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# Saliva sampling: methods and devices. An overview

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## Abstract

The continuous exchange of chemicals with blood and the non-invasive collection make saliva an interesting specimen for clinical applications, from the detection of biomarkers to the new *-omic* sciences in medicine. However, saliva sampling is challenging because the suitability of the collection method for the analyte of interest is either poorly investigated or, more often, not mentioned at all in most publications. This review reports a critical evaluation of the most common procedures for saliva sampling and a description of the off-the-shelf sampling devices. Their suitability for bioanalytical applications **and salivary biomarkers detection**, e.g. representativeness of the sample, sampling feasibility, analyte recovery, and sample amount, is discussed.

**Keywords:** Saliva; saliva analysis; saliva sampling methods; saliva sampling devices; saliva composition, **salivary biomarkers**.

## Highlights

- 1) Overview on commercially available sampling devices for saliva.
- 2) Description of the procedure for saliva sampling in clinical applications.
- 3) Importance of the sampling device in clinical applications.

47 **1. Introduction**

48 In the last decades, unobtrusive monitoring of health conditions by non-invasive fluids analysis  
49 (e.g. breath, saliva, sweat, and wound exudate) has attracted attention in the field of medical  
50 diagnosis and management [1–10] and drug monitoring [11], both for therapeutic and forensic  
51 purposes [12–14], as well as for environmental exposure monitoring (e.g. monitoring of exposure  
52 level to toxic substances) [15–17]. Saliva is an “ultra-filtrate” of blood and a potential source of  
53 clinical information since salivary biomarkers can virtually mirror the status of a pathology such as  
54 oncological, cardiovascular, autoimmune, viral and bacterial diseases [18–20]. Saliva-based  
55 diagnostics can be applied to personalized medicine to evaluate patient’s physiological conditions,  
56 trace the progression of a disease and monitor the efficacy of therapies [21]. Since sampling is non-  
57 invasive and can be performed by the patient himself or untrained caregivers, the analysis of saliva  
58 is a potential substitute of blood, especially for long-term monitoring (e.g. therapeutic drugs  
59 monitoring) or for screening a large number of patients [13,18,22], as well as for developing of  
60 salivary point-of-care technology [23,24].

61 Nevertheless, saliva sampling can be challenging. The results depend on which type of saliva is  
62 sampled, i.e. whole saliva or saliva produced by specific glands, and whether the sample is  
63 collected after stimulation or not. The choice of the device depends on the volume of saliva and the  
64 capability to recover the biomarkers. Furthermore, there are several parameters that can affect the  
65 salivary composition, such as age (high variability for newborns, children and elderly people), flow  
66 rate, diet, temperature and pH [25–27].

67 ~~The literature reports several studies on saliva analysis and most applications have a clinical (e.g.~~  
68 ~~diagnosis and monitoring of diseases) [14,17,18] or environmental (e.g. monitoring of exposure~~  
69 ~~level to toxic substances) [19–21] target.~~ Nowadays, the composition of saliva is also examined in –  
70 omics sciences, such as metabolomics and proteomics [19,21]. The salivary “omics” methodologies  
71 can currently be identified as “Salivaomics” [28–32].

72 However, in most of these works, the suitability of the method for sampling saliva is poorly  
73 investigated. Only a few papers assess the impact of sampling devices and procedures on the  
74 analytes concentration, recovery and chemical forms. The variability associated with the sampling  
75 procedures is worsened in simultaneous analysis of salivary compounds, which would rather need  
76 specific analytical procedures. Furthermore, the lack of standardized sampling procedures makes it  
77 difficult to compare results of different laboratories.

78 This review describes and compares the methods for saliva sampling, and the suitability of off-the-  
79 shelf devices for clinical purposes. This review is divided in three parts: the first one addresses the  
80 production, composition and properties of what we usually call “saliva” (whole saliva). The  
81 second part is a critical review of the state-of-art of saliva sampling and devices for clinical  
82 applications. The last part describes the main salivary biomarkers of clinical interest (e.g. steroids,  
83 peptides, proteins and drugs) and the analytical performances of detection methods.

86 **2. Saliva production, composition and properties**

87 The term saliva refers to the clear, slightly acidic, hypotonic and mucoserous exocrine biological  
88 medium composed of secretions from the major salivary glands (i.e. submandibular, parotid and  
89 sublingual) as well as from the multitudes of minor salivary glands (300 – 1000 unit) distributed  
90 throughout the oral mucosa, which can be divided into labial, buccal, palatal, lingual and retromolar  
91 glands [30,33,34]. The contribution to the total amount of saliva of submandibular, parotid and  
92 sublingual glands is 65, 23 and 4%, respectively, whereas the remaining 10% is produced by the  
93 minor glands [35].

94 **2.1 Production**

96 Nominally healthy adults typically produce 500 – 1500 mL/day of serous and mucinous saliva with  
 97 a basal flow rate of about 0.5 mL/min. Because of the secretions from both the major and minor  
 98 salivary glands, whole saliva is a mixture of oral fluids rich in water (approximately 99%) and  
 99 endogenous substances such as inorganic compounds (Table 1), organic compounds (volatile, non-  
 100 protein and lipids), proteins, polypeptides, and hormones [25,30,33,36,37].

101 The composition of unstimulated saliva differs from stimulated one, which resembles plasma in  
 102 composition [1,38]. Although there is a great individual variability, a normal salivary flow rates for  
 103 unstimulated saliva is above 0.1 mL/min, whereas under stimulation, the flow rate may increases up  
 104 to about 4 mL/min [39].

105 Saliva has a slight buffer capacity [1] due to the presence of three buffer systems: bicarbonate,  
 106 phosphate and proteins. The normal pH range is between 6 and 7 for unstimulated saliva, whereas it  
 107 can extend from 5.3 to 7.8 when the flow rate changes [33,40]. In stimulated saliva, the pH  
 108 increases since the concentration of bicarbonate ions in saliva is higher, i.e. from  $2.4 \pm 1.5$  mM to  
 109  $15 \pm 7$  mM [41]. High salivary flow rates increase the concentrations of sodium and chloride and  
 110 decrease the concentrations of potassium and phosphate [42], thus increasing the saliva tonicity.  
 111 Therefore, the buffering action of saliva is more efficient during stimulated high flow rates, but is  
 112 almost ineffective for unstimulated saliva. At rest and without exogenous or pharmacological  
 113 stimulation, there is a small and continuous salivary flow called *basal unstimulated secretion*. This  
 114 flow covers, moisturizes and lubricates the oral tissues [26].  
 115  
 116

## 117 2.2 Composition

118 Salivary flow and composition are regulated mainly by the activity of the autonomic nervous  
 119 system (ANS):

- 120 – A parasympathetic stimulation leads to high flow of watery (less viscous) saliva with low levels  
 121 of organic and inorganic components [43].
- 122 – A sympathetic stimulation produces mucoid (more viscous) saliva secretions [44].
- 123 – An  $\alpha$ -adrenergic stimulation accounts for low volume of saliva with high concentration of  
 124 proteins and low concentration of mucins. The viscosity of saliva is low [25].
- 125 – A  $\beta$ -adrenergic stimulation accounts for saliva with high content of protein and mucin, high  
 126 viscosity and foamy appearance [1,25].

127 Thus, the parasympathetic and sympathetic stimulation changes the salivary flow rate and volume,  
 128 as well as the levels of organic and inorganic compounds [33]. Table 1 reports a list of inorganic  
 129 compounds and the comparison of their salivary and plasmatic levels. Salivary secretion is thus the  
 130 result of endogenous, mechanical (e.g. high-frequency chewing and high bite force), gustatory (e.g.  
 131 strong acidic stimulus), and olfactory stimuli. For example, pain pregnancy-related hormonal  
 132 changes, sympathomimetic and para-sympathomimetic drugs can increase the salivary secretion,  
 133 whereas hormonal changes related to menopause, stress, and or pharmacological stimulus, such as  
 134 anti-adrenergic and anticholinergic drugs can inhibit secretion [25,33,36,44,45].  
 135  
 136  
 137

**Table 1.** Comparison between salivary and plasmatic levels of inorganic compounds (adapted from [25])

Inorganic compound	Concentration in unstimulated saliva [mmol/L]	Concentration in stimulated saliva [mmol/L]	Concentration in plasma [mmol/L]
Na <sup>+</sup>	5	20 – 80	145
K <sup>+</sup>	22	20	4
Cl <sup>-</sup>	15	30 – 100	120
Ca <sup>2+</sup>	14	14	2.2
HCO <sub>3</sub> <sup>-</sup>	5	1580	25

Mg <sup>2+</sup>	0.2	0.2	1.2
NH <sub>3</sub>	6	3	0.05

138

139

140 Glycoproteins (e.g., mucins and proline-rich glycoproteins), enzymes (e.g.,  $\alpha$ -amylase and carbonic  
 141 anhydrase) and a wide range of peptides (e.g. cystatins, statherin, histatins, and proline-rich  
 142 proteins) are the main constituents of whole saliva. The major sources of proteins in saliva are the  
 143 contra-lateral major (parotid, submandibular, sublingual) and minor (von Ebner) salivary glands  
 144 [46]; however, oral tissues and microorganisms can contribute to the salivary proteome. The  
 145 amount and type of proteins in saliva depend on several factors such as circadian rhythm, diet, age,  
 146 gender and physiological status [47]. **Table S1 (Supplemental files) lists the main proteins in saliva.**  
 147 It is easy to see that concentration can vary for the same compounds, as well as a standard unit of  
 148 measurement is missing.

149

~~Table 2. Main proteins in saliva: origin, function, and mean salivary concentrations in nominally healthy subjects.~~

<del>Salivary protein</del>	<del>Origin</del>	<del>Function</del>	<del>Mean concentration in saliva</del>	<del>References</del>
			<del>0.47 ± 0.19 mg/mL</del>	<del>[54]</del>
<del>Total contents</del>			<del>0.9 ± 0.2 mg/mL</del>	<del>[55]</del>
			<del>4.3 — 710.0 mg/dL</del>	<del>[56]</del>
			<del>2.67 ± 0.54 mg/mL</del>	<del>[57]</del>
			<del>3257 ± 1682 U/mL</del>	<del>[55]</del>
<del><math>\alpha</math>-amylase</del>	<del>Serous acinar cells</del>	<del>Starch digestion</del>	<del>1080.0 ± 135.6 U/L</del>	<del>[17]</del>
			<del>476 ± 191 U/mL</del>	<del>[57]</del>
<del>Adrenomedullin</del>	<del>Oral epithelial cells</del>	<del>Antimicrobial</del>	<del>55 ± 65 pg/mL</del>	<del>[59,60]</del>
<del>Albumin</del>	<del>Mainly from plasma leakage</del>		<del>0.2 ± 0.1 mg/dL</del>	<del>[55]</del>
			<del>0.8 — 192.0 mg/dL</del>	<del>[56]</del>
<del>Alkaline phosphatase (ALP)</del>	<del>Tissue degradation</del>	<del>Metabolic process of cells</del>	<del>24.85 ± 12.14 IU/L</del>	<del>[60]</del>
<del>Acid Phosphatase (ACP)</del>	<del>Tissue degradation</del>	<del>Metabolic process of cells</del>	<del>7.32 ± 4.01 IU/L</del>	<del>[60]</del>
<del>Cathelicidins (LL37)</del>	<del>Oral cavity and respiratory tract</del>	<del>Antimicrobial</del>	<del>0.3 — 33.6 µg/mL</del>	<del>[59,6]</del>
			<del>14.3 kDa form</del>	
<del>Cystatin group</del>	<del>Submandibular → sublingual</del>	<del>Antimicrobial (cystein-proteinase inhibitor)</del>	<del>58 ± 25 µg/mL</del>	<del>[57]</del>
			<del>14.2 kDa form</del>	
			<del>91 ± 46 µg/mL</del>	
<del>Defensins (DEF)</del>	<del>Blood, plasma</del>	<del>Antimicrobial</del>	<del>DEF-1 0.8 ± 0.6 µg/mL</del>	<del>[59,61,62]</del>
			<del>DEF-<math>\alpha</math>1-3 0.094 — 9.4 µg/mL</del>	
<del>Hystatin</del>	<del>Parotid</del>	<del>Antifungal</del>	<del>1190 ± 313 µg/mL</del>	<del>[57,63]</del>
<del>Lactate</del>	<del>Oral epithelium</del>	<del>Cell necrosis</del>	<del>497.00 ± 51.75 U/L</del>	<del>[64]</del>

<del>dehydrogenase (LDH)</del>				<del>[65]</del>
<del>Lactoferrin</del>	<del>Mucous &gt; serous</del>	<del>Antimicrobial</del>	<del>3.7 ± 2.5 µg/mL</del>	<del>[55]</del>
<del>Lysozyme</del>	<del>Sublingual &gt; Submandibular, Parotid</del>	<del>Antimicrobial</del>	<del>3.5 – 92.0 µg/mL</del>	<del>[56]</del>
			<del>21.8 ± 2.5 mg/dL</del>	<del>[17]</del>
			<del>59.7 – 1062.3 µg/mL</del>	<del>[66]</del>
<del>MMP-2</del>	<del>Expressed by neutrophils</del>	<del>Inflammatory mediators</del>	<del>3.1 ng/mL</del>	<del>[67]</del>
<del>MMP-8</del>	<del>Expressed by neutrophils</del>	<del>Inflammatory mediators</del>	<del>543 ± 398 ng/mL</del>	<del>[68]</del>
<del>MMP-9</del>	<del>Expressed by neutrophils</del>	<del>Inflammatory mediators</del>	<del>264 ± 217 ng/mL</del>	<del>[68]</del>
<del>Myeloperoxidase</del>	<del>Expressed by neutrophils</del>	<del>Antibacterial</del>	<del>1899 ± 1447 ng/mL</del>	<del>[68]</del>
<del>Mucins group</del>	<del>Mucous glands</del>	<del>Lubrication</del>	<del>MUC5B: 2.4 ± 1.7 U/mL</del>	<del>[55]</del>
<del>Proline-rich proteins</del>	<del>Parotid</del>	<del>Binding to bacteria and with dietary tannins</del>	<del>Acidic: 456 ± 139 µg/mL</del>	<del>[57]</del>
			<del>Basic: 165 ± 69 µg/mL</del>	
<del>Secretory IgA</del>	<del>B-lymphocytes</del>	<del>Antimicrobial</del>	<del>124.3 – 335.3 µg/mL</del>	<del>[66]</del>
<del>Statherin</del>	<del>Secreted by parotid and submandibular glands</del>	<del>Ca<sup>++</sup>-binding</del>	<del>4.93 ± 0.61 µmol/L</del>	<del>[69]</del>
			<del>36 ± 18 µg/mL</del>	<del>[57]</del>
<del>Transferrin</del>	<del>Plasma</del>	<del>Free iron control</del>	<del>0.58 ± 0.20 mg/dL</del>	<del>[17]</del>

150

151 The chemical composition of saliva also depends on the contribution of several constituents such as  
 152 gingival fold, oral mucosa transudate, intraoral bleeding (serum and cells), gingival crevicular fluid  
 153 (GCF), expectorated bronchial and nasal secretions (e.g. mucous of the nasal cavity and pharynx),  
 154 serum and blood derivatives from oral wounds, non-adherent oral bacterial, viruses and fungi,  
 155 desquamated epithelial and blood cells, and extrinsic substances (e.g. food debris) [48]. Traces of  
 156 medications or chemical products can also be found in saliva (i.e. tooth paste and mouth rinse  
 157 components) [26,33].

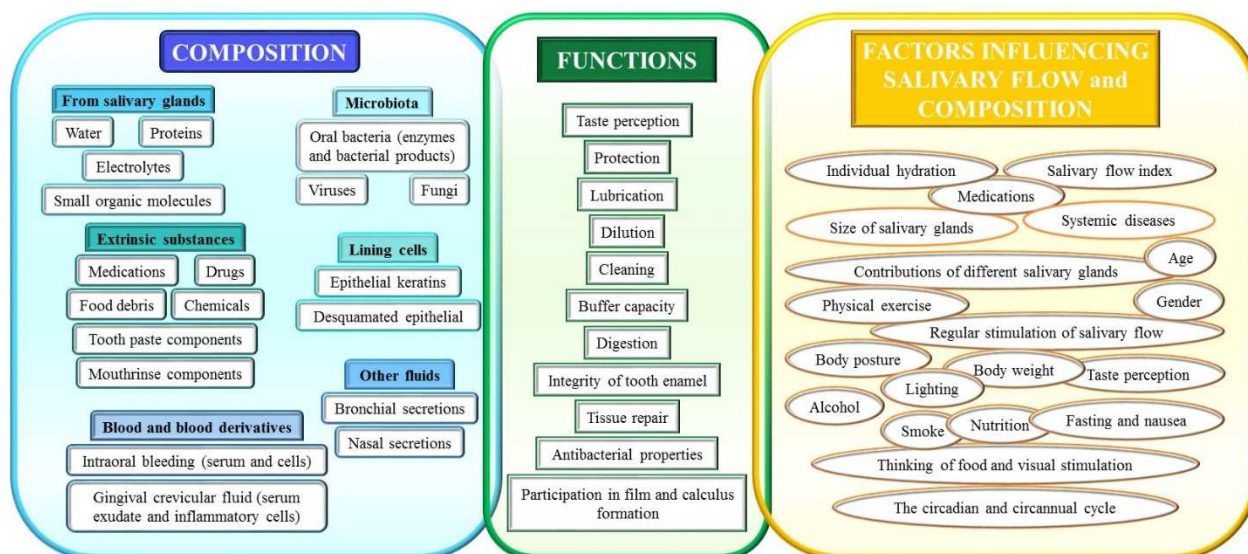
158

### 159 2.3 Properties and functions

160 Saliva plays a key role in initiating and facilitating digestion, and maintaining the oral health and  
 161 homeostasis. Saliva protects the oral cavity against pathogens or mechanical injuries (e.g. friction),  
 162 and lubricates and moistens the oral tissues to support swallowing, chewing, speech, and taste [26].  
 163 The maintenance of oral health largely depends on saliva's cleansing actions and intrinsic  
 164 antipathogenic characteristics. In fact, saliva inhibits demineralization, promotes remineralization  
 165 and has an antibacterial and antiviral effect [49].

166 Fig. 1 summarizes the whole saliva constituents, functions, and the main factors that affect its  
 167 composition [26].

168



169  
170 **Fig. 1.** The whole saliva composition, functions, and main factors affecting salivary flow and composition.

171

## 172 2.4 Transfer mechanisms of analytes from blood

173 Different processes are involved into the movement of compounds from plasma to oral cavity  
174 [13,34,50].

175 The most common transfer mechanism from blood to oral fluid is ultrafiltration, which involves  
176 only molecules with molecular weight lower than 1900 Da (e.g. water, ions, and hormones such as  
177 catecholamines and steroids) [1]. In ultrafiltration, analytes can cross the salivary glands through  
178 the gap junctions among the cells of secretory units (intercellular nexus). Another transfer  
179 mechanism is the transudation of plasma compounds into oral cavity from crevicular fluid or  
180 directly from the oral mucosa. The presence in saliva of some plasmatic molecules (e.g. albumin)  
181 depends on transudation. Analytes are also transferred by passive diffusion through the salivary  
182 membranes of high lipophilic molecules ( $\log(P) > 5$  where  $P$  is the partition coefficient), such as  
183 steroid hormones and some drugs. Drugs usually pass from blood to oral fluid by passive diffusion  
184 through lipid membranes [33,51,52]. Therefore, the concentration of drugs in saliva only reflects  
185 the free concentration (unbound) of drugs in plasma. Active transport and ultrafiltration through  
186 pores in cell membranes are additional transfer mechanisms for drugs and metabolites from blood to  
187 oral fluid [53]. The active transport depends on lipid solubility, molecular weight and  $pK_a$  of drugs,  
188 flow-rate and pH of saliva, and binding proteins in plasma [54,55].

189

190

## 191 3. Methods and devices for saliva sampling

192 ~~Saliva secreted from individual gland is less contaminated by food debris and micro-organisms and~~  
193 ~~thus might be more suitable for diagnostic purposes than whole saliva [29,78,79]. However, this~~  
194 ~~approach needs long sampling time, skilled personnel and invasive custom-made collection devices.~~  
195 ~~The fabrication of custom-made devices is time-consuming, lacks standardization and has to be~~  
196 ~~approved by ethical committees or national agencies. A possible compromise is sampling whole~~  
197 ~~saliva using micropipettes or absorbent materials that are approved for clinical studies and placed~~  
198 ~~close to the salivary glands [80,81].~~

199

### 200 3.1 Saliva sampling from specific salivary glands

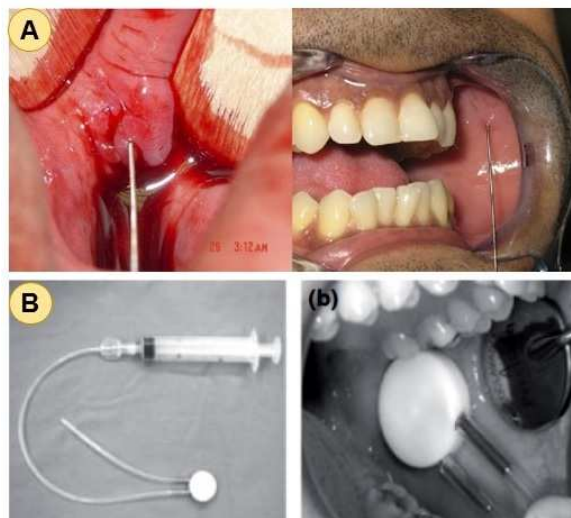
201 Saliva can be collected from specific salivary glands (e.g. parotid glands) or sampling the whole  
202 liquid (whole saliva) secreted from all the glands (mixed sample). In both cases, the samples have  
203 the same chemical composition, although the concentration of analytes can vary from one gland to

204 another [56]. The submandibular and the parotid glands are the main contributors to unstimulated  
205 and stimulated saliva, respectively. The contribution of sublingual glands to unstimulated and  
206 stimulated whole saliva is low [36]. Saliva secreted from individual gland is less contaminated by  
207 food debris and micro-organisms and thus might be more suitable for diagnostic purposes than  
208 whole saliva [36,57]. However, this approach needs long sampling time, skilled personnel and  
209 invasive custom-made collection devices. The fabrication of custom-made devices is time-  
210 consuming, lacks standardization and has to be approved by ethical committees or national  
211 agencies. A possible compromise is sampling whole saliva using micropipettes or absorbent  
212 materials that are approved for clinical studies and placed close to the salivary glands [80,81].  
213 Table 2 summarises pros and cons of sampling procedures of saliva from specific salivary glands.  
214

### 215 3.1.1 Parotid saliva

216 The analysis of saliva secreted by the parotid glands dates back to the Sixties [58]. The parotid  
217 glands are the most voluminous salivary glands and secrete serous saliva. These glands are beneath  
218 and in front of both ears, and are traversed by the facial nerve, the retromandibular vein and the  
219 external carotid artery. The parotid duct opening, called *Stensen's duct*, is on the buccal vestibule,  
220 opposite to the first and second molars. The Stensen's duct drains saliva from the parotid gland into  
221 the mouth at the upper cheeks. This salivary secretion depends on the autonomic functioning of the  
222 glossopharyngeal nerve. Since the parotid glands are symmetrical in the oral cavity, their secretions  
223 are equally distributed into the mouth; however, the amount of saliva depend on gland size. Since  
224 the unstimulated parotid salivary flow is very low (< 0.2 mL/min) or even absent, saliva is usually  
225 stimulated applying few drops of citric acid (2 – 4 % w/v) [59].

226 Parotid saliva can be collected by intraductal cannulation, which is performed inserting a  
227 polyethylene tube or a tapered sialographic cannula into each gland (Fig. 2A). However, this  
228 approach is slow and invasive and not suitable for routine clinical use [43]. Alternative non-  
229 invasive procedures use the *Lashley cup* (also known as *Carlson-Crittenden collector*, introduced in  
230 1910) [58]. This device avoids the leakage and the frequent sticking out of the cannula from the  
231 duct. The Lashley cup consists of two concentric chambers communicating with the exterior by  
232 means of two metal cannulae (Fig. 2B). The inner chamber is a cup that surrounds the opening of  
233 *Stenson's duct* whereas the air is evacuated from the outer chamber by a suction pump. A needle  
234 connected to the inner cup provides an exit for the free-flowing parotid saliva. Anyway, the  
235 homogeneity of the sample is not guaranteed and sample pre-treatment (i.e. filtration) would be  
236 necessary before analysis.  
237



238  
239 **Fig. 2.** (A) Example of parotid duct cannulation using a 0.025 inch Spring Wire. (Figure reprinted from [60]); (B) A  
240 Lashley cup (also named Carlson–Crittenden collector) on the left, and the device placement to both parotid glands'  
241 orifices on the right (Figure reprinted from [61]).



242  
243  
244  
245  
246

~~Fig. 3. (a) A Lashley cup (also named Carlson-Crittenden collector); (b) Device placement to both parotid glands orifices (Figure reprinted from [95]).~~

247 **3.1.2 Submandibular and sublingual saliva**

248 The submandibular and sublingual salivary glands are beneath the floor of the mouth and their  
249 excretory ducts are called *Wharton's* and *Bartholin's ducts*, respectively. Although in the literature  
250 the analysis of saliva is mostly focused on parotid or whole saliva, several studies have reported the  
251 potential role of the submandibular and sublingual glands. In particular, submandibular and  
252 sublingual glands were involved in studies on HIV-1 infection [62], Alzheimer's disease [63],  
253 Sjogren's syndrome [64], and medication intake [65].

254 Submandibular and sublingual secretions can be collected by intraoral duct cannulation [60], gentle  
255 suction [66] or using custom-made devices placed at the openings of the *Wharton's* and *Bartholin's*  
256 *ducts*, such as Pickerill's device [67], Lashley cups [68], Block-Brottman collection devices [69],  
257 polyethylmethacrylate devices [70], custom-made Wolff saliva collector [36].

258

259 **3.1.3 Collection of saliva from minor glands**

260 Salivary secretions from minor glands have a few clinical applications because the collection  
261 procedures are laborious and the amount of saliva is often not adequate for chemical analysis  
262 [36,71]. Kutscher et al. used some capillary tubes for collecting saliva, whereas Eliasson et al. and  
263 Wang et al. investigated the normal range and characteristics of saliva secretion from minor glands  
264 [72–74].

265 Eliasson et al. used the Periotron<sup>®</sup>, which is a flowmeter introduced for the first time in 1979 by  
266 Garnick et al. [75] to quantify sub-microliter volumes of fluid on a paper-strip filter. The Periotron<sup>®</sup>  
267 6000 model 2 (Proflow<sup>™</sup>) was used to measure the unstimulated flow of saliva from minor glands  
268 on 127 subjects. After drying the mucosa with a cotton pad, saliva was sampled using pre-cut (10 ×  
269 15 mm) pieces of standard filter paper (Munktell) and applying light finger pressure to ensure  
270 mucosal contact. In 2015, after carefully drying of the mucosa with a gauze, Wang et al.

271 ~~investigated the flow rates from minor salivary glands in healthy subject. A gently placed~~ A paper-  
272 strip filters (Whatman No. 41, 1 × 2 cm<sup>2</sup> in size) ~~was gently placed~~ for 30 s onto the mucosa (~~Fig.~~  
273 ~~4~~) and, independently of sex, ~~found out that~~ mean flow rates ~~resulted~~ 2.10 ± 0.66 μL/(min·cm<sup>2</sup>)  
274 from lower labial glands, 2.14 ± 0.62 μL/(min·cm<sup>2</sup>) from upper labial glands, 2.88 ± 0.72  
275 μL/(min·cm<sup>2</sup>) from buccal glands, and 2.15 ± 0.51 μL/(min·cm<sup>2</sup>) from palatal glands [74].

276

277

278 ~~Fig. 4. An example of collecting saliva from minor glands. Paper-strip filters are placed on (a) Upper labial glands (b)~~  
279 ~~Lower labial glands (c) Buccal glands, and (d) Palatal glands (Figure reprinted from [110]).~~

280

281

**Table 2.** Summarizing table of pros and cons of approaches for saliva sampling from specific salivary glands.

Sample type	Sampling method	Pros	Cons
Parotid saliva	Intraductal cannulation	• Selective sampling	<ul style="list-style-type: none"> <li>• Invasive</li> <li>• Possible Salivary gland injuries</li> <li>• Complex procedure</li> <li>• Time consuming</li> <li>• Required skilled personnel</li> </ul>
	Lashley cup	<ul style="list-style-type: none"> <li>• Selective sampling</li> <li>• Non-invasive procedures</li> </ul>	<ul style="list-style-type: none"> <li>• Complex procedure</li> <li>• Time consuming</li> </ul>

			<ul style="list-style-type: none"> <li>• Required skilled personnel</li> </ul>
Submandibular and sublingual saliva	Intraoral duct cannulation	<ul style="list-style-type: none"> <li>• Selective sampling</li> </ul>	<ul style="list-style-type: none"> <li>• Invasive</li> <li>• Possible Salivary gland injuries</li> <li>• Complex procedure</li> <li>• Time consuming</li> <li>• Required skilled personnel</li> </ul>
	Suction	<ul style="list-style-type: none"> <li>• Selective sampling</li> </ul>	<ul style="list-style-type: none"> <li>• Inhomogeneity of the sample</li> </ul>
	Using custom-made devices	<ul style="list-style-type: none"> <li>• Selective sampling</li> </ul>	<ul style="list-style-type: none"> <li>• Personalized form may be needed</li> </ul>
Saliva from minor glands	Application of pieces of standard filter paper	<ul style="list-style-type: none"> <li>• Non-invasive sampling</li> </ul>	<ul style="list-style-type: none"> <li>• Not so selective sampling</li> <li>• No large volume collected</li> <li>• Possible interaction with collecting paper</li> </ul>

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### 284 3.2 Whole saliva sampling

285 The sampling of whole saliva is the most common and less invasive procedure, and the main  
 286 distinction concerns the collection of unstimulated and stimulated whole saliva. Table 3 summarizes  
 287 pros and cons of sampling procedures of whole saliva.

288

#### 289 3.2.1 Unstimulated whole saliva sampling

290 Unstimulated whole saliva (UWS) is the mixture of secretions that enters the mouth in the absence  
 291 of exogenous stimuli and depends on the daily basal salivary flow rate in the oral cavity. The  
 292 sampling of unstimulated saliva is often preferred because it minimizes the dilution of analytes  
 293 [59,76]. Nevertheless, standardized sampling procedures are needed because the composition of  
 294 unstimulated saliva can be affected by the degree of hydration, position of head during collection,  
 295 body posture, light exposure, drugs and circadian rhythm [59].

296

##### 297 3.2.1.1 Passive drooling and draining method

298 Practiced since 1934, the passive drooling of unstimulated whole saliva is often referred as the gold  
 299 standard for biological assays because the effect of flow rate on the saliva composition can be ruled  
 300 out. Passive drooling is usually performed by asking the subject to "deburr" (let the saliva drop) into  
 301 plastic tubes (e.g. polypropylene tubes to avoid sample retention or contamination).

302 In 2007, Granger et al. highlighted the advantages of passive drooling such as large sample volume,  
 303 and small influence of materials and substances used to sample or stimulate the salivary flow [77].  
 304 However, passive drooling is not suitable for subjects who are not capable to collaborate properly  
 305 (e.g. very young children, sleepers, and frail elderly). For these subjects, the sampling of saliva by  
 306 absorption is preferable [78].

307 Salimetrics proposes several devices for passive drooling such as the Saliva Collection Aid (SCA,  
 308 polypropylene) where saliva is pooled in the mouth and driven into the vial with the head tilted  
 309 forward (~~Fig-5A~~). Oasis Diagnostic® proposes several devices for saliva collection, e.g. the  
 310 UltraSal-2™ kit that samples up to 24 mL of saliva (~~Fig-5B~~). This device is characterized by two  
 311 12 mL tubes connected to one buccal to split saliva into two aliquots. Although the supplier  
 312 suggests to rotate or tilt the device, the homogeneity of the aliquots is difficult to achieve.

313 Proflow Sialometer™ (Proflow Incorporated) allows saliva to be sampled by drooling or draining.  
 314 Saliva drips off the lower lip into a funnel attached to a graduated collection vessel. **Even then,**  
 315 **sample homogeneity is not guaranteed.**

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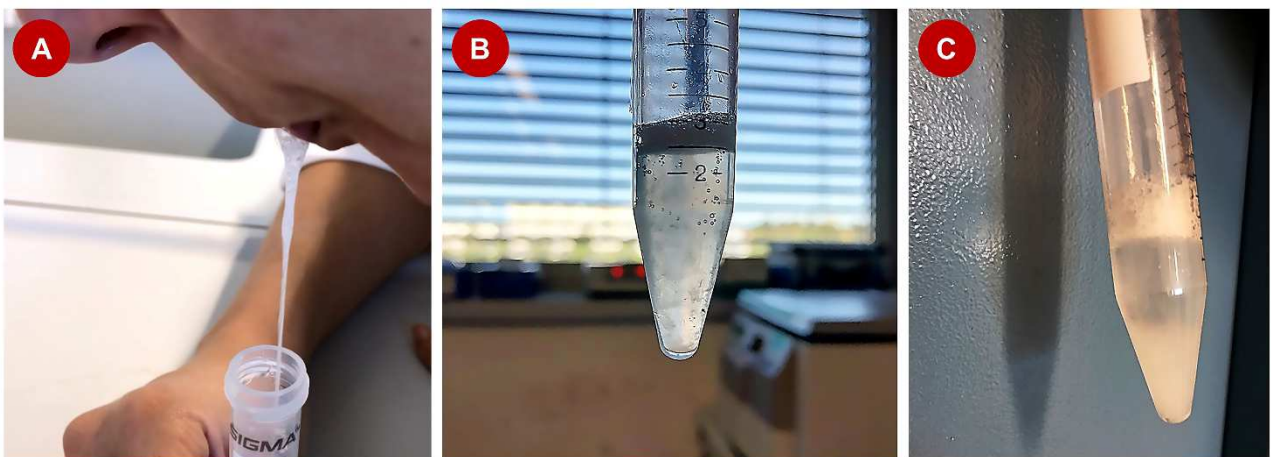
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~~Fig. 5. Examples of devices for sampling saliva by passive drooling: (A) the Saliva Collection Aid by Salimetrics; (B) UltraSal-2™ saliva collection kit by Oasis Diagnostic®.~~

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### 3.2.1.2 Spitting

Spitting is the accumulation of saliva in the floor of the mouth followed by spitting it into a pre-weighed or graduated container, e.g. a funnel connected to a tube/container. This method minimizes the evaporation of saliva in case of long-time samplings and can be used when the flow rate is very low; however, it might have some stimulatory effects [59,79]. **Therefore, since this sampling approach involves a stimulation degree, samples collected by spitting cannot be considered real unstimulated ones.** At the same time, Fig. 3A shows a subject spitting saliva into a 15 mL Falcon tube. **In addition to an appropriate material, size and ease of use are also important factors in choosing the device (i.e. in the case of people with reduced abilities).** Figures 3B and 3C show a clear separation of emulsion (foam), mucin aggregates and aqueous phase in two of saliva samples let rest for a few minutes after sampling by spitting. Because of the low homogeneity of the sample, spitting should be followed by a homogenization step such as filtration and recollection of the filtrate [80]. **However, analyte recovery has to be investigate to identify the most suitable filter.**



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**Fig. 3. (A)** Example of the spitting method into a polypropylene centrifuge tube; **(B)** and **(C)** Examples of untreated saliva samples after spitting. The separation of emulsion (foam), mucin aggregates and aqueous phase is clearly visible.

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~~Norgen Biotek Corp. sells a device for salivary deoxyribonucleic acid (DNA) collection and preservation at ambient temperature. This device consists of three parts, namely i) a saliva collection funnel and collection tube, ii) a sealed squeezable ampoule with the Norgen's saliva DNA preservative within, and iii) a collection tube cap. After spitting, the collection funnel is removed and saliva is mixed with the preservative in the ampoule. Hence, the Saliva Collection Tube is ready to be sent to a laboratory for DNA isolation and analysis.~~

~~The Norgen's device was used to investigate the differences in the quantity of DNA found in the urine and saliva of smokers versus non-smokers [118], the genetic associations with stress fracture injury [119,120], the association of premenstrual/menstrual symptoms with perinatal depression in genomic DNA extracted from saliva [121], the natives' genomic data from Pacific Northwest in British Columbia and South East Alaska with a well-documented history of contacts with European and Asian traders [122], dental genetic disorders [123,124], the effect of the risk factor on familiarity with Alzheimer's disease in cognitively normally aging individuals [125], the relation between attention deficit/hyperactivity disorder and urinary nonylphenol levels [126], the exposure to organophosphate pesticide [127], and the sugar-sweetened beverage consumption [128]. More recently, the Norgen's device has been used for the detection of novel integrons in the metagenome~~

355 ~~of human saliva [129] and for identifying food preference, familiarity and choice among Italians in~~  
356 ~~“The Italian Taste project” [130].~~

357

### 358 **3.2.1.3 Swab-based sampling**

359 Unstimulated whole saliva can be sampled by placing swabs or other absorbent materials in the  
360 mouth. The choice of the material should depend on the subject’s tolerability (dimensions, taste and  
361 allergy) and the capability to preserve the analytes of interest. For some analytes, e.g. salivary  $\alpha$ -  
362 amylase, the retention of analytes is so strong that there is an inverse correlation with the amount of  
363 adsorbed saliva [81].

364 Although swallowing must be avoided and undesired stimulation could be present, swab-based  
365 sampling is particularly suitable for less- or non-collaborative subjects, such as unable people, kids,  
366 newborns and elder people. However, the swab must not be chewed or sucked while saliva is  
367 sampled.

368 If the maximum absorptive capacity of the swab is not exceeded, the salivary flow rate can be  
369 estimated by measuring the sampling time and weighing the swab before and after the sampling  
370 [27,82,83].

371 There are several devices that sample unstimulated whole saliva by passive drooling such as the  
372 Super·Sal™ (Fig. 4A) and Versi·Sal™ (Fig. 4B) by Oasis Diagnostic. The Super·Sal™ is a  
373 universal sampler for biological fluids, e.g. saliva, vaginal specimens, urine, and amniotic fluid.  
374 Super·Sal™ was used with cows, horses, pigs, cats, non-human primates, dogs, and humans to  
375 sample hormones, bacteria, viruses, certain drug molecules, and proteins [84,85]. The Versi·Sal™  
376 has a disposable non-cellulosic absorbent pad, which is placed under the tongue and is capable of  
377 sampling up to about 1.2 mL in 1 – 2 min. Versi·Sal™ allows saliva to be recovered by squeezing  
378 the pad in a compression tube until saliva is pushed towards a vessel such as an Eppendorf tube. In  
379 2012, Fortes et al. used Versi·Sal™ to collect saliva for antimicrobial proteins analysis (e.g.  
380 secretory IgA (SIgA),  $\alpha$ -amylase, and lysozyme) in order to investigate the combination of exercise-  
381 induced dehydration and overnight fluid restriction [86]. Rai et al. used Versi·Sal™ to screen  
382 participants to interplanetary missions [87,88]. Saliva was sampled from six subjects at the Mars  
383 Desert Research Station (Utah, USA) to assess the change in salivary stress biomarkers (cortisol  
384 and salivary  $\alpha$ -amylase) before and after extravehicular activity. Oasis Diagnostic sells another  
385 device, the Micro·SAL™, which is similar to Super·Sal™ and is particularly indicated for  
386 controlled and standardized saliva sampling from small animals. Like Versi·Sal™, Micro·SAL™  
387 allows for an easy and safely transportation of samples.

388 The Quantisal™ saliva collection device supplied by Immalysis Corporation is commonly used to  
389 test illicit and prescription drugs (Fig. 4C). Quantisal™ is similar to Versi·Sal™ and consists of a  
390 cellulosic pad attached to a stem to harvest a maximum of 1 mL saliva. The pad is placed into the  
391 mouth and is removed when a volume indicator turns from white to blue. The pad is stored into a  
392 tube with a stabilizing buffer (whose composition is not specified) to ensure the safe transportation  
393 of the sample. In 2006, Quintela et al. tested Quantisal™ with synthetic saliva for analysing drugs  
394 [89]. After sampling, the recovery of amphetamine and methamphetamine was at least 93%,  
395 whereas it ranged from 91.9% to 100% for morphine and codeine, respectively.

396 The Orasure Technologies Inc. proposes the Intercept® (Fig. 5D), which is specifically designed for  
397 determining the abuse of THC/marijuana, cocaine, opiates, methamphetamines, and phencyclidine  
398 [90,91]. The sampling is performed by swabbing the device in the oral cavity time for about 2 – 5  
399 min.

400 In the literature, the most cited **saliva swab-based sampling** devices are Salivette® (Sarstedt, Fig.  
401 4E) and SalivaBio Oral Swab (SOS, Salimetrics, Fig. 4F). Salivette® is sold in three versions that  
402 include a polypropylene (PP)/low density polyethylene (LD-PE) swab container. The three versions  
403 have the same shape and size, but a different swab material that is recognizable by the color of the  
404 container cap. The white cap corresponds to a cotton swab, the green cap to a cotton swab with

405 citric acid, and the blue cap to a synthetic (polyester) swab. The cotton swab is for general purpose  
 406 sampling, whereas the synthetic swab is specifically designed to determine salivary cortisol. SOS  
 407 consists of a swab storage tube and a synthetic swab. Salivette<sup>®</sup> and SOS can be used to sample  
 408 unstimulated saliva from specific regions or stimulated saliva by simply moving it in the oral  
 409 cavity. For both devices, saliva can be recovered by centrifugation for a few minutes. SOS offers a  
 410 second option to recover saliva by squeezing the swab using a syringe. In 1994, Lamey and Nolan  
 411 tested the recovery of saliva in Salivette<sup>®</sup> [92]. Cotton and polyester swab were used to sample  
 412 different volumes of saliva (from 0.2 mL to 3 mL with a step of 0.2 mL for a total of fifteen  
 413 samples) at different centrifugation speed (600 g for 2 min and 2000 g for 2 min). The recovery  
 414 from the cotton swab linearly depended on the volume of saliva, whereas the recovery was almost  
 415 constant for the polyester swab (more than  $80 \pm 6.3\%$ ).  
 416



417  
 418 **Fig. 4.** A selection of different swab-based devices for sampling saliva: (A) Super Sal<sup>™</sup> (Oasis Diagnostic  
 419 Corporation); (B) Versi Sal<sup>™</sup> (Oasis Diagnostic Corporation); (C) Quantisal<sup>®</sup> (Immunalysis Corporation); (D)  
 420 Intercept<sup>®</sup> (Orasure Technologies Inc.); (E) Salivette<sup>®</sup> (blue cap, Sarstedt); (F) SOS (Salimetrics); (G) **Toothette-Plus**  
 421 **swabs (Sage Products Inc.); (H) OraQuick Advance HIV-1/2 (Orasure Technologies Inc.); (I) BBL CultureSwab orange**  
 422 **and white cap.**

423  
 424 **However, although several kinds of swabs are commercially available and some swabs are sold for**  
 425 **sampling specific salivary analytes (e.g. Salivette<sup>®</sup> Cortisol is specially designed for cortisol**  
 426 **determination in saliva), most manufacturers do not provide details neither on analyte recovery nor**  
 427 **on sampling reproducibility. Therefore, to optimize the sampling procedure, the analyte recovery**  
 428 **must be carefully investigated.**  
 429

430 **3.2.2 Stimulated whole saliva collection**

431 Stimulated saliva is physiologically secreted in response to either masticatory or gustatory  
432 stimulations during food intake. Its composition depends on the gland size, food intake, smoking,  
433 gag reflex and type of stimulation given. Various stimulants such as paraffin wax, unflavored  
434 chewing gum base, cotton puff and rubber bands can be used to sample saliva by masticatory  
435 stimulation, whereas gustatory stimulation can be obtained using citric acid and sour candy drops.  
436 Mastication does not only increase flow rate but has also been shown to increase the protein output  
437 and the salivary pH, but gustatory stimuli have a greater effect on salivary composition than  
438 masticatory stimulants [93]. In fact, Polland et al. studied how a prolonged sugar-free gum-chewing  
439 stimulation affects the salivary flow rate and pH in twenty-eight nominally healthy subjects [94].  
440 The salivary flow and pH were significantly higher even after 90 min of chewing (interrupted with  
441 some moments of rest). Flow rate was  $0.39 \pm 0.16$  mL/min at rest and increased up to  $2.7 \pm 0.52$   
442 mL/min during the stimulation. The pH value was  $6.7 \pm 0.24$  pH units at rest and increased up to  
443  $7.35 \pm 0.22$  pH units. Dawes et al. found similar results for pH but noticed that flow rate reached a  
444 plateau of 0.94 mL/min after 35 – 40 min of chewing [95].

445 Some papers report that mechanical masticatory stimulation can modify the saliva composition.  
446 Dong et al. reported that the level of sucrose in saliva depended on the chewing rate [96]. Higashi et  
447 al. reported a reduced salivary concentration (at least two-fold) of homovanillic acid (HVA) and 3-  
448 methoxy-4-hydroxyphenylglycol (MHPG) caused by an increase of salivary secretion by chewing a  
449 gum [97], whereas chenodeoxycholic acid (CDCA) levels did not change [98].

450 These data highlight the need to standardize the sampling procedures to make saliva analysis more  
451 robust and accurate. Standard-size stimulants could be used (e.g. gum base or paraffin wax) as well  
452 as chewing the gum at a constant frequency using a metronome [83].

453 In 2004, Holm-Hansen et al. compared the absorption and release of saliva in eight devices [99].  
454 Whole saliva was stimulated by chewing neutral gum-based pellets and then collected into iced 15  
455 mL Falcon tubes. Samples were pooled and centrifuged at 1935 g for 15 min. After, the supernatant  
456 was divided into aliquots. Each device has then been submerged both into water and supernatant  
457 that represented clarified saliva sample. The absorbed fluid was recovered by centrifugation at  
458 4,000 rpm for 10 min. Sampling devices were weighted dry, wet, and after centrifugation, as well as  
459 the volumes of water or saliva absorbed and released after centrifugation, were recorded and  
460 compared with each other. The eight devices were capable of picking up and releasing  
461 approximately an equal volume of water and saliva, with no significant differences among any of  
462 the devices in terms of their abilities to absorb and deliver the two fluids. However, there were  
463 significant differences for the sampled volumes: Salivette collected the highest volume, followed by  
464 Toothette (product for oral hygiene, designed to moisten and clean the oral cavity, produced by  
465 Sage Products Inc. Fig. 4H), OraSure (adsorbent pad on a plastic stick, OraSure Technologies Inc.,  
466 Fig. 4I) and UpLink (sampler that absorbs a metered dose, OraSure Technologies Inc.), Transorb  
467 (bonded cellulose acetate fibre for diagnostic devices such as membrane enzyme immunoassays,  
468 fluorescence polarization, and microparticle immunoassays, from Filtrona Richmond, Inc.), and  
469 BBL white (swab to sample aerobic organisms from throat, vagina, skin, and wound specimens,  
470 Becton Dickinson and Co., Fig. 4J). Compared with other devices, Salivette<sup>®</sup> absorbed a volume  
471 that was about four times higher. This result could depend on the big size of the pad (10 mm  
472 diameter and 35 mm length).

473

474

475 ~~Table 4. The eight commercially available devices compared by Holm-Hansen et al. to test their absorption and release~~  
476 ~~of saliva [152].~~

<del>Device</del>	<del>Device characteristics</del>	<del>Supplier</del>
<del>OraSure HIV-1</del>	<del>Adsorbent pad on a plastic stick</del>	<del>OraSure Technologies Inc.</del>

<del>UpLink</del>	<del>Unique sampler that absorbs a metered dose. It can be easily transferred from the plastic handle</del>	<del>OraSure Technologies Inc.</del>
<del>Salivette®</del>	<del>Cotton swab</del>	<del>Sarstedt</del>
<del>Toothette Plus swabs</del>	<del>Product for oral hygiene, designed to moisten and clean the oral cavity. It consists of a sponge-like pad on a plastic stick</del>	<del>Sage Products Inc.</del>
<del>BBL CultureSwab orange cap</del>	<del>Swabs to sample aerobic organisms from male urethral samples, ear, nose, throat, and eye specimens</del>	<del>Becton Dickinson and Co.</del>
<del>BBL CultureSwab white cap</del>	<del>Swabs to sample aerobic organisms from throat, vagina, skin, and wound specimens</del>	
<del>BBL CultureSwab red cap</del>	<del>General purpose swabs for laboratory</del>	
<del>TRANSORB wicks</del>	<del>Bonded cellulose acetate fibre for diagnostic devices such as membrane enzyme immunoassays, fluorescence polarization, and microparticle immunoassays</del>	<del>Filtrona Richmond, Inc.</del>

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~~Fig. 8. Some of the devices tested by Holm-Hansen et al. in terms of sample (water or saliva) transfer capacity (A) Toothette Plus swabs (Sage Products Inc.); (B) OraQuick Advance HIV 1/2 (OraSure Technologies Inc.); (C) BBL CultureSwab orange and white cap.~~

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482 In 2006, Rohleder found that salivary flow rate did not affect the salivary  $\alpha$ -amylase (sAA) activity  
483 when saliva was collected by passive drool and Salivette [100]. In 2010, Beltzer et al. studied the  
484 dependence of sAA activity on collection time, sampling method (i.e. passive drooling, a  
485 ~~micro~~micro-sponge, a cotton pledget, and a synthetic oral swab) and type of saliva (unstimulated  
486 whole saliva and saliva sampled from specific salivary glands) [57]. The authors reported a linear  
487 increase in the volume of saliva collected by passive drool, while this did not occur in the case of  
488 use of absorbent devices. In contrast to Rohleder et al. [100], a decreased sAA activity was  
489 observed when the sample was collected by passive drooling due to the influence of salivary flow  
490 rate. In 2013, Arhakis et al. found a similar influence of the salivary flow rate on sAA activity  
491 [101]. In 2018, Lomonaco et al. used a synthetic Salivette® swab to study the effect of the sampling  
492 procedure in twenty-two nominally healthy volunteers for the determination of uric acid and lactate  
493 in non-stimulated and stimulated whole saliva samples [83]. The analytes recovery was tested at  
494 four different pH values (5, 6, 7, and 8) by analyzing saliva samples spiked with 50  $\mu\text{g/mL}$  of  
495 lactate and 100  $\mu\text{g/mL}$  of uric acid. To sample unstimulated saliva, the subjects were asked for  
496 placing the swab in the mouth between a gum and the cheek for 2 min. Stimulated whole saliva was  
497 sampled at 50, 100 and 150  $\text{min}^{-1}$  by moving the swab in the oral cavity for 1 min. Quantitative  
498 recovery of uric acid and lactate was obtained independently of pH. For increasing flow rate, the  
499 change in the concentration of lactate was not statistically relevant, whereas the concentration of  
500 uric acid decreased from  $70 \pm 20 \mu\text{g/mL}$  to  $30 \pm 10 \mu\text{g/mL}$ .

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### 502 3.2.3 Dried saliva spot (DSS)

503 Abdel-Rehim et al. have recently proposed a technique called dried saliva spot (DSS) to determine  
504 the amount of lidocaine in saliva [102]. In DSS, a few drops of saliva are spotted onto collection  
505 card and dry at room conditions. DSS needed a low volume of saliva (50  $\mu\text{L}$ ) and allowed for a  
506 quantitative recovery of the analyte from a filter paper. Other advantages of DSS are the  
507 transportation, storage and pre-treatment of samples.

508 Numako et al. used DSS for the determination of the D- and L-lactic acid in diabetic patients, pre-  
509 diabetic and nominally healthy persons [103]. The author pointed out that DSS has 1) high detection  
510 sensitivity to the target molecule because of low spotted volume; 2) high accuracy and precision; 3)  
511 high recovery of the target molecule from the spot; 4) high stability of the target molecule because

512 of a relatively long storage before analysis. Zheng et al. used DSS to measure the concentration of  
 513 BMS-927711, a drug for the treatment of migraines, by spotting 15  $\mu$ L of saliva onto two regular  
 514 cards, i.e. Whatman FTA™ and DMPK-C [104]. The amount of spotted saliva was well-controlled  
 515 and fully analyzed in order to minimize sample-to-sample variation. Krone et al. analyzed the  
 516 *Streptococcus pneumoniae* [105] using an aliquot of 100  $\mu$ L saliva on a filter paper card (Whatman  
 517 903 Protein Saver Card) dried at room temperature for 2 h. The DNA of the bacteria was stable in  
 518 saliva spots for 35 days and the concentration agreed with that found in raw saliva samples.  
 519 Although its advantages, DSS is still a recent technique that needs further studies on  
 520 standardization, stability of analytes and interactions with the absorbing material to confirm its  
 521 efficacy for clinical purpose.

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### 523 3.2.4 Microextraction techniques for saliva sampling

524 Solid phase microextraction (SPME) and micro-extraction by packed sorbent (MEPS, a  
 525 miniaturized and sophisticated version of SPE) combine sample pre-treatment/purification and  
 526 sample pre-concentration and extraction [106]. The main advantage of these techniques is the  
 527 reduction of the amount of organic solvents, which is similar or better compared to SPE and liquid–  
 528 liquid extraction (LLE). However, SPME and MEPS also allow the sample manipulation to be  
 529 reduced, improve sensitivity, can be easily automated and adapted for in vivo and onsite  
 530 applications, and can be coupled to liquid chromatography (LC) and gas chromatography (GC)  
 531 coupled to mass spectrometry (LC-MS, GC-MS) instrumentation [52,107–109]. An example of  
 532 SPME application for saliva sampling is described by Bessonneau et al. [52]. The authors  
 533 investigated the efficiency of SPME in the simultaneous extraction of forty-nine prohibited  
 534 substances (e.g. cannabinoids, steroids and narcotics) in saliva using LC-MS separation. SPME  
 535 allowed for a fast extraction of a large number of metabolites with a wide range of polarity and  
 536 basicity, and prevented the loss of hydrophobic compounds. Although there are a few studies,  
 537 SPME can be a potential tool for multidetection of a wide range of salivary compounds with  
 538 different characteristics.

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**Table 3.** Summarizing table of pros and cons of approaches for whole saliva sampling.

Sampling method	Pros	Cons
Passive drooling and draining	<ul style="list-style-type: none"> <li>• No effect of flow rate</li> <li>• More representative of basal unstimulated secretion</li> </ul>	<ul style="list-style-type: none"> <li>• Inhomogeneity of the sample</li> <li>• Sampling duration</li> <li>• The subject has to be collaborative</li> </ul>
Spitting	<ul style="list-style-type: none"> <li>• Evaporation of saliva for long-time samplings minimized</li> <li>• Suitable with very low flow rates</li> </ul>	<ul style="list-style-type: none"> <li>• Possible stimulatory effects</li> <li>• The subject has to be collaborative</li> </ul>
Swab-based	<ul style="list-style-type: none"> <li>• Low cost</li> <li>• Easy availability</li> <li>• Easy use</li> <li>• Particularly suitable for less- or non-collaborative subjects</li> <li>• Possibility to estimate the flow rate (weight vs sampling time)</li> <li>• Homogeneity of samples recovered by centrifugation</li> </ul>	<ul style="list-style-type: none"> <li>• Possible retention of analytes by the swab</li> <li>• Possible stimulatory effects</li> <li>• Risk of swallowing</li> </ul>
DSS	<ul style="list-style-type: none"> <li>• Very small volume of sample required</li> </ul>	<ul style="list-style-type: none"> <li>• Possible issues related to analyte stability</li> </ul>



		<ul style="list-style-type: none"> <li>• Possible interactions of analyte with the absorbing material</li> </ul>
Microextraction sampling	<ul style="list-style-type: none"> <li>• Sampling, analyte extraction, and sample introduction in a single step</li> <li>• Easily automated and adapted for in vivo and onsite applications</li> <li>• Easily coupled to LC-MS and GC-MS instrumentation</li> </ul>	<ul style="list-style-type: none"> <li>• Greater volume of sample needed</li> <li>• Not yet tested on clinical studies</li> </ul>

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#### 4. Salivary biomarkers for clinical applications

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##### 4.1 Salivary steroids

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Steroids are lipophilic, low-molecular weight compounds derived from cholesterol. Steroids play key physiological roles by acting on both the peripheral target tissues and the central nervous system (CNS). Although their relatively simple chemical structure, steroids are in a wide variety of biologically active forms. In biological fluids, steroids are usually found in a conjugated form (i.e. linked to a hydrophilic moiety), whereas unconjugated steroids are mostly bound to carrier proteins in plasma. In steroids, the "free fraction" is the hormone that is directly available for action and accounts for the 1 – 10% of total plasma concentration.

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The transfer mechanism from plasma to saliva have been reported by Gröschl [110] and Zolotukhin [111]. The lipid-soluble steroids, aldosterone and cortisol, enter saliva by passive diffusion through the acinar cells of the salivary gland. Since salivary concentration of aldosterone and cortisol is independent of the salivary flow rate [112], their levels in saliva can be used to obtain information of unbound levels in plasma [113–115]. Steroids can also enter saliva from blood or plasma via oral abrasions or directly from foodstuffs by contamination with exogenous steroids.

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The first measurement of steroids in saliva dates back to the late 70s and reported the determination of testosterone and cortisol [116]. The analysis of salivary hormones is used for assessing the ovarian function (progesterone and estradiol) [117], for the diagnosis of congenital adrenal hyperplasia (17 $\alpha$ -OH progesterone) [118], for investigating adrenal function and for the screening of Cushing's disease (cortisol) [119] (Table 4). A more exhaustive list of main steroids detected in saliva for clinical applications is reported in Table S2 with their salivary levels in nominally healthy subjects.

Many unconjugated steroid hormones can be found at similar concentrations in unstimulated and stimulated saliva [115]. In 2013, Durdiaková et al. studied the concentration of testosterone in unstimulated and stimulated whole saliva samples [120]. Unstimulated saliva was sampled by asking the subjects to drop down the head, let the saliva run naturally to the front of mouth, hold saliva for a short time and spit it into a sterile polypropylene tube. Stimulated saliva was sampled by moving in the mouth with the tongue a cotton swab soaked in 2% citric acid. The concentrations of testosterone were neither influenced by stimulation nor by repeated samplings.

Conjugated steroid hormones (charged steroids, e.g. dehydroepiandrosterone sulfate, DHEA-s) diffuse through tight junctions between epithelial cells and are present in stimulated saliva at lower concentrations than in unstimulated saliva since their concentration is inversely related to saliva flow rate [164,165]. Passive drooling is the most suitable sampling method for the determination of DHEA and DHEA-S. In fact, the correlation between salivary and plasma levels of DHEA occurs only for unstimulated saliva [121], whereas the DHEA-S activity seems to be inversely proportional to the salivary flow rate [122].

For charged steroids, salivary pH has to be carefully controlled since their concentration is pH-dependent [168].

**Table 5.** Main steroids found in saliva with their salivary levels in nominally healthy subjects.

Compound	As biomarker of	Salivary levels in nominally healthy subjects	Saliva collection method
<del>17<math>\alpha</math>-OH progesterone</del>	<del>Congenital adrenal hyperplasia (CAH) [172,178,179]</del>	<del>Morning: 20–55 ng/L</del>	<del>Direct spitting (for children) [180]</del>
		<del>Morning: 623.1 <math>\pm</math> 481.4 pg/mL</del>	<del>Three times a day with Salivette<sup>®</sup> or modified medical pacifiers (for newborns) [181]</del>
		<del>Midday: 497.0 <math>\pm</math> 323.3 pg/mL</del>	
		<del>Evening: 397.5 <math>\pm</math> 260.6 pg/mL</del>	<del>Salivette<sup>®</sup> (for post-menarche girls) [182]</del>
		<del>Follicular phase: 20.5 <math>\pm</math> 1.8 pg/mL</del>	
<del>Luteal phase: 45.8 <math>\pm</math> 4.5 pg/mL</del>	<del>3 mL of saliva were collected in a glass tube by direct spitting in 15 min after an initial mouth rinse with water [183]</del>		
<del>17<math>\beta</math>-estradiol</del>	<del>Menstrual cycle [184,185], breast cancer [186], and bone metabolism [187]</del>	<del>2.009 <math>\pm</math> 1.037 pg/mL</del>	<del>Spitting unstimulated whole saliva for 10 min [188]</del>
		<del>2.447 <math>\pm</math> 0.621 pg/mL</del>	<del>Stimulated whole saliva collected for 5 min by habitual chewing of 1 g gum [188]</del>
<del>Aldosterone</del>	<del>Primary aldosteronism (PA) [189,190], cardiovascular risk [191], hypogonadism [192], depression [193] and stress [194]</del>	<del>44–117 ng/L</del>	<del>Salivette<sup>®</sup> (for nominally healthy volunteers) [194]</del>
		<del>1–7 ng/dL</del>	<del>Parotid saliva was sampled by Lashley cup.</del>
		<del>25–1085 pmol/L</del>	<del>Expectorated saliva was collected into a plastic cup for 5 min while sucking sour lemon [195]</del>
<del>Androstenedione</del>	<del>Congenital adrenal hyperplasia</del>	<del>78 <math>\pm</math> 30 pg/ml in 3 mL</del>	<del>Saliva was collected into small plastic tubes [196]</del>
			<del>Unstimulated saliva sampled from women between 9 am and 2 pm in</del>

	(CAH) [172,178,179], body composition in young girls [197], hyperandrogenism [198]		sterile plastic tubes [198]
Cortisol	<del>Cushing's syndrome [199,200], hypothalamic-pituitary-adrenal axis (HPA) response to stress [201–203], post-traumatic stress disorder [204], anxiety [205,206], aggressive behaviour [207], panic attacks [208], cognitive deficits [200], depression [202,210,211], cardiovascular risk (e.g. hypertension, insulin-resistance) [191,200,212–214], and psoriasis [215].</del>	<del>3.6–35.1 mmol/L</del>	<del>1 mL saliva was collected in 5 mL polystyrene bottle immediately after awakening [216]</del>
		In the morning: 1–12 ng/mL, in the evening: 0.1–3 ng/mL	Salivette <sup>®</sup> with an insert containing a sterile cotton wool swab or a swab treated with citric acid [217]
Dehydroepiandrosterone sulfate (DHEA-s)	HPA response to stress [218,219]	291.21 ± 294.81 pg/mL	Whole saliva sampled by Salivette <sup>®</sup> four times in a single working day [220]
Estradiol	Menstrual cycle [221]	Follicular phase 22.1 ± 2.7 pmol/L	Whole stimulated saliva was collected in a vial from healthy women by chewing a sugarless gum [221]
		Luteal phase 20.6 ± 2.4 pmol/L	
		Follicular phase: 5–18 pg/mL	Spitting in a plastic cup [222]
		Luteal phase: 8–35 pg/mL	
		Luteal phase: 20.6 ± 0.4 pmol/L	5 mL of stimulated saliva by chewing a gum [223]
		6–62 pmol/L	Saliva sampled from women in reproductive age during the

			menstrual cycle [224]
Progesterone	Menstrual cycle [185,225], imminent delivery in pregnant women [226], early preterm birth in asymptomatic high-risk women [227], and physical stress [203]	Luteal phase: $436 \pm 34$ pmol/L; Follicular phase: $22.1 \pm 2.7$ pmol/L	5 mL of stimulated saliva sampled by chewing a gum [223]
		Luteal phase: $436 \pm 34$ pmol/L	Stimulated whole saliva was collected in a vial from nominally healthy women by chewing a sugarless gum [221]
Testosterone	Hypogonadism [192,228,229], menstrual cycle [185], and stress response [230]	$140.30 \pm 154.15$ pg/mL	Salivette <sup>®</sup> used four times in a single working day [220]
		$0.190 \pm 68$ nmol/L	Two Salivette <sup>®</sup> used between 8 am and 9 am and placed under the tongue [231]

585 To date, the saliva sampling procedure for analysing steroids are not standardized. However, in the  
586 literature there are several studies that assess the interaction between steroids and the materials used  
587 for sampling (Table S2). Krüger et al. used a plastic tube and a cotton Salivette® to determine by  
588 spitting the salivary levels of 17 $\alpha$ -OH progesterone [123]. The concentration found using Salivette®  
589 was higher than that in plastic tube with a mean difference of about 40.9 ng/L (SD: 18.8; range: 7.9  
590 – 81.5 ng/L). The hypothesis was that the cotton swabs contained an unknown material that either  
591 cross-reacted with the antibody in the assay or affects binding affinity.

592 In 2001, Shirtcliff et al. used cotton swabs (Salivette®) for the determination of salivary levels of  
593 cortisol, DHEA, DHEA-s, estradiol, testosterone, and progesterone [124]. Subjects expectorated 6 –  
594 10 mL of saliva through a short plastic straw into a collection vial. Some samples were left  
595 untreated, whereas other samples were absorbed using cotton swabs. With cotton swabs, the  
596 concentration of DHEA, estradiol and testosterone were about 13, 4.5 and 2.2 times respectively  
597 higher than those found in untreated samples regardless the measuring method (radio-immunoassay  
598 and enzyme-immunoassay). Analogously to Krüger et al., the presence of interfering substances in  
599 the cotton swabs was hypothesized but no blank analysis was performed.

600 In 2005, Strazdins et al. compared three saliva collection methods (passive collection into a sterile  
601 container, the Sarstedt Salivette® cotton swab devices, and an absorbent ‘eyespear’ cellulose-cotton  
602 tip 0.5  $\times$  1.5 cm, on a 5 cm plastic stick supplied from De Fries, Australia) to investigate their  
603 potential impact on the measurement of salivary cortisol and sIgA [125]. In this study, thirteen  
604 adults (25 – 59 years) provided either one or two 5 mL samples of saliva by passive drooling (head  
605 is tilted forward, collecting saliva at the front of the mouth, and then spat into a sterile container),  
606 for a total of fifteen samples. They found that samples obtained with Salivette® provided  
607 significantly lower values for cortisol ( $0.14 \pm 0.07$   $\mu$ g/dL) if compared to passive ( $0.09 \pm 0.05$   
608  $\mu$ g/dL;  $p = 0.001$ ) and eyespear ( $0.11 \pm 0.06$   $\mu$ g/dL;  $p = 0.002$ ) collection methods. The authors  
609 suggested that the material used in the cotton Salivette® devices may have an absorbance affinity  
610 for sIgA.

611 In 2006, Gröschl and Rauh tested two versions of the Salivette® (cotton or polyester swabs),  
612 “Sterile Foam-Tipped Applicators” (for sampling oral DNA, Whatman Inc.), and blood collection  
613 paper cards (Whatman Inc.), to analyze 17 $\alpha$ -OH progesterone, androstenedione, cortisol, cortisone,  
614 and testosterone [126]. The best sample recovery was achieved using the polyester Salivette® swab  
615 (91.8% for 17 $\alpha$ -OH progesterone, 98.9% for androstenedione, 99.8% for cortisol, 98.7% for  
616 cortisone, and 96.3% for testosterone), which had an almost quantitative volume recovery ( $98 \pm$   
617 1%). The paper strips had slightly lower recoveries for all the analytes (72.0% for 17 $\alpha$ -OH  
618 progesterone, 77.1% for androstenedione, 92.0% for cortisol, 89.1% for cortisone, and 70.3% for  
619 testosterone), and a recovery of the sample volume of  $95 \pm 2\%$ . The cotton Salivette® showed low  
620 recoveries for all the analytes (60.9% for 17 $\alpha$ -OH progesterone, 72.4% for androstenedione, 88.7%  
621 for cortisol, 86.2% for cortisone, and 62.0 % for testosterone) with a low reproducible volume  
622 recovery ( $89 \pm 8\%$ ). Although a volume recovery of  $97 \pm 1\%$ , the foam-tips had the worst  
623 performance (76.2% for cortisol, only 41.8% for cortisone, 31.1% for 17 $\alpha$ -OH progesterone, 38.5%  
624 for testosterone, and 36.1% for androstenedione). These results agreed with other studies [127,128].

625 Gallagher et al. compared passive drooling with citric acid-treated Salivette® swab to measure  
626 cortisol and DHEA [121]. No difference were found for the cortisol levels, whereas only DHEA  
627 levels sampled by passive drooling correlated with plasma levels. Poll et al. studied the correlation  
628 between salivary and serum cortisol levels using the Salivette® cotton swab or passive drooling into  
629 plastic tubes [129]. They found lower salivary cortisol concentrations (about 15%) in Salivette®  
630 than in plastic tubes, in agreement with previous studies [124,125].

631 Ogawa et al. tested passive drool using a straw, cotton swab rolls and polymer swab rolls for  
632 measuring cortisol, DHEA and DHEA-S [130]. The highest concentration of DHEA-S was found  
633 with the cotton swabs. The same result was found by Atkinson et al. [131], thus suggesting that the  
634 use of Salivette® cotton swab affects the salivary testosterone and DHEA levels.

635 Whetzel and Klein contradict the previous results as they found that DHEA-s levels were similar  
636 between passive drool and Salivette<sup>®</sup> cotton swabs methods [132]. However, since the cotton swab  
637 was rolled onto the tongue for 2 min, stimulated saliva cannot be excluded.

638 The effect of sample volume, exposure time and temperature on the recovery of 17 $\alpha$ -OH  
639 progesterone, androstenedione, cortisol, cortisone, and testosterone from commercial saliva  
640 collection devices was also investigated by Gröschl et al. [240]. Salivette<sup>®</sup> swab (e.g. cotton,  
641 polyester, and polyethylene), a Quantisal<sup>®</sup> saliva collection device, and the liquid based approach  
642 Saliva-Collection-System<sup>®</sup> (SCS<sup>®</sup>, Greiner-BioOne) were used for sampling saliva. A reference  
643 sample was obtained by draining the saliva from the mouth directly into a low-binding  
644 polypropylene tube. The results obtained with the two synthetic Salivette<sup>®</sup> swabs and the  
645 Quantisal<sup>®</sup> did not differ significantly from the reference sample, whereas lower steroids levels  
646 were found in the cotton Salivette<sup>®</sup> and the SCS<sup>®</sup>. The volume recovery was excellent for the  
647 polyester and polyethylene Salivette<sup>®</sup> and the Quantisal<sup>®</sup> (> 95%). For the synthetic swabs, a  
648 storage for more than 4 days at 4 °C of the sample was not recommended, whereas it should be  
649 preferred an immediate centrifugation followed by freezing. Gröschl et al. concluded that synthetic  
650 Salivette<sup>®</sup> swabs and the Quantisal<sup>®</sup> saliva collection device are the most suitable collection devices  
651 for steroids analysis. However, Quantisal<sup>®</sup> needs the sample to be processed immediately without  
652 any storage. The SCS<sup>®</sup> is more complicated and unsuitable for immediate use by untrained people,  
653 whereas the cotton Salivette<sup>®</sup> swabs seemed to alter the salivary steroids levels.

654 In 2012, Celec and Ostatníková found that Salivette<sup>®</sup> significantly alters the salivary concentrations  
655 of sex steroids such as testosterone and estradiol [134]. They collected saliva samples by asking to  
656 300 young healthy volunteers (231 women and 69 men) to spit into sterile tubes (whole saliva  
657 sample), using cotton swabs. Using the cotton swabs, testosterone and estradiol concentrations  
658 increased (9% in women and 33% in men). The authors hypothesized that constituents of the cotton  
659 material interact with the antibodies used in the enzyme-linked immunosorbent assay (ELISA) as  
660 reported for other steroids [123,125].

661 Granger et al. summarized how cotton swabs could erroneously increase the testosterone level  
662 [135]. Their work discussed how salivary flow rate and pH (stimulated by powdered drink-mix  
663 crystals placed in subjects' mouths or by chewing) could increase the testosterone level in saliva. It  
664 was suggested to exclude saliva samples at pH < 6. A transient rise of the testosterone level was  
665 observed in saliva sampled during the first few minutes after chewing a gum, thus it was suggested  
666 to start sampling saliva after 3 min at least.

667

## 668 **4.2 Peptides and proteins**

669 In recent years, especially due to the progress in analytical techniques, the analysis of saliva has  
670 gained popularity in proteomics [136]. Saliva contains more than 2000 proteins and peptides, which  
671 are involved in different biological functions [30]. For example, interleukins (ILs), tumor necrosis  
672 factor- $\alpha$  (TNF- $\alpha$ ) and matrix metalloproteinases (MMPs) are associated with inflammation and used  
673 as biomarkers in clinical practice [22,137,138]. Salivary proteomics has been recently applied for  
674 the diagnosis and monitoring of several diseases, e.g. oral squamous cell carcinoma, oral  
675 leukoplakia, chronic graft-versus-host disease Sjögren's syndrome, SAPHO (i.e. synovitis, acne,  
676 pustulosis, hyperostosis, and osteitis), schizophrenia, bipolar disorder, and genetic diseases such as  
677 Down's Syndrome and Wilson disease [139]. However, the effect of sampling methods on proteins  
678 and peptides levels are seldom discussed in the literature. Table 4 shows an example of how the  
679 lack of standardization in sampling and analytical procedure might affect the concentration of  
680 salivary levels for ILs and TNF- $\alpha$  in nominally healthy subjects. **At the same time, the presented**  
681 **values may make us reflect on the analytical method sensibility.**

682

683

684 **Table 4.** Mean salivary concentration of cytokines in nominally healthy subjects.

Cytokine	Sample	Collection method	Mean salivary concentration in nominally healthy subjects [pg/mL]	n	[Reference]	
<b>IL-1<math>\beta</math></b>	UWS	Drooling	14.1	20	[140]	
		Spitting	64.5 $\pm$ 89.6	20	[141]	
		Not reported	71.5 (28.8 – 158.9)	27	[142]	
	SWS	Spitting	24.7 $\pm$ 49.2	20	[141]	
<b>IL-2</b>	UWS	Expectoration	7.3 $\pm$ 3.0	30	[143]	
			11.91 $\pm$ 1.70	30	[144]	
<b>IL-6</b>	UWS	Drooling	4.61 $\pm$ 4.42	34	[145]	
			1.80 $\pm$ 4.25	45	[146]	
		Expectoration	5.2 $\pm$ 2.8	30	[143]	
			2.5 $\pm$ 1.3	9	[147]	
			10 – 25	20	[148]	
			259.68 $\pm$ 12.838	25	[149]	
			16.6 $\pm$ 1.88	50	[150]	
			Spitting	27.6 $\pm$ 26.3	20	[141]
			Not reported	3.7 (2.6 – 5.9)	27	[142]
			SWS	Spitting	10.0 $\pm$ 8.6	20
<b>IL-8</b>	UWS	Drooling	250	32	[151]	
			250.0	20	[140]	
			300 – 785	20	[148]	
		Spitting	755.3 $\pm$ 700.4	20	[141]	
		Expectoration	700.7 $\pm$ 1031.5	9	[147]	
			210.096 $\pm$ 142.302	25	[152]	
			319.49 (10.48 – 592.6)	100	[153]	
			738.5 $\pm$ 98.5	50	[150]	
		Not reported	478.0 (230.5 – 1067.2)	27	[142]	
SWS	Spitting	392.1 $\pm$ 440.4	20	[141]		
<b>IL-10</b>	UWS	Passive drooling	4.86 (IQR = 7.50)	92	[154]	
		Spitting	1.72 $\pm$ 1.33	28	[155]	
		Expectoration	12.02 $\pm$ 7.23	41	[156]	
		Not reported	2.5 (0.45 – 5.1)	40	[157]	
		0.1 $\pm$ 0.04	44	[158]		
<b>TNF-<math>\alpha</math></b>	UWS	Drooling	4.06 $\pm$ 7.46	34	[145]	

	Spitting	11.2 ± 8.5	20	[141]
		4.1 ± 2.1	9	[147]
	Expectoration	8.5 ± 1.74	50	[150]
		1.39 (IQR = 1.27 – 1.64)	20	[159]
		8.7	30	[160]
	Not reported	8.1 (3.8 – 11.9)	27	[142]
		0.131 (0.116 – 0.213)	25	[161]
	SWS Spitting	6.5 ± 5.6	20	[141]

685 Legend: n = number of subjects; UWS = unstimulated whole saliva; SWS = stimulated whole saliva.

686

687 In 2006, Neyraud et al. investigated the changes in human salivary proteome in whole saliva and  
688 saliva samples from parotids after stimulation with four different tastes, namely sweet, umami,  
689 bitter, and sour [162]. Although this study was carried out on a limited number of subjects, Neyraud  
690 et al. found that each taste changed the concentration of the whole saliva proteome. High levels of  
691 sour flavor increased the concentrations of Annexin-A2 and  $\beta$ -2-microglobulin in whole saliva, and  
692 PRH2 protein and  $\alpha$ -amylase in parotid saliva. High concentrations of umami, bitterness, and sweet  
693 flavors increased the levels of calgranulin-A and annexin-A1). Cystatin-S and enolase-1 decreased  
694 after a strong stimulation with a bitter flavor.

695 In 2006, Michishige et al. compared three methods for the determination of proteins in saliva:  
696 suction using an aspiration set, spitting into a sterile plastic dish, and cotton Salivette<sup>®</sup> swab placed  
697 under the tongue [163]. The saliva samples were obtained from 10 non-smoker female volunteers  
698 (20 – 22 years) and the analyzed proteins were S-IgA, kallikrein activity, trypsin-like activity, and  
699 human airway trypsin-like protease (HAT). The content of total protein, S-IgA, trypsin-like activity  
700 and HAT was higher in the samples collected by Salivette<sup>®</sup> than by suction and spitting, which had  
701 similar values. However, the high standard deviations suggest a deeper analysis of these results.  
702 Gröschl et al. investigated the sample recovery from Salivette<sup>®</sup>, Quantisal<sup>®</sup> and SCS<sup>®</sup> [240]. The  
703 authors focused on the hormones associated with the proliferation of the oral mucosa, such as leptin  
704 and ghrelin (acylated and des-acylated), the inflammatory and tumor markers IL-8 and EGF, s-IgA,  
705 amylase, insulin and melatonin. Gröschl et al. found that cotton Salivette<sup>®</sup> devices provided very  
706 poor recoveries (e.g. <10% for IL-8, leptin, insulin, and acylghrelin), whereas the recovery using  
707 polyethylene Salivette<sup>®</sup> was in good accordance with the reference samples for insulin, EGF, and  
708 amylase. Melatonin, IL-8 and acyl-ghrelin showed a recovery of 59 ± 6%, 10 and 23%,  
709 respectively. The polyester Salivette<sup>®</sup> provided acceptable recoveries for the panel of small salivary  
710 peptides, but not for the amine melatonin (59 ± 6%). The Quantisal<sup>®</sup> device had recoveries > 85%  
711 for the entire panel of proteins/peptides, whereas SCS<sup>®</sup> recovered more than 90% only for amylase,  
712 amine melatonin and s-IgA. The authors assumed that analytical performance of SCS<sup>®</sup> was  
713 influenced by the presence of dye and/or the citric acid.

714 In 2012, Williamson et al. measured the concentration of twenty-seven cytokines sampled from  
715 venous blood and saliva using a filter paper (1 min) and passive drooling (30 s). No or low  
716 correlation was found between the concentrations of the cytokines in blood and saliva, whereas the  
717 saliva samples from passive drooling and filter paper were correlated for sixteen cytokines [164].  
718 Mohamed et al. studied the effect of the methods for saliva sampling and processing of C-reactive  
719 protein (CRP), immunoglobulin E (IgE), and myoglobin [165]. They compared the analyte levels  
720 and the salivary total protein concentrations in three different samples: unstimulated saliva collected  
721 by passive drooling and saliva obtained by mechanical and acid stimulation. The total protein  
722 concentrations were significantly ( $p < 0.05$ ) lower in acid stimulated saliva compared with



723 unstimulated saliva, whereas no significant difference was found between mechanically stimulated  
 724 and unstimulated samples. Table 5 summarizes the results obtained by Mohamed et al. and Topkas  
 725 et al. who analyzed the same analytes [166]. Topkas et al. tested five sampling methods: passive  
 726 drooling, SOS, Salivette® (cotton and synthetic swabs), and SCS®. Unstimulated and stimulated  
 727 saliva samples were collected from seventeen healthy volunteers (7 women and 10 men, 25 years  
 728 old on average). The differences between the results of Mohamed et al. and Topkas et al. could  
 729 depend on the use of assays, which were originally designed for blood or plasma samples, without  
 730 reporting any test on their applicability to saliva analysis.

731  
 732 **Table 5.** Mean concentrations and IQR of total proteins, CRP, IgE, and myoglobin in saliva samples collected from 25  
 733 healthy volunteers (12 women and 13 men in the 18–34 years old range) by Mohamed et al. [165] vs concentrations  
 734 found by Topkas et al. [166] from 17 healthy volunteers (7 women and 10 men, 25 years old on average).

Analyte	Mohamed et al. [165]			Topkas et al. [166]			
	Passive drooling	Mechanically stimulated saliva	Acid stimulated saliva	Passive drooling	SOS	Cotton Salivette®	Synthetic Salivette®
Total proteins [µg/mL]	1286 (954 – 2709)	1206 (870 – 2053)	1026 (821 – 1680)	-	-	-	-
CRP [pg/mL]	105 (35 – 217)	97 (32 – 213)	66 (38 – 171)	28 (9 – 51)	9 (5 – 30)	19 (7 – 46)	14 (5 – 44)
IgE [pg/mL]	142 (56 – 368)	152 (42 – 246)	139 (46 – 221)	72 (38 – 202)	72 (35 – 123)	43 (30 – 153)	37 (30 – 164)
Myoglobin [pg/mL]	181 (132 – 320)	134 (102 – 202)	147 (111 – 195)	98 (31 – 140)	23 (2 – 54)	23 (12 – 52)	59 (34 – 136)

735  
 736  
 737 In 2013, Takagi et al. fabricated Muddler, a saliva sampler that could be used with a small portable  
 738 luminescent spectrometer [167]. There were two versions of the Muddler, namely Muddler A and  
 739 Muddler B. Each Muddler is made of transparent plastic and can be inserted in a cuvette. Muddler  
 740 A and B differ from the number and diameter of the holes. Muddler A had more holes (13) and a  
 741 smaller inner diameter (1.75 mm) than Muddler B (7 and 2 mm, respectively). Takagi et al. enrolled  
 742 nominally healthy volunteers and compared Muddler with eye sponge by BD Visitec™, and cotton  
 743 and synthetic Salivette®. In the first part of the study, the participants were adult females and  
 744 infants. Muddler A and B were placed on the tongue for 15 – 30 s to collect saliva from healthy  
 745 adult females, whereas the plastic shaft of the Muddler B was held by mothers and introduced into  
 746 the mouth of each infant. The eye sponges were held on the tongue without chewing, whereas  
 747 Salivette® swabs were moved and rolled for 60 s in the mouth, thus stimulated saliva could have  
 748 been sampled. The authors pointed out that commercial devices were not designed to collect a  
 749 constant amount of saliva; thus, the main achievement of this study was that, although Muddler A  
 750 and B were capable of collecting constant amounts of saliva (B slightly better than A), volumes  
 751 were much smaller those sampled by eye sponge and swabs. In the second part of the study, passive  
 752 drooling was used to sample whole saliva at two different times from the oral cavity of six healthy.  
 753 All these samples were mixed and divided into five aliquots. One aliquot was analyzed without any  
 754 further treatment, whereas the other four aliquots were separately pipetted onto the Salivette® swabs  
 755 and the Muddlers A and B. For proteins recovery, passive drooling and Muddlers had similar results  
 756 and outperformed Salivette® swabs, thus suggesting the potential use of Muddlers for saliva  
 757 analysis.

758 Golatowski et al. measured saliva volume, protein concentration and salivary protein patterns  
759 (proteome profile) in unstimulated saliva sampled by passive drooling and stimulated saliva  
760 sampled using a paraffin gum (Ivoclar Vivadent) or cotton Salivette<sup>®</sup> [168]. For each method,  
761 sample collection was performed on three consecutive days. Samples collected using paraffin gum  
762 showed the highest saliva volume ( $4.1 \pm 1.5$  mL) followed by cotton Salivette<sup>®</sup> swab ( $1.8 \pm 0.4$  mL)  
763 and passive drooling ( $1.0 \pm 0.4$  mL). No significant differences between the three sampling  
764 procedures were observed for the saliva total protein concentrations. One hundred and sixty proteins  
765 were identified, but variations in proteins composition were observed depending on the sampling  
766 procedure. Therefore, the authors suggested to use the same sampling method (which has to be  
767 standardized) for large-scale or multi-centric studies.

#### 768 769 **4.3 Drug monitoring**

770 Probably, drugs monitoring is currently the main application of saliva analysis [11,13,169–171].  
771 Nevertheless, there are some limitations because of the variability of the saliva/plasma  
772 concentration ratio [27,172]. Ideally, the best analytical condition is when the saliva/plasma ratio is  
773 approximately constant and equal to about 1.0 [289]. However, salivary flow rate and pH can affect  
774 the analysis. Salivary concentrations of drugs depend on pH: the salivary concentration of weakly  
775 acidic and basic drugs correlates with plasma concentrations at normal plasma pH for non-ionized  
776 drugs but not for ionized drugs. Commercial adulterants and other similar products such as  
777 mouthwashes had no substantial effect on drugs monitoring after 30 min [169]. Regarding the  
778 collection techniques, Drummer has published an interesting review in 2008 [80] reviewing an  
779 updated version of the saliva collection devices. ~~Table 7 shows an updated version of the saliva~~  
780 ~~collection devices reported by Drummer [117].~~ Table S3 reports these sampling devices together  
781 with the targeted analytes.

782 **Table 6.** Example of collection devices reported in the literature for drug monitoring.

Device	Supplier	Sampling procedure	Analyte	Reference
DrugWipe® (Fig. 9A)	Securitec	Swipe only (tongue or skin)	<del>COD</del>	<del>[291]</del>
			<del>MDMA</del>	<del>[292]</del>
			<del>THC</del>	<del>[293]</del>
			<del>AMP, MAMP, COC, MOR, THC, THC-COOH, Benzodiazepines</del>	<del>[294]</del>
Cozart® collector	Cozart Bioscience Ltd.	Absorbent foam pad with diluent	<del>AMP, MAMP, COC, MOR, THC, THC-COOH, Benzodiazepines</del>	<del>[294]</del>
			<del>MTDN, OPI</del>	<del>[295]</del>
			<del>COC, OPI</del>	<del>[296,297]</del>
			<del>COD, OPI</del>	<del>[298]</del>
Dräger DrugTest® (Fig. 9B)	Dräger	Absorbent foam pad with diluent	<del>COC, BZE, ecgonine methyl ester, MOR, 6-AM, dihydrocodeine, COD, MTDN, AMP, MAMP, MDA, MDMA, MBDB, THC, THC-COOH, THCA</del>	<del>[299]</del>
			<del>AMP, MAMP, MDA, MDMA, COC, BZE, MOR, 6-AM, COD</del>	<del>[300]</del>
			<del>Cannabinoids</del>	<del>[293]</del>
			<del>Alprazolam, 7-aminoclonazepam, 7-aminoflunitrazepam, Bromazepam, Clobazam, Diazepam, Lorazepam, Lormetazepam, Midazolam, Nordiazepam, Oxazepam, Temazepam, Tetrazepam, Triazolam, Zaleplon, Zopiclone, Zolpidem</del>	<del>[301]</del>
Interecept®	OraSure Technologies Inc.	Absorbent foam pad with diluent	<del>AMP, MAMP, MDMA, Alprazolam, Clonazepam, Diazepam, Nordiazepam, Oxazepam, Ntrazepam, Flunitrazepam, Zopiclone, MOR, COD, MTDN, Carisoprodol, Meprobamate, THC</del>	<del>[302]</del>
			<del>THC</del>	<del>[303-305]</del>
Interecept® DOA Oral Specimen Collection Device	OraSure Technologies Inc.	Absorbent foam pad with diluent	<del>AMPs, THC, BZE, MOR, 6-AM, COD</del>	<del>[306]</del>
OralLab	Ansys Technologies Inc.	Absorbent foam pad; the collector is squeezed to push oral fluid into test cartridge	<del>AMP, MAMP, COC, MOR, THC, THC-COOH, Benzodiazepines</del>	<del>[294]</del>
OraLine®	OraLine	Direct application to	<del>THC</del>	<del>[305]</del>

		<del>oral cavity, or use of other collectors</del>		
<del>OralScreen<sup>®</sup></del>	<del>Avitar Inc.</del>	<del>Absorbent foam pad only; drops applied to device</del>	<del>COC, OPI, MET, THC</del>	<del>{307}</del>
<del>Oratect (Fig. 9C)</del>	<del>Branan Medical Corporation</del>	<del>Absorbent directly connected to the device</del>	<del>AMP, MAMP, COC, MOR, THC, THC-COOH</del>	<del>{308}</del>
			<del>COC, MET, DAMP, OPI, PCP, THC</del>	<del>{309}</del>
<del>Quantisal<sup>®</sup></del>	<del>Immunoanalysis Corporation</del>	<del>Absorbent foam pad with diluent</del>	<del>THC</del>	<del>{310}</del>
<del>SalivaScreen<sup>®</sup></del>	<del>Ulti-Med</del>	<del>Absorbent foam pad</del>	<del>AMP, MAMP, COC, MOR, THC, THC-COOH, Benzodiazepines</del>	<del>{294}</del>
			<del>COD</del>	<del>{311}</del>
<del>Salivette<sup>®</sup></del>	<del>Sarstedt</del>	<del>Cotton wool swab</del>	<del>THC</del>	<del>{312}</del>
			<del>AMPs, COC, OPI, THC</del>	<del>{313}</del>
<del>Toxiquick<sup>®</sup></del>	<del>Biomar Systems GmbH</del>	<del>Absorbent bud</del>	<del>AMP, MAMP, COC, BZE, OPI, MTDN, THC, THC-COOH</del>	<del>{314}</del>
<del>Uplink</del>	<del>OraSure Technologies Inc.</del>	<del>Absorbent pad</del>	<del>AMP, MAMP, COC, MOR, THC, THC-COOH</del>	<del>{308}</del>

783 Legend: ~~6-AM~~ = ~~6-acetylmorphine~~; ~~AMP~~ = ~~Amphetamine~~; ~~BZE~~ = ~~Benzoylcegonine~~; ~~COC~~ = ~~Cocaine~~; ~~COD~~ = ~~Codeine~~; ~~DAMP~~ = ~~Dextroamphetamine (D-~~  
784 ~~amphetamine)~~; ~~MAMP~~ = ~~Methamphetamine~~; ~~MBDB~~ = ~~3,4-methylenedioxy-N-methylbutanamine~~; ~~MDA~~ = ~~3,4-Methylenedioxyamphetamine~~; ~~MDMA~~ = ~~3,4-~~  
785 ~~Methylenedioxymethamphetamine~~; ~~MET~~ = ~~D-methamphetamine~~; ~~MOR~~ = ~~Morphine~~; ~~MTDN~~ = ~~Methadone~~; ~~OPI~~ = ~~Opiates~~; ~~PCP~~ = ~~Phencyclidine~~; ~~THC~~ = ~~Δ9-~~  
786 ~~tetrahydrocannabinol (cannabis, marijuana)~~; ~~THC-COOH~~ = ~~11-carboxy-THC~~; ~~THCA~~ = ~~11-nor-9-carboxy-THC~~.

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~~Fig. 9. Examples of saliva sampling devices for drugs monitoring. (A) DrugWipe<sup>®</sup> (Securitec); (B) Dräger DrugTest<sup>®</sup> (Dräger); (C) Oratect (Branan Medical Corporation).~~

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792 In 2014, Lomonaco et al. investigated the impact of the sampling method on the salivary  
793 concentration of an anticoagulant drug, warfarin (WAR), and its main metabolites (RS/SR-  
794 and RR/SS warfarin alcohols, WAROHs) [27]. The recovery of a standard solution of WAR  
795 and WAROHs was studied for three different Salivette<sup>®</sup> devices (cotton swab, cotton swab  
796 with citric acid, and synthetic swab). The recovery was also tested for the Salivette<sup>®</sup> synthetic  
797 swab at four different pH values (5, 6, 7 and 8) by analyzing saliva samples spiked with 5  
798 ng/mL of WAROHs. Unstimulated and stimulated whole saliva samples were sampled using  
799 the Salivette<sup>®</sup> synthetic swab from fourteen patients (9 males, 5 females) undergoing WAR  
800 therapy. Unstimulated samples were collected by asking the subjects to place a swab in the  
801 mouth, between the gum and cheek, and to keep it steady for 10 min. Two protocols were  
802 adopted for sampling stimulated saliva: rolling a swab on the tongue for 2 min and chewing a  
803 sugar-free chewing gum for 6 min. Soon after, the whole saliva was collected by rolling  
804 another Salivette<sup>®</sup> synthetic swab for 2 min. Stimulation increased pH values from  $6.6 \pm 0.4$   
805 (range 5.7–7.2) to  $7.5 \pm 0.3$  (range 6.9–8.1). The WAR and WAROHs recovery from  
806 Salivette<sup>®</sup> devices was  $88\% \pm 10\%$ ,  $91\% \pm 6\%$ ,  $96\% \pm 5\%$  using cotton swab;  $62\% \pm 3\%$ ,  
807  $76\% \pm 3\%$ , and  $75\% \pm 1\%$  with cotton swab with citric acid;  $98\% \pm 1\%$ ,  $98\% \pm 1\%$ , and  
808  $100\% \pm 0.3\%$  with synthetic swab. The recovery at different pH values of RS/SR- and RR/SS  
809 WAROHs was:  $93\% \pm 5\%$  and  $94\% \pm 1\%$  at pH 5.1;  $96\% \pm 3\%$  and  $97\% \pm 1\%$  at pH 6.1;  
810  $98\% \pm 0.3\%$  and  $100\% \pm 0.3\%$  at pH 6.9;  $98\% \pm 0.3\%$  and  $99\% \pm 0.3\%$  at pH 8.2. The  
811 concentration of WAR and RS/SR-WAROH increased with the pH value, whereas the  
812 concentration of RR/SS WAROH was not affected. By comparison with WAR plasmatic  
813 levels, the authors highlighted that when salivary pH was close to blood pH, a strong  
814 correlations were observed between the concentrations of both WAR and RS/SR-WAROH in  
815 the stimulated saliva samples and their unbound plasma fractions.

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## 818 5. Conclusions

819 The analysis of saliva can be a powerful alternative to blood for clinical applications.  
820 Although standardization is still far to be achieved, this review highlights that the studies  
821 published so far have paved the way towards the choice of the most suitable methods and  
822 devices for saliva sampling.

823 Saliva collected from specific salivary glands is the most suitable sampling method if the  
824 analyte of interested is mainly secreted by a specific gland, but the user (either clinician or  
825 researcher) should be aware that this collection procedure is invasive and needs custom-made  
826 devices.

827 Whole saliva is easier and faster to sample than glandular saliva. Although the concentration  
828 of analytes is low, pre-concentration can improve the performance of the whole saliva  
829 analysis.

830 Since exogenous and endogenous factors and salivary parameters such as pH and flow rate  
831 affect the saliva composition, it is critical to choose between the collection of stimulated and  
832 unstimulated saliva. For example, unstimulated saliva is less dependent of flow rate and pH,  
833 but sample volumes are mostly lower than those obtained by stimulation. However,  
834 stimulated samples are much diluted, thus the choice between stimulated and unstimulated  
835 saliva largely depends on the analyte of interest. **Consequently, since many factors can**

836 influence on salivary secretion and composition, a precise standard for saliva collection must  
837 be established to obtain reliable and comparable data.

838 The choice of the sampling device is also analyte-dependent. The literature reports that  
839 commercial devices differently perform to recover the analyte of interest, e.g. the cotton swab  
840 by Salivette® that seems have a strong interaction with biological molecules. Since there is no  
841 standard, the intra-dependencies of collection methods, type of saliva and devices should be  
842 carefully consider in advance.

843 Unfortunately, several publications do not report details such as the reagents or the analytical  
844 techniques used for analysis. This lack of information could make it difficult to compare inter-  
845 laboratory data or multi-center results. ELISA seems the most used method, but it is worth  
846 remembering that immunoassays are designed and validated for plasma and blood. Except a  
847 few exception, e.g. cortisol, ELISA kits still need to be validated for saliva analysis.

848 Saliva analysis as routinely approach in a clinical setting is still hindered by the absence of  
849 standardized procedures and analytical information. Future efforts should focus to improve  
850 our knowledge or discover the transport mechanisms from blood to saliva and the analyte  
851 correlation between blood and salivary levels. Several papers reported the detection of  
852 salivary biomarkers, but the lack of standardization procedures does not allow comparing data  
853 obtained from different laboratories. The analysis of saliva will be comparable with blood  
854 only when these limitations will be overcome.

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