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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 ± 1.5 mM to
 109 15 ± 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 α -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 β -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 ⁺	5	20 – 80	145
K ⁺	22	20	4
Cl ⁻	15	30 – 100	120
Ca ²⁺	14	14	2.2
HCO ₃ ⁻	5	1580	25

Mg ²⁺	0.2	0.2	1.2
NH ₃	6	3	0.05

138

139

140 Glycoproteins (e.g., mucins and proline-rich glycoproteins), enzymes (e.g., α -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.~~

Salivary protein	Origin	Function	Mean concentration in saliva	References
			0.47 ± 0.19 mg/mL	[54]
Total contents			0.9 ± 0.2 mg/mL	[55]
			4.3 — 710.0 mg/dL	[56]
			2.67 ± 0.54 mg/mL	[57]
			3257 ± 1682 U/mL	[55]
α-amylase	Serous acinar cells	Starch digestion	1080.0 ± 135.6 U/L	[17]
			476 ± 191 U/mL	[57]
Adrenomedullin	Oral epithelial cells	Antimicrobial	55 ± 65 pg/mL	[59,60]
Albumin	Mainly from plasma leakage		0.2 ± 0.1 mg/dL	[55]
			0.8 — 192.0 mg/dL	[56]
Alkaline phosphatase (ALP)	Tissue degradation	Metabolic process of cells	24.85 ± 12.14 IU/L	[60]
Acid Phosphatase (ACP)	Tissue degradation	Metabolic process of cells	7.32 ± 4.01 IU/L	[60]
Cathelicidins (LL37)	Oral cavity and respiratory tract	Antimicrobial	0.3 — 33.6 µg/mL	[59,6]
			14.3 kDa form	
Cystatin group	Submandibular → sublingual	Antimicrobial (cystein-proteinase inhibitor)	58 ± 25 µg/mL	[57]
			14.2 kDa form	
			91 ± 46 µg/mL	
Defensins (DEF)	Blood, plasma	Antimicrobial	DEF-1 0.8 ± 0.6 µg/mL	[59,61,62]
			DEF-α1-3 0.094 — 9.4 µg/mL	
Hystatin	Parotid	Antifungal	1190 ± 313 µg/mL	[57,63]
Lactate	Oral epithelium	Cell necrosis	497.00 ± 51.75 U/L	[64]

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

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

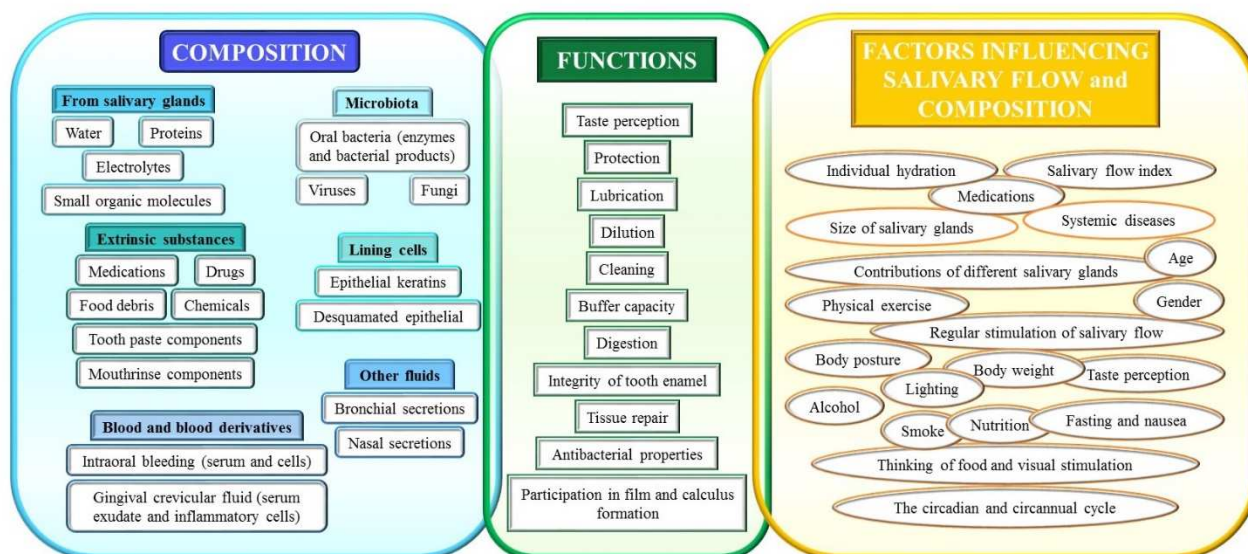
158

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

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

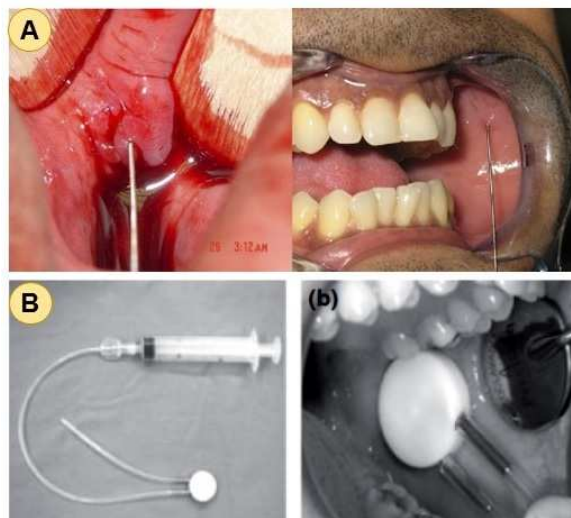
201 Saliva can be collected from specific salivary glands (e.g. parotid glands) or sampling the whole
202 liquid (whole saliva) secreted from all the glands (mixed sample). In both cases, the samples have
203 the same chemical composition, although the concentration of analytes can vary from one gland 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[®], 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[®]
267 6000 model 2 (Proflow[™]) 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 cm² 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·cm²)
274 from lower labial glands, 2.14 ± 0.62 μL/(min·cm²) from upper labial glands, 2.88 ± 0.72
275 μL/(min·cm²) from buccal glands, and 2.15 ± 0.51 μL/(min·cm²) 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	<ul style="list-style-type: none"> • Selective sampling 	<ul style="list-style-type: none"> • Invasive • Possible Salivary gland injuries • Complex procedure • Time consuming • Required skilled personnel
	Lashley cup	<ul style="list-style-type: none"> • Selective sampling • Non-invasive procedures 	<ul style="list-style-type: none"> • Complex procedure • Time consuming

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

282

283

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.**

316

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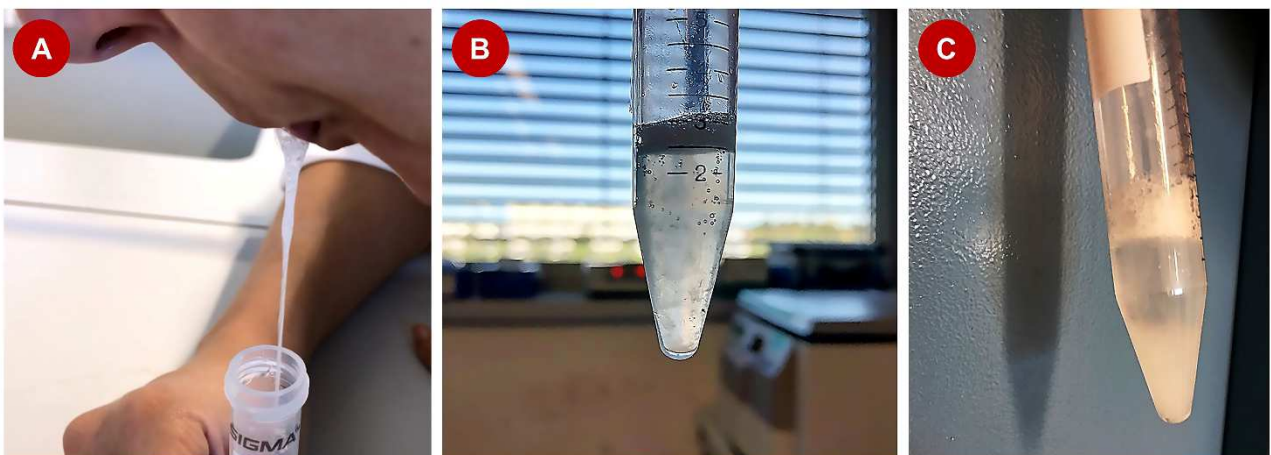
~~Fig. 5. Examples of devices for sampling saliva by passive drooling: (A) the Saliva Collection Aid by Salimetrics; (B) UltraSal-2™ saliva collection kit by Oasis Diagnostic®.~~

321

3.2.1.2 Spitting

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

335



336
337 **Fig. 3.** (A) Example of the spitting method into a polypropylene centrifuge tube; (B) and (C) Examples of untreated
338 saliva samples after spitting. The separation of emulsion (foam), mucin aggregates and aqueous phase is clearly visible.

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

345 ~~The Norgen's device was used to investigate the differences in the quantity of DNA found in the~~
346 ~~urine and saliva of smokers versus non-smokers [118], the genetic associations with stress fracture~~
347 ~~injury [119,120], the association of premenstrual/menstrual symptoms with perinatal depression in~~
348 ~~genomic DNA extracted from saliva [121], the natives' genomic data from Pacific Northwest in~~
349 ~~British Columbia and South East Alaska with a well documented history of contacts with European~~
350 ~~and Asian traders [122], dental genetic disorders [123,124], the effect of the risk factor on~~
351 ~~familiarity with Alzheimer's disease in cognitively normally aging individuals [125], the relation~~
352 ~~between attention deficit/hyperactivity disorder and urinary nonylphenol levels [126], the exposure~~
353 ~~to organophosphate pesticide [127], and the sugar-sweetened beverage consumption [128]. More~~
354 ~~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 α -
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), α -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 α -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® 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® [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™ (Oasis Diagnostic
 419 Corporation); (B) Versi Sal™ (Oasis Diagnostic Corporation); (C) Quantisal® (Immunalysis Corporation); (D)
 420 Intercept® (Orasure Technologies Inc.); (E) Salivette® (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® 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 ± 0.16 mL/min at rest and increased up to 2.7 ± 0.52
 442 mL/min during the stimulation. The pH value was 6.7 ± 0.24 pH units at rest and increased up to
 443 7.35 ± 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[®] 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].~~

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

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

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479 ~~Fig. 8. Some of the devices tested by Holm-Hansen et al. in terms of sample (water or saliva) transfer capacity (A)~~
480 ~~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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483 In 2006, Rohleder found that salivary flow rate did not affect the salivary α -amylase (sAA) activity
484 when saliva was collected by passive drool and Salivette [100]. In 2010, Beltzer et al. studied the
485 dependence of sAA activity on collection time, sampling method (i.e. passive drooling, a
486 ~~micro~~micro-sponge, a cotton pledget, and a synthetic oral swab) and type of saliva (unstimulated
487 whole saliva and saliva sampled from specific salivary glands) [57]. The authors reported a linear
488 increase in the volume of saliva collected by passive drool, while this did not occur in the case of
489 use of absorbent devices. In contrast to Rohleder et al. [100], a decreased sAA activity was
490 observed when the sample was collected by passive drooling due to the influence of salivary flow
491 rate. In 2013, Arhakis et al. found a similar influence of the salivary flow rate on sAA activity
492 [101]. In 2018, Lomonaco et al. used a synthetic Salivette® swab to study the effect of the sampling
493 procedure in twenty-two nominally healthy volunteers for the determination of uric acid and lactate
494 in non-stimulated and stimulated whole saliva samples [83]. The analytes recovery was tested at
495 four different pH values (5, 6, 7, and 8) by analyzing saliva samples spiked with 50 $\mu\text{g/mL}$ of
496 lactate and 100 $\mu\text{g/mL}$ of uric acid. To sample unstimulated saliva, the subjects were asked for
497 placing the swab in the mouth between a gum and the cheek for 2 min. Stimulated whole saliva was
498 sampled at 50, 100 and 150 min^{-1} by moving the swab in the oral cavity for 1 min. Quantitative
499 recovery of uric acid and lactate was obtained independently of pH. For increasing flow rate, the
500 change in the concentration of lactate was not statistically relevant, whereas the concentration of
501 uric acid decreased from $70 \pm 20 \mu\text{g/mL}$ to $30 \pm 10 \mu\text{g/mL}$.

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3.2.3 Dried saliva spot (DSS)

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

Numako et al. used DSS for the determination of the D- and L-lactic acid in diabetic patients, pre-diabetic and nominally healthy persons [103]. The author pointed out that DSS has 1) high detection sensitivity to the target molecule because of low spotted volume; 2) high accuracy and precision; 3) 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 μ 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 μ 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"> • No effect of flow rate • More representative of basal unstimulated secretion 	<ul style="list-style-type: none"> • Inhomogeneity of the sample • Sampling duration • The subject has to be collaborative
Spitting	<ul style="list-style-type: none"> • Evaporation of saliva for long-time samplings minimized • Suitable with very low flow rates 	<ul style="list-style-type: none"> • Possible stimulatory effects • The subject has to be collaborative
Swab-based	<ul style="list-style-type: none"> • Low cost • Easy availability • Easy use • Particularly suitable for less- or non-collaborative subjects • Possibility to estimate the flow rate (weight vs sampling time) • Homogeneity of samples recovered by centrifugation 	<ul style="list-style-type: none"> • Possible retention of analytes by the swab • Possible stimulatory effects • Risk of swallowing
DSS	<ul style="list-style-type: none"> • Very small volume of sample required 	<ul style="list-style-type: none"> • Possible issues related to analyte stability

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

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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 α -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
17α-OH progesterone	Congenital adrenal hyperplasia (CAH) [172,178,179]	Morning: 20–55 ng/L	Direct spitting (for children) [180]
		Morning: 623.1 \pm 481.4 pg/mL	Three times a day with Salivette[®] or modified medical pacifiers (for newborns) [181]
		Midday: 497.0 \pm 323.3 pg/mL	
		Evening: 397.5 \pm 260.6 pg/mL	
		Follicular phase: 20.5 \pm 1.8 pg/mL	Salivette[®] (for post-menarche girls) [182]
Luteal phase: 45.8 \pm 4.5 pg/mL			
		51 \pm 24 (range 17–109) pg/mL	3 mL of saliva were collected in a glass tube by direct spitting in 15 min after an initial mouth rinse with water [183]
17β-estradiol	Menstrual cycle [184,185], breast cancer [186], and bone metabolism [187]	2.009 \pm 1.037 pg/mL	Spitting unstimulated whole saliva for 10 min [188]
		2.447 \pm 0.621 pg/mL	Stimulated whole saliva collected for 5 min by habitual chewing of 1 g gum [188]
Aldosterone	Primary aldosteronism (PA) [189,190], cardiovascular risk [191], hypogonadism [192], depression [193] and stress [194]	44–117 ng/L	Salivette[®] (for nominally healthy volunteers) [194]
		1–7 ng/dL	Parotid saliva was sampled by Lashley cup.
			Expectorated saliva was collected into a plastic cup for 5 min while sucking sour lemon [195]
		25–1085 pmol/L	Saliva was collected into small plastic tubes [196]
Androstenedione	Congenital adrenal hyperplasia	78 \pm 30 pg/ml in 3 mL	Unstimulated saliva sampled from women between 9 am and 2 pm in

	(CAH) [172,178,179], body composition in young girls [197], hyperandrogenism [198]		sterile plastic tubes [198]
Cortisol	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].	3.6–35.1 mmol/L	1 mL saliva was collected in 5 mL polystyrene bottle immediately after awakening [216]
		In the morning: 1–12 ng/mL, in the evening: 0.1–3 ng/mL	Salivette [®] 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 [®] 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 ± 34 pmol/L; Follicular phase: 22.1 ± 2.7 pmol/L	5 mL of stimulated saliva sampled by chewing a gum [223]
		Luteal phase: 436 ± 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 ± 154.15 pg/mL	Salivette[®] used four times in a single working day [220]
		0.190 ± 68 nmol/L	Two Salivette[®] 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 α -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 ± 0.07 μ g/dL) if compared to passive (0.09 ± 0.05
608 μ g/dL; $p = 0.001$) and eyespear (0.11 ± 0.06 μ 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 α -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 α -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 α -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 α -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 α -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[®] 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 α -OH
639 progesterone, androstenedione, cortisol, cortisone, and testosterone from commercial saliva
640 collection devices was also investigated by Gröschl et al. [240]. Salivette[®] swab (e.g. cotton,
641 polyester, and polyethylene), a Quantisal[®] saliva collection device, and the liquid based approach
642 Saliva-Collection-System[®] (SCS[®], 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[®] swabs and the
645 Quantisal[®] did not differ significantly from the reference sample, whereas lower steroids levels
646 were found in the cotton Salivette[®] and the SCS[®]. The volume recovery was excellent for the
647 polyester and polyethylene Salivette[®] and the Quantisal[®] (> 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[®] swabs and the Quantisal[®] saliva collection device are the most suitable collection devices
651 for steroids analysis. However, Quantisal[®] needs the sample to be processed immediately without
652 any storage. The SCS[®] is more complicated and unsuitable for immediate use by untrained people,
653 whereas the cotton Salivette[®] swabs seemed to alter the salivary steroids levels.

654 In 2012, Celec and Ostatníková found that Salivette[®] 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- α (TNF- α) 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- α 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]
IL-1β	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]
IL-2	UWS	Expectoration	7.3 \pm 3.0	30	[143]
			11.91 \pm 1.70	30	[144]
IL-6	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]
		Spitting	259.68 \pm 12.838	25	[149]
			16.6 \pm 1.88	50	[150]
		Not reported	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
IL-8	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]
		Not reported	319.49 (10.48 – 592.6)	100	[153]
		Spitting	738.5 \pm 98.5	50	[150]
			478.0 (230.5 – 1067.2)	27	[142]
		SWS	Spitting	392.1 \pm 440.4	20
IL-10	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]
		Not reported	0.1 \pm 0.04	44	[158]
TNF-α	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 β -2-microglobulin in whole saliva, and
692 PRH2 protein and α -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[®] 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[®] 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[®], Quantisal[®] and SCS[®] [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[®] devices provided very
706 poor recoveries (e.g. <10% for IL-8, leptin, insulin, and acylghrelin), whereas the recovery using
707 polyethylene Salivette[®] 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[®] provided acceptable recoveries for the panel of small salivary
710 peptides, but not for the amine melatonin (59 ± 6%). The Quantisal[®] device had recoveries > 85%
711 for the entire panel of proteins/peptides, whereas SCS[®] recovered more than 90% only for amylase,
712 amine melatonin and s-IgA. The authors assumed that analytical performance of SCS[®] 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[®] [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 ± 1.5 mL) followed by cotton Salivette[®] swab (1.8 ± 0.4 mL)
763 and passive drooling (1.0 ± 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)	Securitee	Swipe only (tongue or skin)	COD	[291]
			MDMA	[292]
			THC	[293]
			AMP, MAMP, COC, MOR, THC, THC-COOH, Benzodiazepines	[294]
Cozart [®] collector	Cozart Bioscience Ltd.	Absorbent foam pad with diluent	AMP, MAMP, COC, MOR, THC, THC-COOH, Benzodiazepines	[294]
			MTDN, OPI	[295]
			COC, OPI	[296,297]
			COD, OPI	[298]
Dräger DrugTest [®] (Fig. 9B)	Dräger	Absorbent foam pad with diluent	COC, BZE, ecgonine methyl ester, MOR, 6-AM, dihydrocodeine, COD, MTDN, AMP, MAMP, MDA, MDMA, MBDB, THC, THC-COOH, THCA	[299]
			AMP, MAMP, MDA, MDMA, COC, BZE, MOR, 6-AM, COD	[300]
			Cannabinoids	[293]
			Alprazolam, 7-aminoclonazepam, 7-aminoflunitrazepam, Bromazepam, Clobazam, Diazepam, Lorazepam, Lormetazepam, Midazolam, Nordiazepam, Oxazepam, Temazepam, Tetrazepam, Triazolam, Zaleplon, Zopiclone, Zolpidem	[301]
Interecept [®]	OraSure Technologies Inc.	Absorbent foam pad with diluent	AMP, MAMP, MDMA, Alprazolam, Clonazepam, Diazepam, Nordiazepam, Oxazepam, Ntrazepam, Flunitrazepam, Zopiclone, MOR, COD, MTDN, Carisoprodol, Meprobamate, THC	[302]
			THC	[303-305]
Interecept [®] DOA Oral Specimen Collection Device	OraSure Technologies Inc.	Absorbent foam pad with diluent	AMPs, THC, BZE, MOR, 6-AM, COD	[306]
OralLab	Ansys Technologies Inc.	Absorbent foam pad; the collector is squeezed to push oral fluid into test cartridge	AMP, MAMP, COC, MOR, THC, THC-COOH, Benzodiazepines	[294]
OraLine [®]	OraLine	Direct application to	THC	[305]

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

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~~.

787
788
789

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

790
791

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[®] devices (cotton swab, cotton swab
796 with citric acid, and synthetic swab). The recovery was also tested for the Salivette[®] 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[®] 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[®] synthetic swab for 2 min. Stimulation increased pH values from 6.6 ± 0.4
805 (range 5.7–7.2) to 7.5 ± 0.3 (range 6.9–8.1). The WAR and WAROHs recovery from
806 Salivette[®] 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.

816
817

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.

855
856

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860
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864 **References**

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