of the lactate concentration measured in pooled oral fluid sample,
793 normalized to the lactate concentration corresponding to the first observation time (t_0). Error bars
794 correspond to the standard deviation of three replicates.

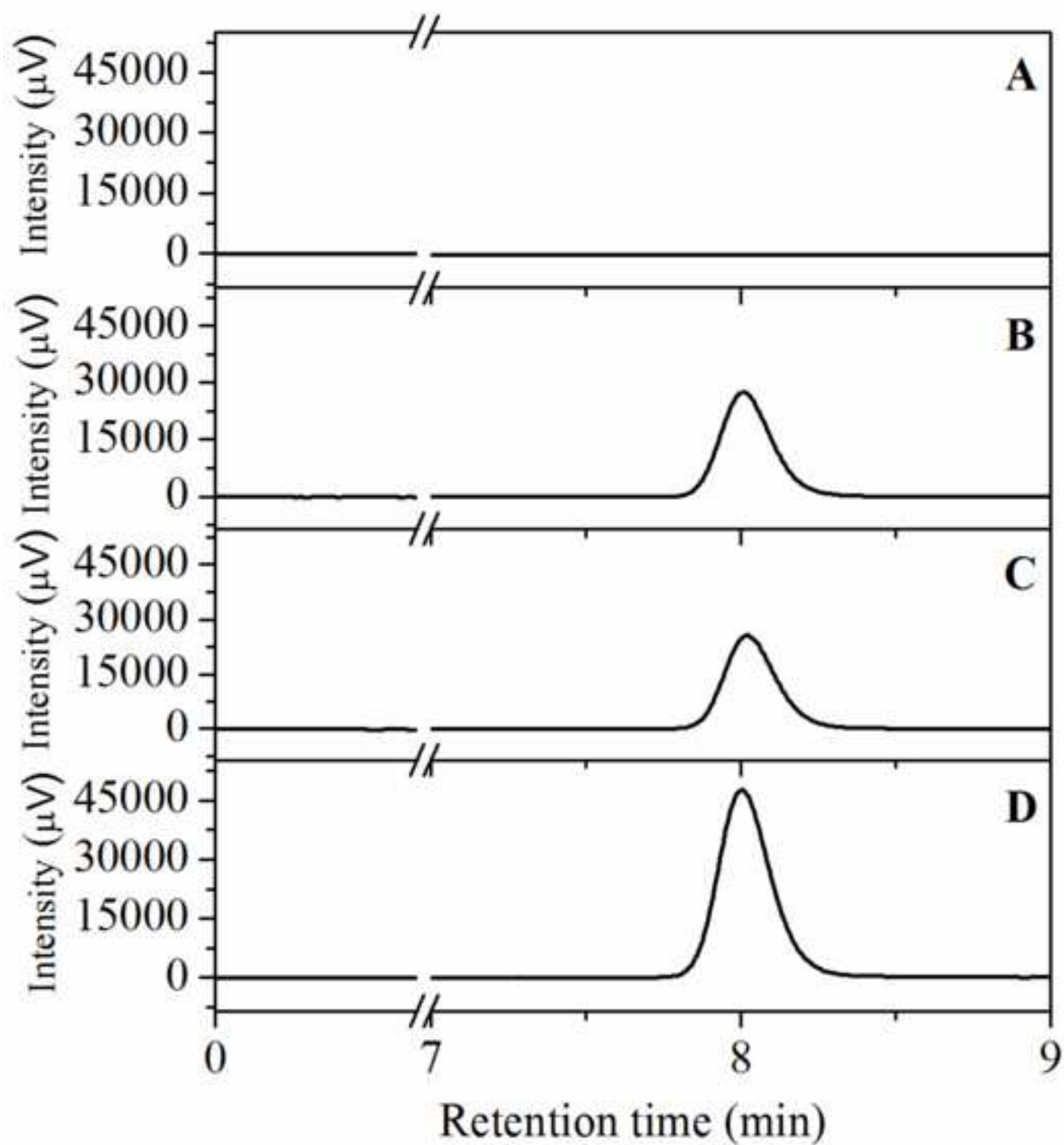
795

796 **Fig. 4.** Box-plot for oral fluid flow rate (A), pH (B) and chloride (C), urate (D) and lactate (E)
797 concentration for non-stimulated (N-S) and stimulated oral fluid samples collected at 50 (S-1), 100
798 (S-2) and 150 (S-3) min^{-1} from 22 healthy volunteers. Note: The box-plot shows: the minimum, the
799 5th and the 25th percentiles, the median, the 75th and 95th percentiles, and the maximum value for
800 each variable investigated. The dot inside the box shows the mean value.

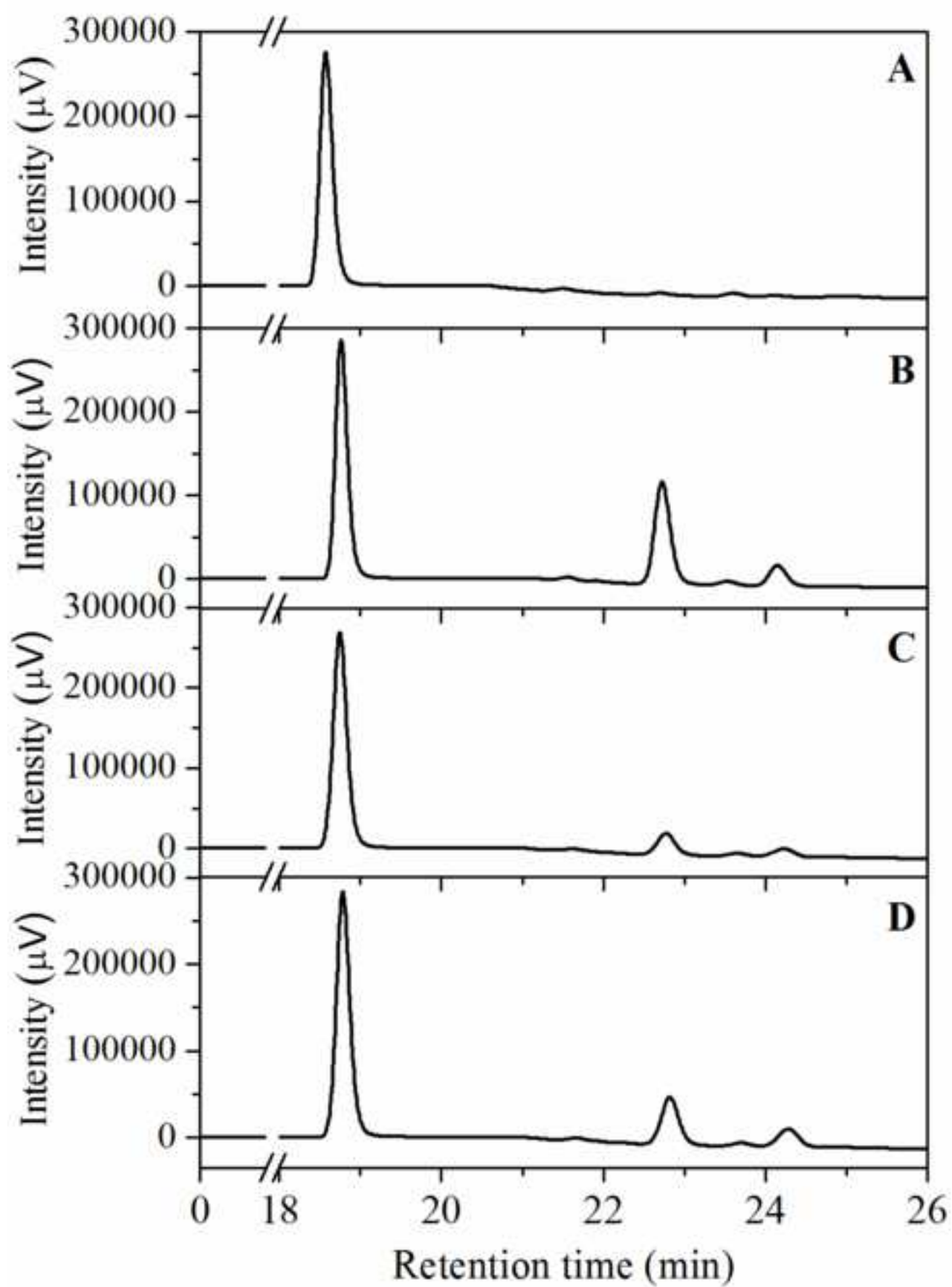
801

802 **Fig. 5.** Effect of different collection protocols (non-stimulated vs stimulated) and frequencies of
803 stimulation (50, 100 and 150 min^{-1}) on the normalized value of flow rate (black circle), pH (black
804 square) and chloride (white up-pointing triangle), urate (black diamond) and lactate (white down-
805 pointing triangle) concentration. Flow rate values was plotted on the right axis.

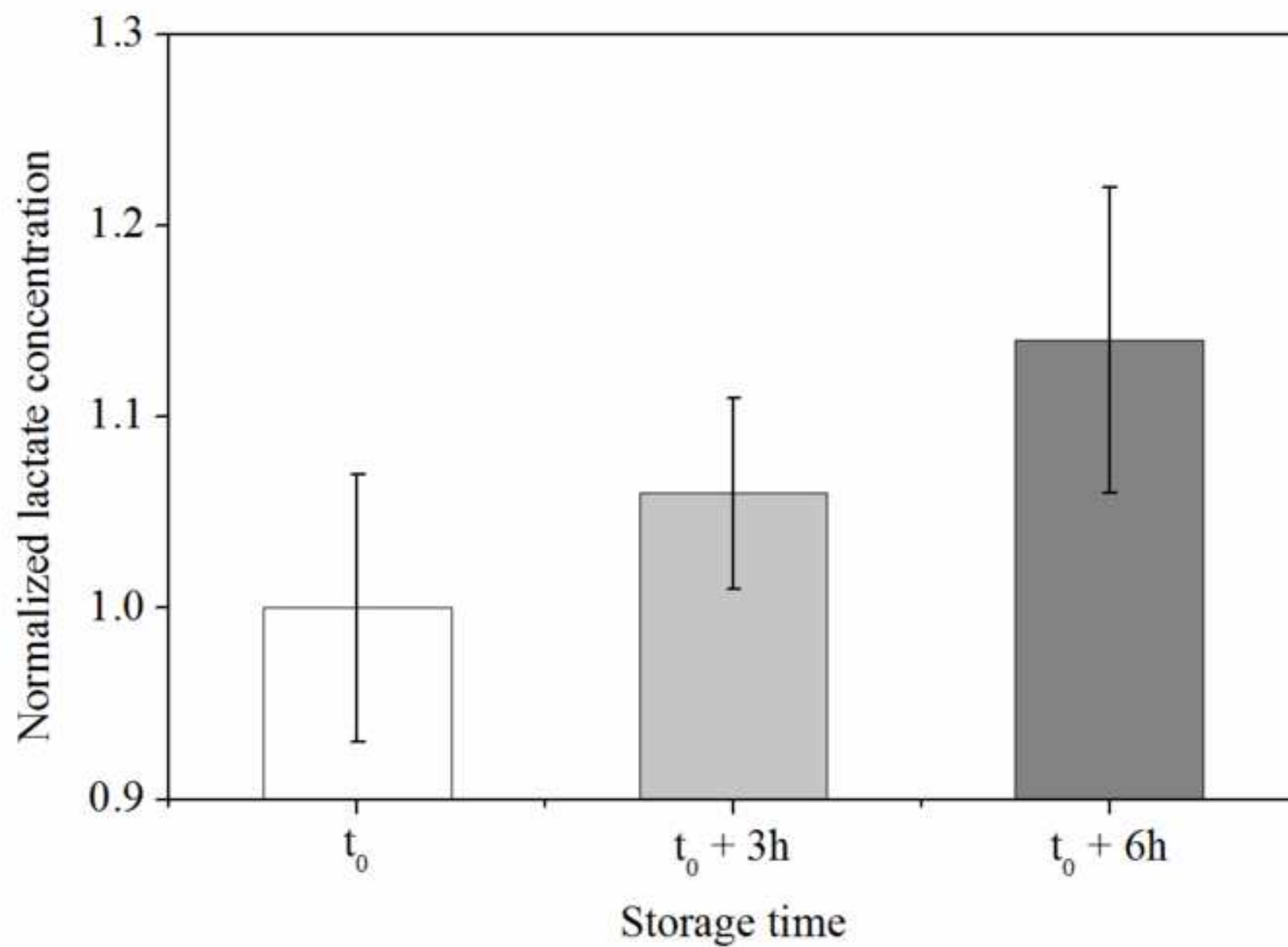
Figure_1
[Click here to download high resolution image](#)



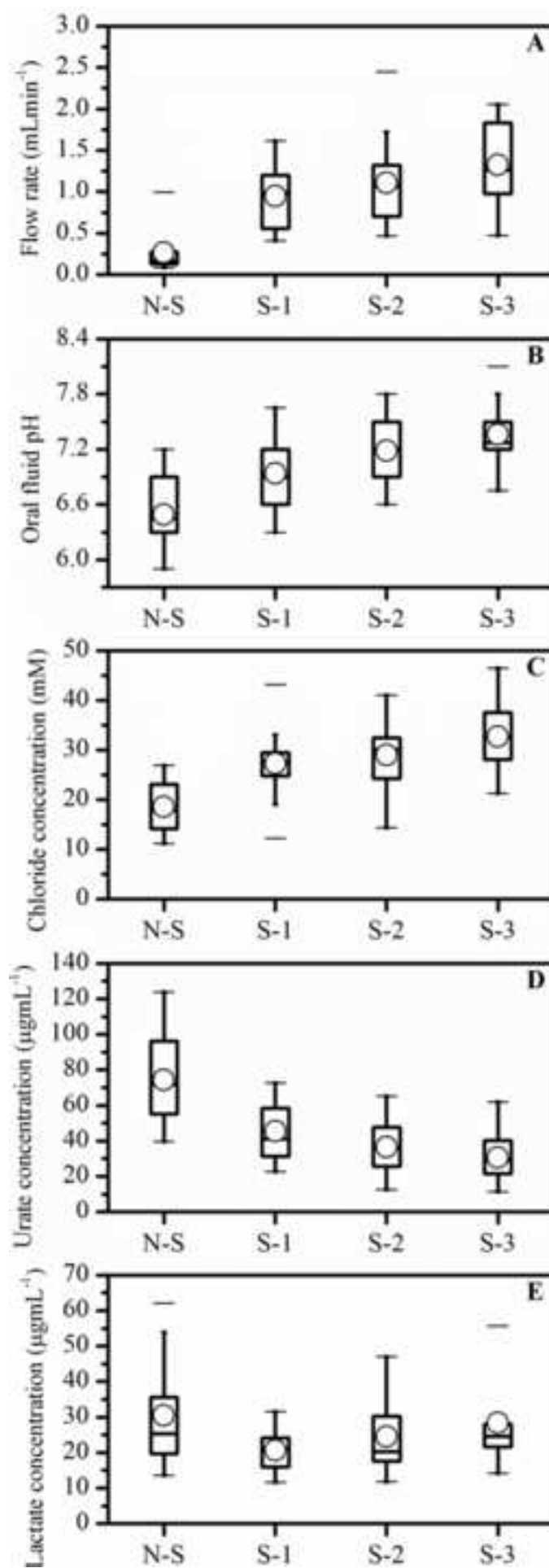
Figure_2
[Click here to download high resolution image](#)



Figure_3
[Click here to download high resolution image](#)



Figure_4
[Click here to download high resolution image](#)



Figure_5
[Click here to download high resolution image](#)

