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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.

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1. Introduction

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

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

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

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

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

2. Saliva production, composition and properties

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

2.1 Production

Nominally healthy adults typically produce 500 – 1500 mL/day of serous and mucinous saliva with a basal flow rate of about 0.5 mL/min. Because of the secretions from both the major and minor salivary glands, whole saliva is a mixture of oral fluids rich in water (approximately 99%) and endogenous substances such as inorganic compounds (Table 1), organic compounds (volatile, non-protein and lipids), proteins, polypeptides, and hormones [25,30,33,36,37].
The composition of unstimulated saliva differs from stimulated one, which resembles plasma in composition [1,38]. Although there is a great individual variability, a normal salivary flow rates for unstimulated saliva is above 0.1 mL/min, whereas under stimulation, the flow rate may increases up to about 4 mL/min [39].
Saliva has a slight buffer capacity [1] due to the presence of three buffer systems: bicarbonate, phosphate and proteins. The normal pH range is between 6 and 7 for unstimulated saliva, whereas it can extend from 5.3 to 7.8 when the flow rate changes [33,40]. In stimulated saliva, the pH increases since the concentration of bicarbonate ions in saliva is higher, i.e. from 2.4 ± 1.5 mM to 15 ± 7 mM [41]. High salivary flow rates increase the concentrations of sodium and chloride and decrease the concentrations of potassium and phosphate [42], thus increasing the saliva tonicity. Therefore, the buffering action of saliva is more efficient during stimulated high flow rates, but is almost ineffective for unstimulated saliva. At rest and without exogenous or pharmacological stimulation, there is a small and continuous salivary flow called *basal unstimulated secretion*. This flow covers, moisturizes and lubricates the oral tissues [26].

2.2 Composition

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

- A parasympathetic stimulation leads to high flow of watery (less viscous) saliva with low levels of organic and inorganic components [43].
- A sympathetic stimulation produces mucoid (more viscous) saliva secretions [44].
- An α-adrenergic stimulation accounts for low volume of saliva with high concentration of proteins and low concentration of mucins. The viscosity of saliva is low [25].
- A β-adrenergic stimulation accounts for saliva with high content of protein and mucin, high viscosity and foamy appearance [1,25].

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

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

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

~~**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**~~
~~**Total contents**~~			~~0.47 ± 0.19 mg/mL~~	~~[54]~~
			~~0.9 ± 0.2 mg/mL~~	~~[55]~~
			~~4.3 – 710.0 mg/dL~~	~~[56]~~
			~~2.67 ± 0.54 mg/mL~~	~~[57]~~
~~**α-amylase**~~	~~Serous acinar cells~~	~~Starch digestion~~	~~3257 ± 1682 U/mL~~	~~[55]~~
			~~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]~~
~~**Cystatin group**~~	~~Submandibular > sublingual~~	~~Antimicrobial (cistein-proteinase inhibitor)~~	~~14.3 kDa form~~ ~~58 ± 25 μg/mL~~ ~~14.2 kDa form~~ ~~91 ± 46 μg/mL~~	~~[57]~~
~~**Defensins (DEF)**~~	~~Blood, plasma~~	~~Antimicrobial~~	~~DEF-1 0.8 ± 0.6 μg/mL~~ ~~DEF-α1-3 0.094 – 9.4 μg/mL~~	~~[59,61,62]~~
~~**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~~ ~~Basic: 165 ± 69 µg/mL~~	~~[57]~~
~~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~~ ~~36 ± 18 µg/mL~~	~~[69]~~ ~~[57]~~
~~Transferrin~~	~~Plasma~~	~~Free iron control~~	~~0.58 ± 0.20 mg/dL~~	~~[17]~~

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

2.3 Properties and functions

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

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

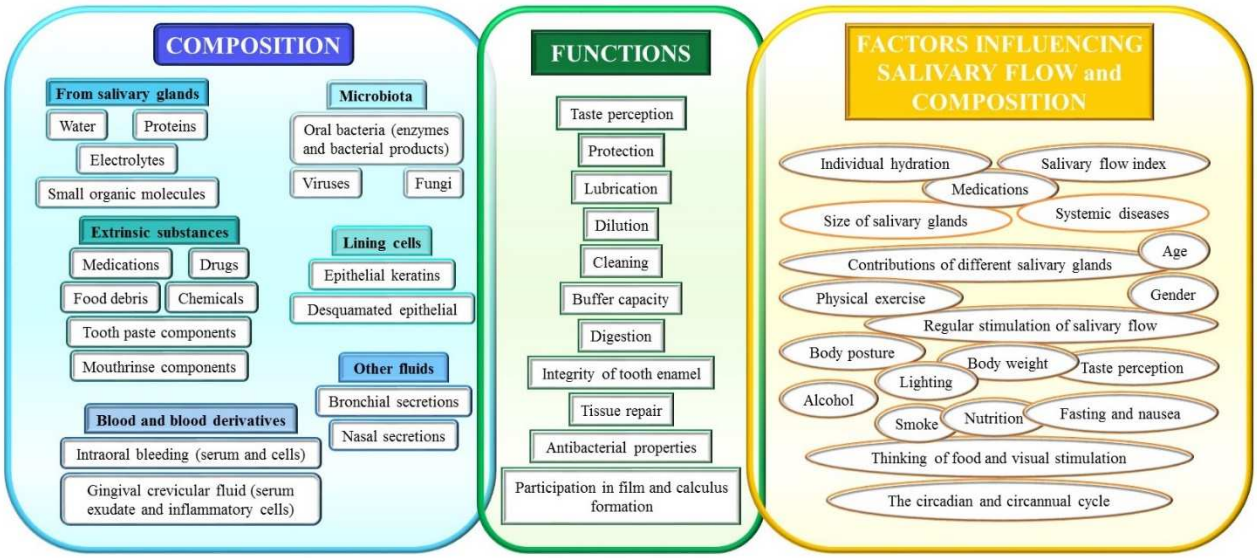

Fig. 1. The whole saliva composition, functions, and main factors affecting salivary flow and composition.

2.4 Transfer mechanisms of analytes from blood

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

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

3. Methods and devices for saliva sampling

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

3.1 Saliva sampling from specific salivary glands

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

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

3.1.1 Parotid saliva

The analysis of saliva secreted by the parotid glands dates back to the Sixties [58]. The parotid glands are the most voluminous salivary glands and secrete serous saliva. These glands are beneath and in front of both ears, and are traversed by the facial nerve, the retromandibular vein and the external carotid artery. The parotid duct opening, called *Stensen's duct*, is on the buccal vestibule, opposite to the first and second molars. The Stensen's duct drains saliva from the parotid gland into the mouth at the upper cheeks. This salivary secretion depends on the autonomic functioning of the glossopharyngeal nerve. Since the parotid glands are symmetrical in the oral cavity, their secretions are equally distributed into the mouth; however, the amount of saliva depend on gland size. Since the unstimulated parotid salivary flow is very low (< 0.2 mL/min) or even absent, saliva is usually stimulated applying few drops of citric acid (2 – 4 % w/v) [59].
Parotid saliva can be collected by intraductal cannulation, which is performed inserting a polyethylene tube or a tapered sialographic cannula into each gland (Fig. 2A). However, this approach is slow and invasive and not suitable for routine clinical use [43]. Alternative non-invasive procedures use the *Lashley cup* (also known as *Carlson-Crittenden collector*, introduced in 1910) [58]. This device avoids the leakage and the frequent sticking out of the cannula from the duct. The Lashley cup consists of two concentric chambers communicating with the exterior by means of two metal cannulae (Fig. 2B). The inner chamber is a cup that surrounds the opening of *Stenson's duct* whereas the air is evacuated from the outer chamber by a suction pump. A needle connected to the inner cup provides an exit for the free-flowing parotid saliva. Anyway, the homogeneity of the sample is not guaranteed and sample pre-treatment (i.e. filtration) would be necessary before analysis.

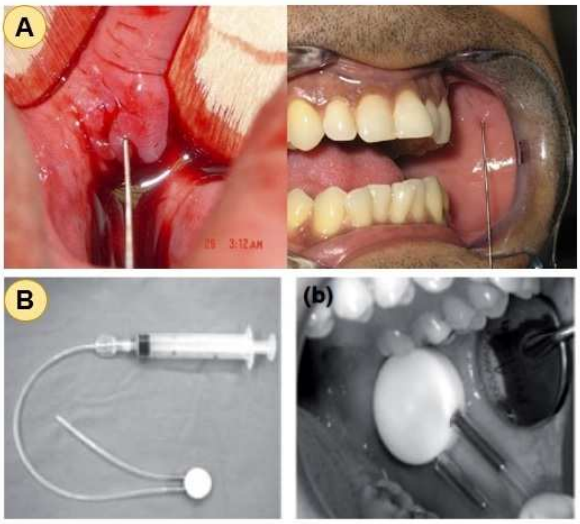

Fig. 2. **(A)** Example of parotid duct cannulation using a 0.025 inch Spring Wire. (Figure reprinted from [60]); **(B)** A Lashley cup (also named Carlson–Crittenden collector) on the left, and the device placement to both parotid glands' orifices on the right (Figure reprinted from [61]).

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

3.1.2 Submandibular and sublingual saliva

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

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

3.1.3 Collection of saliva from minor glands

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

Eliasson et al. used the Periotron®, which is a flowmeter introduced for the first time in 1979 by Garnick et al. [75] to quantify sub-microliter volumes of fluid on a paper-strip filter. The Periotron® 6000 model 2 (Proflow™) was used to measure the unstimulated flow of saliva from minor glands on 127 subjects. After drying the mucosa with a cotton pad, saliva was sampled using pre-cut (10 × 15 mm) pieces of standard filter paper (Munktell) and applying light finger pressure to ensure mucosal contact. In 2015, after carefully drying of the mucosa with a gauze, Wang et al. investigated the flow rates from minor salivary glands in healthy subject. ~~A gently placed~~ A paper-strip filters (Whattman No. 41, 1 × 2 cm^2 in size) was gently placed for 30 s onto the mucosa ~~(Fig. 4)~~ and, independently of sex, ~~found out that~~ mean flow rates resulted 2.10 ± 0.66 μL/(min·cm^2) from lower labial glands, 2.14 ± 0.62 μL/(min·cm^2) from upper labial glands, 2.88 ± 0.72 μL/(min·cm^2) from buccal glands, and 2.15 ± 0.51 μL/(min·cm^2) from palatal glands [74].

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

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	• Invasive • Possible Salivary gland injuries • Complex procedure • Time consuming • Required skilled personnel
	Lashley cup	• Selective sampling • Non-invasive procedures	• Complex procedure • Time consuming

			• Required skilled personnel
Submandibular and sublingual saliva	Intraoral duct cannulation	• Selective sampling	• Invasive • Possible Salivary gland injuries • Complex procedure • Time consuming • Required skilled personnel
	Suction	• Selective sampling	• Inhomogeneity of the sample
	Using custom-made devices	• Selective sampling	• Personalized form may be needed
Saliva from minor glands	Application of pieces of standard filter paper	• Non-invasive sampling	• Not so selective sampling • No large volume collected • Possible interaction with collecting paper

3.2 Whole saliva sampling

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

3.2.1 Unstimulated whole saliva sampling

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

3.2.1.1 Passive drooling and draining method

Practiced since 1934, the passive drooling of unstimulated whole saliva is often referred as the gold standard for biological assays because the effect of flow rate on the saliva composition can be ruled out. Passive drooling is usually performed by asking the subject to "deburr" (let the saliva drop) into plastic tubes (e.g. polypropylene tubes to avoid sample retention or contamination).
In 2007, Granger et al. highlighted the advantages of passive drooling such as large sample volume, and small influence of materials and substances used to sample or stimulate the salivary flow [77]. However, passive drooling is not suitable for subjects who are not capable to collaborate properly (e.g. very young children, sleepers, and frail elderly). For these subjects, the sampling of saliva by absorption is preferable [78].
Salimetrics proposes several devices for passive drooling such as the Saliva Collection Aid (SCA, polypropylene) where saliva is pooled in the mouth and driven into the vial with the head tilted forward ~~(Fig. 5A)~~. Oasis Diagnostic® proposes several devices for saliva collection, e.g. the UltraSal-2™ kit that samples up to 24 mL of saliva ~~(Fig. 5B)~~. This device is characterized by two 12 mL tubes connected to one buccal to split saliva into two aliquots. Although the supplier suggests to rotate or tilt the device, the homogeneity of the aliquots is difficult to achieve.
Proflow Sialometer™ (Proflow Incorporated) allows saliva to be sampled by drooling or draining. Saliva drips off the lower lip into a funnel attached to a graduated collection vessel. Even then, sample homogeneity is not guaranteed.

~~**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®.~~

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.

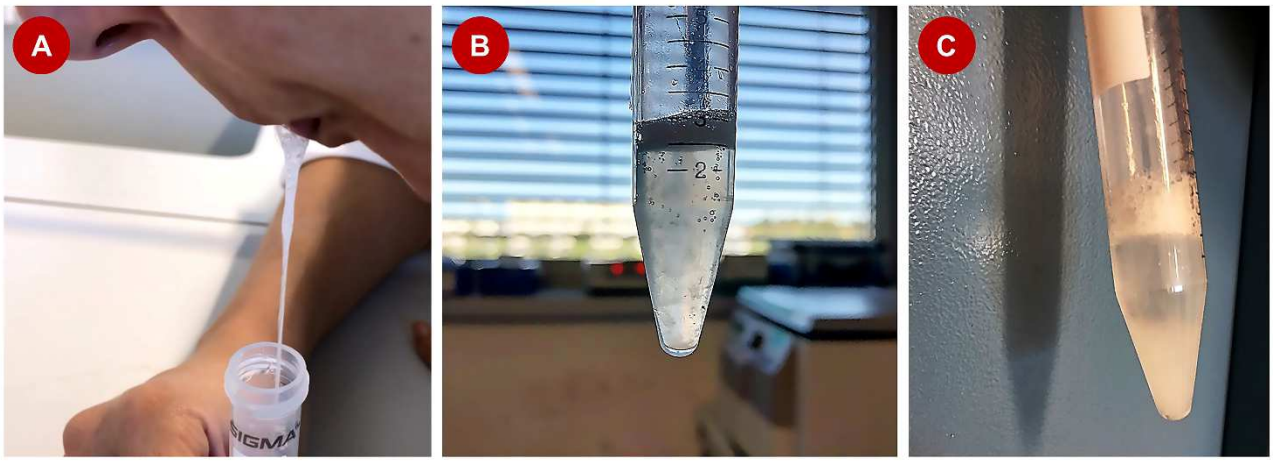

Fig. 3. **(A)** Example of the spitting method into a polypropylene centrifuge tube; **(B)** and **(C)** Examples of untreated saliva samples after spitting. The separation of emulsion (foam), mucin aggregates and aqueous phase is clearly visible.

~~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~~

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

3.2.1.3 Swab-based sampling

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

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

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

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

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

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

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

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

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

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

3.2.2 Stimulated whole saliva collection

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

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

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

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

~~**Table 4.** The eight commercially available devices compared by Holm-Hansen et al. to test their absorption and release 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.~~

~~**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.~~

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

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 µL 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 µL 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.
522
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.
539
540
541 **Table 3.** Summarizing table of pros and cons of approaches for whole saliva sampling.

Sampling method	Pros	Cons
Passive drooling and draining	• No effect of flow rate • More representative of basal unstimulated secretion	• Inhomogeneity of the sample • Sampling duration • The subject has to be collaborative
Spitting	• Evaporation of saliva for long-time samplings minimized • Suitable with very low flow rates	• Possible stimulatory effects • The subject has to be collaborative
Swab-based	• 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	• Possible retention of analytes by the swab • Possible stimulatory effects • Risk of swallowing
DSS	• Very small volume of sample required	• Possible issues related to analyte stability

		• Possible interactions of analyte with the absorbing material
Microextraction sampling	• 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	• Greater volume of sample needed • Not yet tested on clinical studies

4. Salivary biomarkers for clinical applications

4.1 Salivary steroids

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.

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.

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 (17α-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].

584 ~~**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 ± 481.4 pg/mL~~ ~~Midday: 497.0 ± 323.3 pg/mL~~ ~~Evening: 397.5 ± 260.6 pg/mL~~	~~Three times a day with Salivette® or modified medical pacifiers (for newborns) [181]~~
		~~Follicular phase: 20.5 ± 1.8 pg/mL~~ ~~Luteal phase: 45.8 ± 4.5 pg/mL~~	~~Salivette® (for post-menarche girls) [182]~~
		~~51 ± 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 ± 1.037 pg/mL~~	~~Spitting unstimulated whole saliva for 10 min [188]~~
		~~2.447 ± 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 ± 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]~~
~~Dehydroepiandosterone 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~~ ~~Luteal phase 20.6 ± 2.4 pmol/L~~	~~Whole stimulated saliva was collected in a vial from healthy women by chewing a sugarless gum [221]~~
		~~Follicular phase: 5 – 18 pg/mL~~ ~~Luteal phase: 8 – 35 pg/mL~~	~~Spitting in a plastic cup [222]~~
		~~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 17α-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 × 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 µg/dL) if compared to passive (0.09 ± 0.05
608 µg/dL; $p = 0.001$) and eyespear (0.11 ± 0.06 µg/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 17α-OH progesterone, androstenedione, cortisol, cortisone,
614 and testosterone [126]. The best sample recovery was achieved using the polyester Salivette® swab
615 (91.8% for 17α-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 ±
617 1%). The paper strips had slightly lower recoveries for all the analytes (72.0% for 17α-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 ± 2%. The cotton Salivette® showed low
620 recoveries for all the analytes (60.9% for 17α-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 ± 8%). Although a volume recovery of 97 ± 1%, the foam-tips had the worst
623 performance (76.2% for cortisol, only 41.8% for cortisone, 31.1% for 17α-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.

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

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

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

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

4.2 Peptides and proteins

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

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 ± 89.6	20	[141]
		Not reported	71.5 (28.8 – 158.9)	27	[142]
	SWS	Spitting	24.7 ± 49.2	20	[141]
IL-2	UWS	Expectoration	7.3 ± 3.0	30	[143]
			11.91 ± 1.70	30	[144]
IL-6	UWS	Drooling	4.61 ± 4.42	34	[145]
			1.80 ± 4.25	45	[146]
		Expectoration	5.2 ± 2.8	30	[143]
			2.5 ± 1.3	9	[147]
			10 – 25	20	[148]
			259.68 ± 12.838	25	[149]
			16.6 ± 1.88	50	[150]
		Spitting	27.6 ± 26.3	20	[141]
		Not reported	3.7 (2.6 – 5.9)	27	[142]
	SWS	Spitting	10.0 ± 8.6	20	[141]
IL-8	UWS	Drooling	250	32	[151]
			250.0	20	[140]
			300 – 785	20	[148]
		Spitting	755.3 ± 700.4	20	[141]
		Expectoration	700.7 ± 1031.5	9	[147]
			210.096 ± 142.302	25	[152]
			319.49 (10.48 – 592.6)	100	[153]
			738.5 ± 98.5	50	[150]
		Not reported	478.0 (230.5 – 1067.2)	27	[142]
	SWS	Spitting	392.1 ± 440.4	20	[141]
IL-10	UWS	Passive drooling	4.86 (IQR = 7.50)	92	[154]
		Spitting	1.72 ± 1.33	28	[155]
		Expectoration	12.02 ± 7.23	41	[156]
		Not reported	2.5 (0.45 – 5.1)	40	[157]
			0.1 ± 0.04	44	[158]
TNF-α	UWS	Drooling	4.06 ± 7.46	34	[145]

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

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

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

In 2006, Michishige et al. compared three methods for the determination of proteins in saliva: suction using an aspiration set, spitting into a sterile plastic dish, and cotton Salivette® swab placed under the tongue [163]. The saliva samples were obtained from 10 non-smoker female volunteers (20 – 22 years) and the analyzed proteins were S-IgA, kallikrein activity, trypsin-like activity, and human airway trypsin-like protease (HAT). The content of total protein, S-IgA, trypsin-like activity and HAT was higher in the samples collected by Salivette® than by suction and spitting, which had similar values. However, the high standard deviations suggest a deeper analysis of these results.

Gröschl et al. investigated the sample recovery from Salivette®, Quantisal® and SCS® [240]. The authors focused on the hormones associated with the proliferation of the oral mucosa, such as leptin and ghrelin (acylated and des-acylated), the inflammatory and tumor markers IL-8 and EGF, s-IgA, amylase, insulin and melatonin. Gröschl et al. found that cotton Salivette® devices provided very poor recoveries (e.g. <10% for IL-8, leptin, insulin, and acylghrelin), whereas the recovery using polyethylene Salivette® was in good accordance with the reference samples for insulin, EGF, and amylase. Melatonin, IL-8 and acyl-ghrelin showed a recovery of 59 ± 6%, 10 and 23%, respectively. The polyester Salivette® provided acceptable recoveries for the panel of small salivary peptides, but not for the amine melatonin (59 ± 6%). The Quantisal® device had recoveries > 85% for the entire panel of proteins/peptides, whereas SCS® recovered more than 90% only for amylase, amine melatonin and s-IgA. The authors assumed that analytical performance of SCS® was influenced by the presence of dye and/or the citric acid.

In 2012, Williamson et al. measured the concentration of twenty-seven cytokines sampled from venous blood and saliva using a filter paper (1 min) and passive drooling (30 s). No or low correlation was found between the concentrations of the cytokines in blood and saliva, whereas the saliva samples from passive drooling and filter paper were correlated for sixteen cytokines [164].

Mohamed et al. studied the effect of the methods for saliva sampling and processing of C-reactive protein (CRP), immunoglobulin E (IgE), and myoglobin [165]. They compared the analyte levels and the salivary total protein concentrations in three different samples: unstimulated saliva collected by passive drooling and saliva obtained by mechanical and acid stimulation. The total protein 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.

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

4.3 Drug monitoring

Probably, drugs monitoring is currently the main application of saliva analysis [11,13,169−171]. Nevertheless, there are some limitations because of the variability of the saliva/plasma concentration ratio [27,172]. Ideally, the best analytical condition is when the saliva/plasma ratio is approximately constant and equal to about 1.0 [289]. However, salivary flow rate and pH can affect the analysis. Salivary concentrations of drugs depend on pH: the salivary concentration of weakly acidic and basic drugs correlates with plasma concentrations at normal plasma pH for non-ionized drugs but not for ionized drugs. Commercial adulterants and other similar products such as mouthwashes had no substantial effect on drugs monitoring after 30 min [169]. Regarding the collection techniques, Drummer has published an interesting review in 2008 [80] reviewing an updated version of the saliva collection devices. ~~Table 7 shows an updated version of the saliva collection devices reported by Drummer [117].~~ Table S3 reports these sampling devices together 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)~~	~~Securetec~~	~~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, cegonine methyl ester, MOR, 6-AM, dihydrocodeine, COD, MTDN, AMP, MAMP, MDA, MDMA, MBDB, THC, THC-COOH, THCA~~	~~[299]~~
~~Intercept®~~	~~OraSure Technologies Inc.~~	~~Absorbent foam pad with diluent~~	~~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]~~
			~~AMP, MAMP, MDMA, Alprazolam, Clonazepam, Diazepam, Nordiazepam, Oxazepam, Ntrazepam, Flunitrazepam, Zopiclone, MOR, COD, MTDN, Carisoprodol, Meprobamate, THC~~	~~[302]~~
~~Intercept® DOA Oral Specimen Collection Device~~	~~OraSure Technologies Inc.~~	~~Absorbent foam pad with diluent~~	~~THC~~	~~[303–305]~~
			~~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]~~
~~Orateet (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®~~	~~Immunalysis Corporation~~	~~Absorbent foam pad with diluent~~	~~THC~~	~~[310]~~
~~SalivaScreen®~~	~~Ulti-Med~~	~~Absorbent foam pad~~	~~AMP, MAMP, COC, MOR, THC, THC-COOH, Benzodiazepines~~	~~[294]~~
~~Salivette®~~	~~Sarstetd~~	~~Cotton wool swab~~	~~COD~~	~~[311]~~
			~~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]~~

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

~~**Fig. 9.** Examples of saliva smapling devices for drugs monitoring. (A) DrugWipe® (Securetec); (B) Dräger DrugTest® (Dräger); (C) Oratect (Branan Medical Corporation).~~

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

5. Conclusions

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

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

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

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

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

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

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

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

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