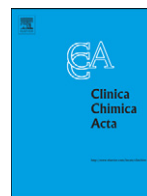




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Evaluation of saliva collection devices for the analysis of proteins

Eleni Topkas^a, Patricia Keith^a, Goce Dimeski^{b,c}, Justin Cooper-White^{a,d}, Chamindie Punyadeera^{a,d,*}

^a The Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Australia

^b Chemical Pathology, Princes Alexandra Hospital, Pathology Queensland, The University of Queensland, Australia

^c School of Medicine, Southside Clinical School, The University of Queensland, Australia

^d The School of Chemical Engineering and the University of Queensland, Australia

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ABSTRACT

Background: Human saliva mirrors the body's health and can be collected non-invasively, does not require specialized skills and is suitable for large population based screening programs. The aims were twofold: to evaluate the suitability of commercially available saliva collection devices for quantifying proteins present in saliva and to provide levels for C-reactive protein (CRP), myoglobin, and immunoglobulin E (IgE) in saliva of healthy individuals as a baseline for future studies.

Methods: Saliva was collected from healthy volunteers ($n = 17$, ages 18–33 years). The following collection methods were evaluated: drool; Salimetrics® Oral Swab (SOS); Salivette® Cotton and Synthetic (Sarstedt) and Greiner Bio-One Saliva Collection System (GBO SCS®). We used AlphaLISA® assays to measure CRP, IgE and myoglobin levels in human saliva.

Results: Significant ($p < 0.05$) differences in the salivary flow rates were observed based on the method of collection, i.e. salivary flow rates were significantly lower ($p < 0.05$) in unstimulated saliva (i.e. drool and SOS) when compared with mechanically stimulated methods ($p < 0.05$) (Salivette® Cotton and Synthetic) and acid stimulated method ($p < 0.05$) (SCS®). Saliva collected using SOS yielded significantly ($p < 0.05$) lower concentrations of myoglobin and CRP, whilst, saliva collected using the Salivette® Cotton and Synthetic swab yielded significantly ($p < 0.05$) lower myoglobin and IgE concentrations respectively.

Conclusions: The results demonstrated significantly relevant differences in analyte levels based on the collection method. Significant differences in the salivary flow rates were also observed depending on the saliva collection method. The data provide preliminary base line values for salivary CRP, myoglobin, and IgE levels in healthy participants and based on the collection method.

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1. Introduction

Saliva is an important biological fluid with many functions in the oral cavity including maintaining oral homeostasis [1]. Saliva contains a wide spectrum of biomolecules that are either synthesized *in situ* or transported from the blood capillaries into salivary acinar cells [2]. Not only can human saliva provide important information about the oral cavity, it also harbors a wealth of information that is derived from tissues/organs in the body. Such information can be used to assess and monitor health, well-being and disease status or alternatively be used to determine response to drug treatments [3–7].

A recent study by Bandhakavi et al. has identified 2340 proteins in human saliva and approximately 20% of these proteins are also found in plasma [8]. This highlights the importance and potential of saliva as

a biological fluid and warrants more comprehensive evaluation as a diagnostic medium. There has been increasing interest in saliva diagnostics due to its non-invasive nature and ease of collection [3–5,9–13]. Much of the literature on the use of saliva research focuses on developing technologies for the detection of biomolecules [5,11–15]. However, important consideration must also be given to the effects of different saliva collection and processing methods and their influence on the accuracy of results as well as reference interval or cut off limits [8–13,15,16].

Currently, saliva assays are routinely used to determine, disease such as HIV [17], illicit drug use [16,17], cortisol levels for diagnosing Cushing's syndrome [18] and to measure many other hormones [19–21]. Saliva has numerous advantages over blood or urine as a diagnostic fluid: a) the non-invasive nature of sample collection and simple, safe, painless, and cost-effective; b) unskilled people can collect saliva samples at multiple time points; c) the total protein concentration is approximately a quarter of what is present in plasma which makes it easier to investigate low abundant proteins [5,6,22]. Human saliva can be collected either under resting conditions or stimulated conditions, such as mechanical stimulation via chewing

* Corresponding author at: Tissue Engineering and Microfluidic Laboratory, the Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Old Cooper Road, St. Lucia, 4072, Queensland, Australia. Tel.: +61 7 3346 3891; fax: +61 7 3346 3973.

E-mail address: c.punyadeera@uq.edu.au (C. Punyadeera).

onto an absorbent pad or acid stimulation [16,23,24]. Various methods of collecting human whole saliva samples, such as draining, spitting, suction and swabbing have been extensively reviewed [13,23]. Currently, there are a number of commercially available saliva collection devices, namely Salivette® (Sarstedt, Germany), Salimetrics® Oral Swab (SOS) (USA), and Greiner Bio-One, Saliva Collection System (GBO SCS®) (Austria).

Biomarkers with clinical relevance [25] were selected and measured in the saliva collected from healthy participants in an attempt to provide baseline concentrations for these biomolecules in saliva. We selected CRP (115 kDa, inflammation marker) [25–27], IgE (160 kDa, allergy status biomarker) [28] and myoglobin (16.7 kDa muscle injury marker) [29].

The aims of our study were to evaluate the suitability of commercially available saliva collection devices (drool; SOS; Salivette® and SCS®) for protein detection using immunoassays and also to provide an indication of the levels of CRP, myoglobin, and IgE in the saliva of healthy individuals.

2. Materials and methods

2.1. Participants

This study was approved by the University of Queensland Medical Ethical Institutional Board and all participants were required to sign an informed consent. The participant population consisted of 17 healthy volunteers: 7 women, mean age 25 years) and 10 men, mean age 25 years. Exclusion criteria included existence of any comorbid and/or oral disease (e.g. periodontal disease and gingivitis), autoimmune, infectious, musculoskeletal, or malignant disease, and recent operation or trauma, identified with a questionnaire. Additionally, participants had to be symptom free of fever and/or cold and had maintained good oral hygiene.

Volunteers were asked to refrain from eating and drinking, 2 h prior to saliva collection. Five saliva collection devices were assessed including commercially available kits according to manufacturers protocols. For all volunteers, the saliva collection order was passive

methods first, which included drool and SOS techniques, followed by mechanically stimulated saliva collected using Salivette® Cotton and Synthetic swabs, and finally acid stimulated saliva was collected using SCS®. Volunteers rinsed their mouth with water in-between collections to minimize cross contamination of collected saliva between collections. Post saliva collection, we deliberately did not add proteases inhibitor cocktails to the collected saliva samples because commercially available protein cocktails contain sodium azide and DMSO and both of these substances interfere with the 5 AlphaLISA® assays (<http://perkinelmerreagents.onconfluence.com/display/ts/DMSO+and+bead+interference>).

2.2. Saliva collection devices

Passive drooling (no oral movements) (Fig. 1A) was used to collect whole saliva from the oral cavity [30]. The participants were asked to rinse their mouth with water before saliva collection, and then to tilt their heads down to pool saliva in the mouth for 2 min. The specimen was collected into a sterile container. The SOS collection device (Fig. 1B) was placed under the participants' tongue for 2 min to collect unstimulated saliva. To extract the saliva, the swab was centrifuged at 2095 rcf for 15 min at 22 °C as per manufacturer's protocol.

Salivette® saliva collection device consists of a polypropylene tube and contains absorbent pads made either from Cotton or Synthetic material (Fig. 1C). These devices will be referred to as "Cotton" and "Synthetic" in the remainder of the manuscript. The swab was placed inside the participants' mouth and they were asked to chew onto these pads for 45 s to stimulate saliva. The stimulated saliva was collected from the swab by centrifugation at 233 rcf for 2 min at 22 °C according to manufacturer's protocol. SCS® saliva collection method used a liquid based approach (Fig. 1D). The participants rinsed their mouths using the rinse solution (27 mM citrate buffer), and this was followed by rinse with the saliva stimulant solution (39 mM citrate buffer and tartrazine) for 2 min and the saliva was collected into a sterile specimen container.

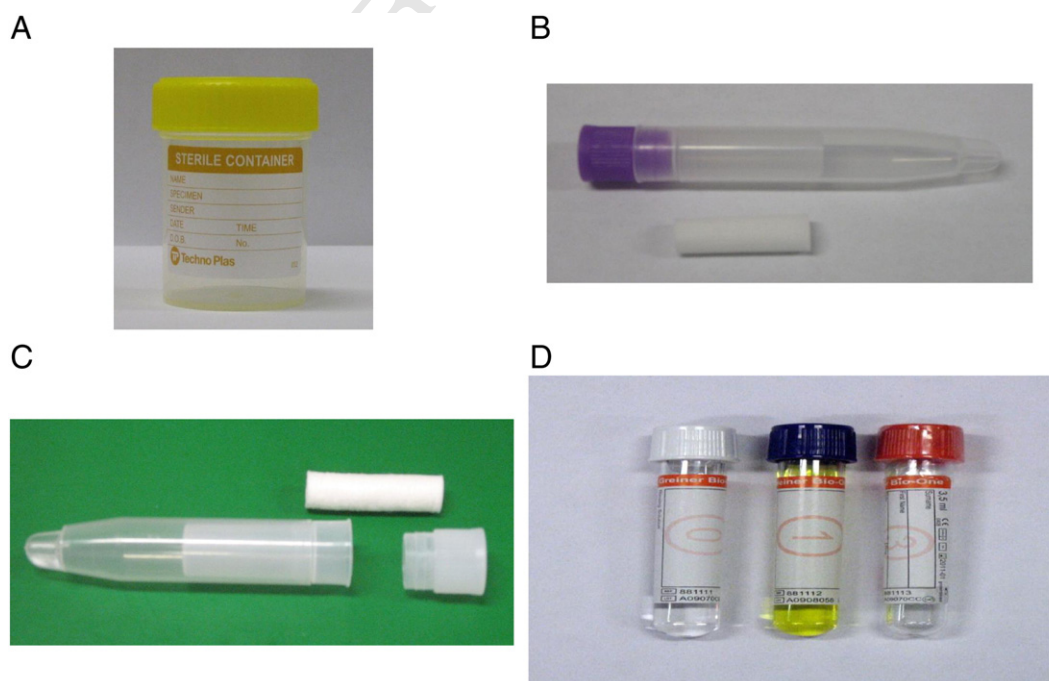


Fig. 1. Commercially available saliva collection devices used in our current study: (A) Drool collected in a sterile specimen container; (B) Salimetrics® Oral Swab (SOS); (C) Salivette® (Sarstedt) Cotton and Synthetic; (D) Greiner Bio-One, Saliva Collection System® (GBO SCS®).

2.3. Greiner Bio-One Saliva Quantification kit

The mixture of stimulant and saliva was quantified using the GBO Saliva Quantification kit. Saliva concentration standards were used to calculate the percentage of saliva present, equal to the OD 450 minus OD 520.

2.4. Protein concentrations in human saliva

The Bicinchoninic acid assay (BCA) kit (Thermo Scientific, IL, USA) was used to determine the total protein concentration in the saliva samples, measured at 562 nm using the SpectroMax (Molecular Devices, CA, USA).

2.5. AlphaLISA® immunoassays

AlphaLISA® kits (Perkin Elmer®, MA, USA) were used to determine CRP, IgE and myoglobin concentrations in human saliva. These protocols have previously been validated by the manufacturer for the optimal concentration of acceptor beads and biotinylated antibody concentrations. Twelve standard assay points were used to generate a standard curve. The samples were analysed in triplicate in 384 well ProxiPlates™ (Perkin Elmer®, MA, USA) as per manufacturer recommendations, except for the decrease in total reaction volumes from 50 µL to 10 µL. In brief, the assay consisted of sample/analyte (1 µL), biotinylated antibody (10 mM) and acceptor bead (40 µg/mL) mix and streptavidin donor beads (50 µg/mL). The total incubation time was 1.5 h at RT in the dark and the plates were read using an EnSpire™ plate reader (PerkinElmer, MA, USA).

2.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism software version 5.04 (GraphPad Software Inc., USA). Standard curves were generated using a 4-parameter logistic equation (sigmoidal dose–response curve with variable slope) and a 1/Y2 data weighting [31].

The measurements obtained for the study were evaluated for normal distribution using the D'Agostino and Pearson omnibus normality test. The Wilcoxon matched-pairs signed rank test was used to compare data generated using the five collection methods. Statistical significance between the collection methods were assessed as $p < 0.05$ and was calculated using the same program.

3. Results

3.1. Salivary flow rates in a healthy population

Unstimulated saliva collection methods namely, drool method and SOS, gave the lowest salivary flow rates 0.60 ± 0.39 and 0.47 ± 0.29 (mL/min) respectively. The mechanically stimulated salivary flow rates for the Cotton and Synthetic swabs resulted in significantly ($p < 0.05$) higher flow rates 1.10 ± 0.57 and 1.22 ± 0.46 (mL/min) when compared with the unstimulated saliva collection methods. Acid stimulated saliva collection method (SCS®) yielded the highest salivary flow rate 2.56 ± 0.95 (mL/min) ($p < 0.05$) when compared with both unstimulated and mechanically stimulated saliva collection methods ($p < 0.05$).

3.2. Salivary protein concentrations within a healthy population

The protein concentration in healthy volunteers ranged between 250 and 4811 µg/mL for all the five collection methods. There was no significant difference between the total protein concentration for the unstimulated collections (drool 1159 ± 654 , SOS 1042 ± 463 µg/mL) and the mechanically stimulated collections (Cotton swab 1039 ± 506 ,

Synthetic swab 995 ± 741 µg/mL). SCS® gave significantly ($p < 0.05$) lower total protein concentration 690 ± 1070 (µg/mL) when compared to all other saliva collection methods.

3.3. Salivary CRP, IgE and myoglobin levels measured using the four different collection methods

Concentration levels using the four collection devices for the three analytes is provided in Table 1, and for the concentration levels based on salivary collection method (passive drool and mechanically stimulated saliva) is provided in Table 2.

3.4. The reference interval of salivary CRP, IgE and myoglobin levels measured in the entire study group

We have provided preliminary estimates of reference intervals in healthy participants. The reference intervals for CRP, IgE and myoglobin were 1.7–234, 9.4–651 and 0.7–682 (pg/mL) respectively (Fig. 2).

The intra and inter assay CVs for the measured analytes in saliva are shown in Table 3.

4. Discussion

In the present study, we investigated methods and commercially available saliva collection devices for their suitability for the detection of proteins present in saliva in an emerging field of saliva diagnostics. We have determined preliminary reference intervals for the three analytes measured in saliva for different collection devices and different collection methods within a healthy population. Although, the IgE median values suggest there may be difference between saliva collection methods (i.e. passive vs drool), however, statistical analysis showed no difference.

The data showed that the salivary flow rate is directly associated with the method of saliva collection, i.e. the lowest salivary flow rate was observed during the collection of unstimulated saliva (drool and SOS). The salivary flow rates significantly increased ($p < 0.05$) upon mechanical stimulation (Salivette® Cotton and Synthetic) and during acid stimulations (SCS®). In contrast, the total salivary protein concentrations in samples collected using different collection methods did not vary significantly, except in the case of SCS® where we observed a significant reduction in the total salivary protein concentration in comparison with other collection methods. We hypothesize that the significant reduction in the total salivary protein concentration with SCS® collection device may be associated with an increase in salivary flow rates. Such relationship has also been reported by others [31].

Our data demonstrated significant differences in salivary flow rates, as well as significant variations in concentration of the proteins assayed, between collection devices. A number of factors such as age, gender, hydration status, diet and medication, as well as diurnal variation can influence the composition of saliva resulting in high biological variances in the proteins measured [32]. In this study, the

Table 1

Summary of concentration levels (median and the interquartile range (IQR) for the three analytes measured using four different collection methods. Significant differences ($*p < 0.05$) were observed for both CRP and myoglobin levels between drool and SOS collection devices. Also, significant differences ($**p < 0.01$) for myoglobin levels was observed between drool and Salivette® Cotton and $*p < 0.05$ was observed for IgE levels between drool and Salivette® Synthetic devices.

	Drool	SOS	Salivette® Cotton	Salivette® Synthetic	
CRP (pg/mL)	28 (9–51)	9 (5–30)*	19 (7–46)	14 (5–44)	t1.4
IgE (pg/mL)	72 (38–202)	72 (35–123)	43 (30–153)	37 (30–164)*	t1.5
Myoglobin (pg/mL)	98 (31–140)	23 (2–54)*	23 (12–52)**	59 (34–136)	t1.6

Table 2

Summary of concentration levels (median and IQR) for the three analytes measured for passive drool methods (includes the drool method and SOS method) and mechanically stimulated saliva (includes Salivette® Cotton and Salivette® Synthetic).

	Passive drool	Mechanically stimulated saliva
CRP (pg/mL)	13 (7–42)	16 (6–43)
IgE (pg/mL)	72 (35–151)	40 (32–153)
Myoglobin (pg/mL)	53 (21–108)	43 (23–110)

Table 3

Performance characteristics of the three assays.

Analyte	Quality control (pg/mL)	Measured mean concentration (pg/mL)	Coefficient of variation (%)	Recoveries (%)
CRP	1000	1153	13.8	115
	200	257	12.8	129
IgE	1000	1021	12.9	102
	200	200	14	100
Myoglobin	1000	1029	13	103
	200	159	13	79

influence of external factors was minimised by asking the volunteers to refrain from eating and drinking 2 h prior to saliva collection, to establish a common base line (Fig. 3).

Salivary secretion rates and composition have shown large inter-individual and intra-individual variations when whole saliva is collected and measured, as well as glandular secretions [33]. Dodds et al. found decreased salivary flow rates in older people and in women compared with men with an average unstimulated salivary flow rate ranging between 0.08 and 0.16 mL/min [34]. Our study demonstrated a higher unstimulated salivary flow rate of 0.47–

0.60 mL/min, which can partially be explained by the younger mean age of our study participants. Using Cotton and Synthetic swabs (stimulated collection methods) resulted in significantly increased salivary flow rates 1.1–1.2 mL/min ($p < 0.05$). Similarly, an increase in salivary flow rates was also observed when volunteers were chewing onto paraffin to stimulate saliva secretion over 5 min, with salivary flow rates between 1.9 and 2.1 mL/min [35]. Acid stimulated saliva yielded higher salivary flow rates; however this method was not suitable for AlphaLISA® immunoassays due to interferences from the yellow tartrazine liquid, so it could not be further evaluated as a suitable saliva collection device.

Previous work from our group has demonstrated that in eight healthy volunteers, collecting four saliva samples throughout the day showed no significant salivary CRP variations throughout the day [24]. One of the limitations in our present study is that we did not investigate diurnal variations of myoglobin and IgE levels in saliva.

The major component of unstimulated saliva is derived from the submandibular gland and is mainly comprised of proline rich proteins [6]. This gland is responsible for producing high concentration of mucins in saliva, which facilitates lubrication and aids in hydration. In contrast, during mechanical stimulation, saliva is predominantly derived from the parotid gland and consists mainly of water [17,21]. Mechanically stimulated saliva collection method (Synthetic only) yielded significantly lower IgE concentrations when compared with the drool method. One possible reason for such a reduction in IgE levels during mechanical stimulation could be due to the type of gland that the saliva is primarily derived from (parotid) or the molecule could be absorbed into the collection device itself. In contrast, CRP levels in saliva were significantly lower in the unstimulated SOS collection method as compared with the rest of the methods. Myoglobin concentration was significantly lower in both the unstimulated SOS and stimulated Cotton collection methods, suggesting possible absorption onto the cotton component of these devices.

Our data demonstrated that when selecting a saliva collection method, it is important to investigate the possibility of device influence on analyte concentrations before measuring the analytes in saliva. The data showed that it is very important to properly evaluate the collection method and collection devices and then standardise this process for meaningful determination of cut off limits or reference intervals and result interpretation. An exception or alternate method may be required for the elderly, disabled, dehydrated, or infants, to provide adequate flow rates and volumes.

The results demonstrated significantly relevant differences in analyte levels based on the collection method. Significant differences in the salivary flow rates were also observed depending on the saliva collection method. The data provide preliminary base line values for salivary CRP, myoglobin, and IgE levels in healthy participants and based on the collection method.

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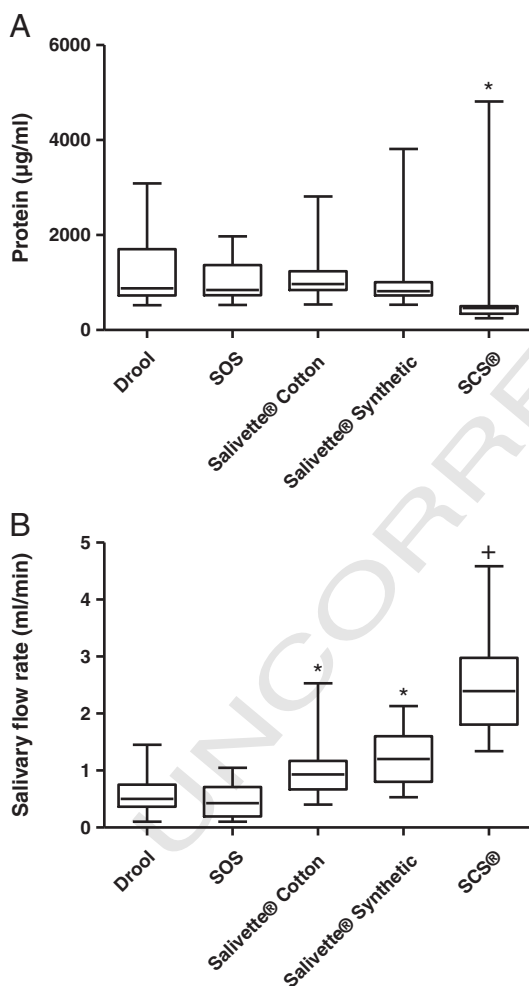


Fig. 2. (A) Total salivary protein concentrations (µg/mL) when using the five saliva collection devices and (B) salivary flow rate (mL/min). Significant ($p < 0.05$) differences in the salivary protein concentration was observed between the standard drool method and SCS® method. The Cotton and Synthetic swabs gave significantly ($p < 0.05$) higher flow rates when compared to unstimulated saliva collection methods. Significant differences ($p < 0.05$) in salivary flow rate was observed also between acid stimulated saliva collection method (SCS®) when compared to both unstimulated and mechanically stimulated saliva collection methods.

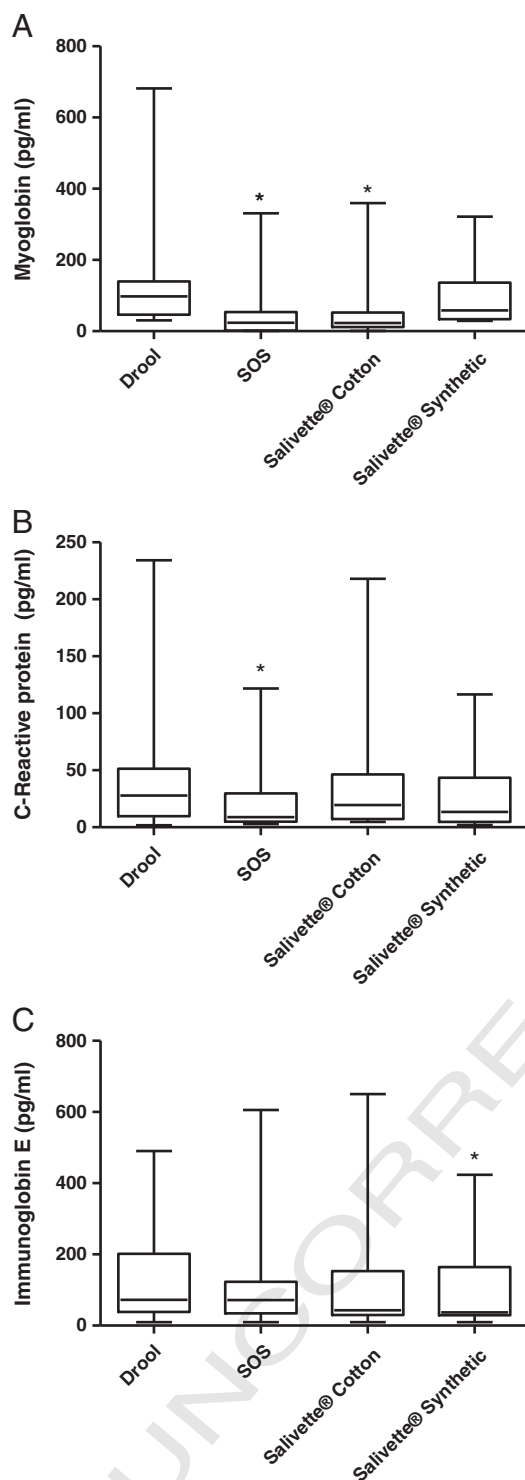


Fig. 3. Comparison of saliva collection techniques for the detection of (A) myoglobin (average LOD 1.4 pg/mL), (B) CRP (average LOD 4.1 pg/mL) and (C) IgE (average LOD 24.1 pg/mL) in the collected saliva samples using AlphaLISA® immunoassays. Significant differences (* $p < 0.05$) were observed for both CRP and myoglobin levels between drool and SOS collection devices. Also, significant differences (** $p < 0.01$) for myoglobin levels were observed between drool and Salivette® Cotton and * $p < 0.05$ was observed for IgE levels between drool and Salivette® Synthetic devices.

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