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Evaluation of saliva collection devices for the analysis of steroids, peptides and therapeutic drugs

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Abstract

The recovery of steroids, peptides and therapeutic drugs from commercial saliva collection devices was investigated. Saliva, spiked with defined concentrations of the analytes was applied to the Quantisal®, three different Salivettes®, and the Saliva-Collection-System® to investigate effects of volume, exposure time and temperature on the recovery. Additionally, saliva was collected from healthy subjects with the same devices.

It was found that glucocorticoids can be measured very well from samples obtained with the synthetic fiber Salivettes® and the Quantisal® (80–100%). For androgens, the Quantisal® and the Saliva-Collection-System® reached recoveries >80%. The Quantisal® and polyester Salivette® achieved best recoveries (>80%) for peptides. The results for the cotton Salivette® were extremely poor for melatonin, insulin or IL-8 (<20%).

The results from the spike-recovery experiments were confirmed by samples collected from healthy volunteers. For most therapeutic drugs the synthetic fiber Salivettes® achieved best recoveries of $100 \pm 10\%$. Longer exposure of saliva on the collection devices must be avoided for most of the analytes, due to their limited stability and increased adsorption.

In conclusion, no device is suitable for all of the salivary compounds. Strict pre-analytical precautions must be considered (e.g. immediate processing of the sample) to guarantee reliable analytical results.
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1. Introduction

Saliva contains various endogenous substances being interesting for both researchers and clinicians. The analysis of salivary hormones in particular is a proven and accepted alternative to plasma analysis. Steroids pass through cell membranes into the salivary glands by diffusion. Consequently, salivary concentrations reflect the non-protein-bound, physiologically active part of the blood concentration [1]. Amines and peptides, such as melatonin and insulin, enter the saliva by either passive diffusion or active transport. For these hormones, excellent correlation was determined between saliva and blood concentrations [2–4].

Of special interest are potential inflammatory or tumor markers, such as IL-8 [5] and EGF [6]. Other peptide hormones, such as leptin [7,8] and ghrelin [9,10], were proved to be expressed by the salivary glands and are involved in the proliferation of the oral mucosa [9,11].

With saliva being a major component in oral antibiotic defense it is not surprising that immunglobulins, such as secretory IgA [12], can be analyzed in saliva offering possibilities in epidemiological and vaccination studies. Finally, salivary enzyme activity, such as amylase [13] or lysozym [14], is used in the assessment of metabolic or infectious diseases.

In addition to these endogenous substances, various drugs can be measured in saliva as an excellent option for therapeutic drug monitoring (TDM) [15] or in the assessment of drugs of abuse [16]. In most cases a very strong correlation between salivary and plasma concentrations could be demonstrated. Anticonvulsants in particular, but also cytostatic drugs are promising candidates for salivary TDM [17].

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The non-invasive character of sample collection and the retention of the patient's privacy are especially convenient in children or out-patient sampling. Chewing absorbent materials may be preferable to the simpler but less esthetic spitting into tubes or passive drool [18], due to a better standardization of saliva flow and comparability of flow dependent components.

Several collection devices exist, with the Salivette® being commonly used in Europe. In North America, the Quantisal® is used for drug monitoring. In comparison to these adsorption-based systems, Greiner-BioOne introduced an alternative approach using rinsing and collection liquids (Saliva-Collection-System®).

In the present study, we investigated the utility of these devices for the above-mentioned panel of salivary analytes combining spike-recovery experiments, variability in the measurement of endogenous concentrations and an evaluation of practicability for the user.

2. Materials and methods

2.1. Collection devices

(a) Salivette® (Sarstedt, Fig. 1A) consists of a polypropylene tube with perforated inlay. It contains an absorbent wad produced in three different versions, cotton, polyester, and polyethylene. The wad is taken into the mouth, preferably in the cheek, to collect mixed saliva. Although Sarstedt currently does not provide the polyester version, we added this material from our stock as a reference since we have had considerable experience with it over the years.
(b) For the Quantisal® (Immunalysis, Fig. 1B), saliva is collected by placing a cellulose pad affixed to a polypropylene stem under the tongue or, for yielding saliva with the same glandular origin as with the Salivette, in the cheek. The volume absorbed by the pad is indicated by blue coloration in a window on the stem. The collector is transferred into a container with preservative buffer.
(c) The SCS® (Greiner-BioOne, Fig. 1C) is a liquid based approach. Rinsing of the oral cavity with the saliva extraction solution gives a mixture of saliva and this extraction solution. The saliva extraction solution contains an internal standard, allowing determination of the volume of saliva spectrophotometrically. After rinsing, the sample is spat into a collection beaker. The collected saliva is filled under vacuum into the transfer tube that contains preservative chemicals.

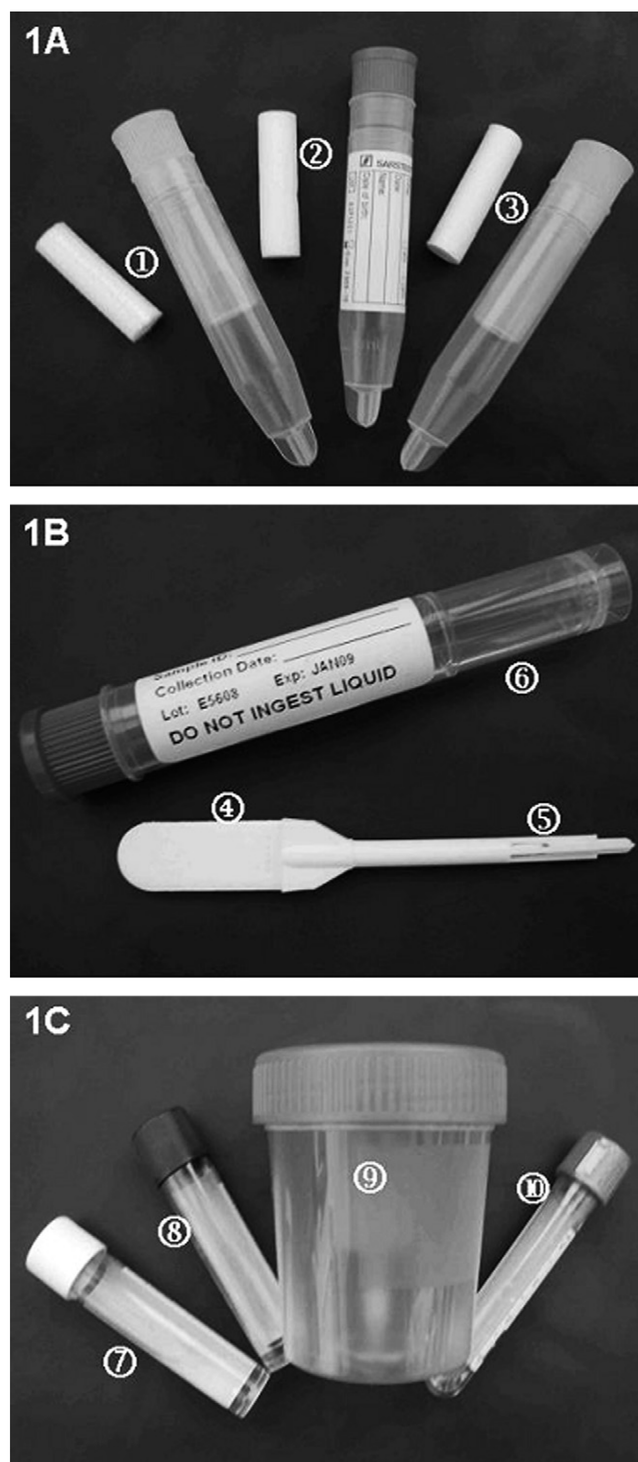

Fig. 1. Different collection devices for saliva evaluated in this study:A three versions of the Salivette® (Sarstedt) with inlays of either polyester ①, polyethylene ② or cotton ③ and the accompanying collection tubes; B: the Quantisal® (Immunalysis) designed for salivary drug testing consists of a cellulose pad ④ on a plastic stem with a flag window⑤ , signaling adequate volume by a color shift. A sample container with preservative buffer ⑥ is part of the system for stabilization during transport; C: the SCS® (Greiner Bio-One) the only liquid based collection system in our study, and consists of a mouth rinsing solution ,⑦ the yellow colored collection solution ⑧, a collection container for draining the saliva mix ⑨ and a storage unit coated with preservative powder ⑩, which is filled from ⑨ by vacuum.

2.2. Experimental design

A pool of stimulated saliva (pH 8.1) was collected by slight movement of the jaws, in order to yield a big volume of mixed saliva, which was not feasible by passive drool. The saliva was collected in polypropylene tubes (Nunc) and centrifuged (4000 g/20 min) to separate out mucins and cell debris. Donors were healthy males and females (age 30–45; 3♀, 3♂). Collection time was in the late afternoon for relatively low endogenous hormone concentrations [19]. The fractions were mixed and the

saliva pool was then divided into five fractions with the first fraction being spiked with defined concentrations of the parameters of interest. The total volume of the analyte-mix, prepared in phosphate buffered saline (PBS), was kept at 1% of the volume of the sample matrix, keeping the dilution of the saliva matrix by the added components to a minimum.

Then a linear dilution raw was built, spanning the entire range of the respective standard curves. The lower range of these standard curves overlaps the physiological/therapeutic concentrations in saliva as described in the literature.

We added the steroids cortisol, cortisone, 17α-hydroxyprogesterone, testosterone, androstendione (Sigma–Aldrich), the peptides leptin (Sigma–Aldrich), ghrelin (acylated and des-acylated; Bachem), IL-8 and EGF (R&D Systems), and the amine melatonin (Sigma–Aldrich) as typical salivary hormones. All therapeutic drugs (busulfan, ethosuximid, lamotrigin, phenobarbital, phenytoin, carbamazepin, caffeine, and theophylline) were purchased from Sigma–Aldrich. The concentrations of the parameters are provided in Table 1.

2.2.1. Experiment 1: effect of sample volume on recovery

The experiment was run in duplicate using 0.7 ml as an average volume collected in patients in our hospital and sufficient to perform the entire spectrum of analyses, and using 2.0 ml as the maximum volume effectively absorbed by the solid-phase devices.

Two series with five pieces of each device were prepared by applying either 0.7 or 2.0 ml of each of the five dilutions onto the wads of the Salivettes® and the Quantisal®. The same volumes were mixed with the extraction liquid of the SCS®, giving a 1 + 2 dilution. Additionally, one reference sample per dilution step was stored for analysis without application to a collection device. This procedure yielded 30 samples per series.

After 5–10 min soaking/equilibration, the sample was obtained by centrifugation (4000 × *g* for 10 min) or by transfer to the transport tube (SCS®). The volume was quantified and transferred to low-bind polypropylene vials (Eppendorf).

2.2.2. Experiment 2: effect of exposure time and temperature on recovery

As saliva is often used for out-patient collection with subsequent mailing to a laboratory, an experiment was designed to investigate the influence of temperature and exposure to the collection devices on the recovery of the analytes. The experimental setting was performed as described above, now limited to 0.7 ml volume, with the saliva remaining in the collection devices for one and four days either at room temperature or under cooled conditions. At the end of this exposure, the saliva was centrifuged and frozen (−80 °C) until analysis, together with the immediately centrifuged samples from Exp. 1. Some of the parameters, such as ghrelin, are known to have an extremely low stability ($t_{1/2} < 20$ min). Others, such as the cytostatic drug busulfan are analyzed only from samples prepared and analyzed contemporaneously during therapeutic drug monitoring. A longer storage of samples before analyzing these parameters, therefore, cannot be intended. Consequently these parameters were excluded from this experimental setting. Total number of samples in this experiment was $n = 120$.

2.2.3. Experiment 3: comparison of native saliva concentrations collected with the different devices

In addition to the spike-recovery experiments, we collected saliva from healthy adults (4♀/8♂; age 20–45 years) using all five devices for each volunteer. In addition to each collection using one of the devices, a reference sample was obtained by draining the saliva from the mouth directly into a low-binding polypropylene tube. For this purpose, volunteers were asked to rinse the mouth with water, two minutes later stimulating salivation by slightly moving the jaws, yielding mixed glandular saliva. The devices were placed in the mouth (cheek) immediately after collecting the reference sample.

This procedure was performed at home in the early morning after awakening, in anticipation of high steroid [20], high ghrelin [10] and leptin [8], and measurable melatonin values [21], and also one hour after lunch to observe the rise in insulin and the decline in ghrelin after food ingestion [22]. As recommended for out-patient use, samples were frozen after collection in household freezers before transportation to the lab in a cool box. Since for ethical reasons only healthy volunteers were enrolled to the study, no pharmaceuticals were analyzed in these samples. However, in addition to the steroids and peptides assessed in the spike-recovery experiments, some larger salivary proteins, such as secretory IgA and amylase, were determined.

2.3. Analysis

We analyzed steroids, ghrelin, and busulfan by LC-MS/MS, as described in detail elsewhere [23–25]. For sample preparation before injection, 10 μl (per 100 μl sample) of the respective internal standard was added to each sample aliquot. Protein precipitation occurred by adding 25 μl acetonitrile and 25 μl sulfosalicylic acid (100 g/l) per 100 μl sample. After vigorous vortexing, the mixture was centrifuged (36,000 × *g*/4 °C/10 min). The clear supernatants were transferred to polypropylene microtiter plates (Greiner-BioOne) and placed in the autosampler. For analysis, an on-line extraction method with a column-switching technique, combined with analytical LC–MS/MS was used. An API 4000TM (Applied Biosystems) tandem mass spectrometer fitted with either APCI or ESI source was used for detection, operated in positive ionization mode and was directly coupled to the HPLC without split.

The high selectivity and specificity of LC–MS/MS excludes any cross-reacting signals. Lowest limits of detection were 0.06 μg/l for steroids, 0.03 μg/l for ghrelin, and 2 μg/l for busulfan, intra-assay variation was <10% for steroids, 8% for ghrelin, and 2–4% for busulfan.

The panel of anticonvulsants was determined using a commercial isocratic HPLC with UV–detection (204 nm) requiring only 20 μl sample volume (chromsystems). Intra-assay CVs were <3% and inter-assay CVs were <5%. LOQ of this method was 2.5 mg/l for ethosuximid and 0.5 mg/l for the other drugs.

Table 1
Recovery of hormones and drugs from saliva measured in % directly and after absorption of 2 ml spiked saliva by different collection devices, these being the Sarstedt Salivette® (cotton, polyethylene, and polyester version), the Quantisal® device, and the "SCS®" from Greiner Bio-One

Analyte	Added amounts	Cotton	Polyethylene	Polyester	Quantisal	SCS	Direct
Melatonin %	40 – 2.5 ng/l	31.4 ± 11.1	65.1 ± 3.6	59.2 ± 6.4	85.0 ± 3.7	91.8 ± 11.4	100 ± 3.4
IL-8 %	1000 – 62 ng/l	< 10	22.6 ± 3.1	90.8 ± 4.3	83.9 ± 3.3	105.2 ± 3.2	100 ± 0.2
Leptin %	16 - 1 μg/l	< 10	66.5 ± 4.3	91.5 ± 2.8	95.8 ± 3.1	141.2 ± 5.1	100 ± 3.0
EGF %	250 – 15 ng/l	49.7 ± 2.9	113.1 ± 5.1	88.7 ± 2.3	99.5 ± 1.4	42.1 ± 1.9	100 ± 1.3
Insulin %	160 – 10 mU/l	< 10	93.3 ± 7.7	110.3 ± 9.5	89.2 ± 3.0	93.7 ± 6.6	100 ± 1.9
Acyl-Ghrelin %	50 – 3.1 μg/l	< 10	< 10	84.4 ± 1.0	85.0 ± 4.5	17.0 ± 1.2	100 ± 2.3
Desacyl-Ghrelin %	50 – 3.1 μg/l	16.3 ± 1.2	57.3 ± 2.5	97.8 ± 0.6	103.4 ± 2.1	68.0 ± 4.5	100 ± 0.8
s-IgA	Ø	94.0 ± 34.5 **a**	105.2 ± 47.4 **a**	99.0 ± 10.0	96.9 ± 10.7	97.3 ± 18.6	100 ± 9.2
Amylase	Ø	98.0 ± 5.0	93.4 ± 9.8	114.0 ± 4.8	101.6 ± 6.0	91.3 ± 8.7	100 ± 4.4
Cortisol %	50 –3.1 μg/l	81.0 ± 1.9	98.2 ± 3.7	103.3 ± 5.8	85.0 ± 4.3	82.9 ± 4.8	100 ± 0.9
Cortison %	50 –3.1 μg/l	77.8 ± 3.0	93.6 ± 6.3	98.0 ± 9.7	84.4 ± 5.6	86.1 ± 7.0	100 ± 1.1
17OHP %	50 –3.1 μg/l	43.6 ± 1.4	77.0 ± 4.8	73.8 ± 6.1	91.0 ± 4.3	84.8 ± 6.0	100 ± 4.3
Testosteron %	50 –3.1 μg/l	38.5 ± 4.3	83.6 ± 4.4	77.7 ± 4.3	84.2 ± 5.4	80.7 ± 5.4	100 ± 3.4
Androstendion %	50 –3.1 μg/l	56.5 ± 2.5	79.0 ± 4.0	78.3 ± 4.8	85.4 ± 3.9	76.9 ± 5.7	100 ± 2.0
Busulfan %	100 – 6.2 mg/l	86.2 ± 5.5	105.1 ± 9.9	103.5 ± 8.9	106.4 ± 2.3	73.1 ± 11.5	100 ± 1.0
Ethosuximid %	50 –3.1 mg/l	94.9 ± 5.1	100.0 ± 5.9	100.5 ± 3.0	82.1 ± 6.3	**b**	100 ± 1.1
Lamotrigin %	20 – 1.2 mg/l	58.4 ± 1.4	96.7 ± 2.4	99.7 ± 1.6	77.8 ± 2.1	93.4 ± 1.4	100 ± 0.3
Phenobarbital %	100 – 6.2 mg/l	100.3 ± 4.3	100.5 ± 3.9	102.2 ± 2.4	87.0 ± 4.2	100.5 ± 2.6	100 ± 0.6
Phenytoin %	20 – 1.2 mg/l	79.3 ± 3.1	99.0 ± 4.4	91.8 ± 2.0	85.6 ± 3.4	99.3 ± 3.4	100 ± 0.6
Carbamazepin %	20 – 1.2 mg/l	72.1 ± 3.4	91.6 ± 3.6	97.0 ± 3.1	80.5 ± 3.5	96.6 ± 3.5	100 ± 1.1
Caffeine %	20 – 1.2 mg/l	82.6 ± 3.1	104.5 ± 3.8	94.6 ± 3.8	71.8 ± 8.5	84.1 ± 5.7	100 ± 2.2
Theophylline %	20 – 1.2 mg/l	73.9 ± 2.0	106.7 ± 5.3	104.4 ± 4.6	82.2 ± 6.0	75.2 ± 5.7	100 ± 1.1

Data are provided as mean S.D. Acceptable recovery was defined as 100 ± 15% of the concentrations obtained in the directly collected reference samples (direct). Best results are highlighted in dark grey, 2nd choice in medium grey and third alternative in light grey. If equivalent results were obtained with more than one device, same color was used for these devices. (a) Not recommended due to high variability. (b) False high background (see text); (Ø) recovery calculated in relation to endogenous content of reference samples.

Amylase activity in 100-fold diluted saliva (sensitivity 3 IU/l) was determined with the Cobas Integra 800 (Roche) using 4,6-Ethyliden-*p*-nitrophenyl-α,D-maltoheptaosid as substrate.

Leptin was determined by radioimmunoassay [26]; ELISAs were purchased for melatonin (Bühlmann); insulin (DSL/Beckman-Coulter) IL-8 and EGF (R&D Systems), and secretory IgA (Salimetrics). The immunoassays were preformed with standards prepared in a low-protein standard diluent (R&D Systems) keeping the total protein content of the standards equivalent to the samples. This is important to avoid shifts in the values as known for using high-protein (serum-like) standards [27–29]. The immunoassays had CVs of <5–8%. Sensitivities of the immunoassays were 20 ng/l for leptin, 0.5 mU/l for insulin, 1 ng/l for melatonin, 2 ng/l for EGF, and 4 ng/l for IL-8.

Recovery was calculated by linear regression in percent of amounts added, based on the analysis of the original spiked material and was defined to be acceptable based on a symmetric accuracy of 100 ± 15% [30]. Influence of time and temperature on the recovery was assessed by ANOVA. The differences between endogenous values obtained from the different devices and the native reference samples were calculated by ANOVA (Friedman statistic for repeated measurements) with Dunn's multiple comparison test. Differences were considered to be significant when $p < 0.05$.

3. Results

3.1. Convenience and practicality of the collection devices

Both the participants and the contributors rated the Salivettes® to be very practical and convenient. Although the new polyethylene wad seems relatively hard in the mouth compared to the soft cotton version, this device was considered best for out-patient collection, e.g. for sports medicine and drug monitoring, as the small containers can be opened easily and used by the patient in training conditions, and in the field.

The Quantisal® is also practical, although a little more elaborate. The plastic stem affixed to the collection tissue does not allow the use under sports conditions (risk of injury), but has the tremendous advantage signaling the quantity collected to be sufficient. The system provides a container with a defined volume of preservative buffer, which may not be an optimal solution for sample preservation as the dilution effects require back calculation of the results.

Finally, Greiner's SCS® uses a completely different method to the other devices. Our volunteers found the succession of mouth rinse liquid, colored collection liquid, and vacuum storage container quite challenging. Outpatient use may, therefore, be problematic. Moreover, the system requires a "saliva-quantification kit" with the saliva quantity being determined spectrophotometrically by means of defined saliva standards. In comparison to the Quantisal®, the use of a preservative-coated container was more practical, avoiding further dilution (in addition to the dilution caused by the collection liquid) of the sample by liquid preservatives.

3.2. Collection volume and appearance of saliva

The volume obtained with all of the devices was sufficient for analysis of the entire panel of analytes. The maximum amount soaked up was approximately 2 ml in the Salivettes® and the Quantisal® when the blue color showed up in the flag window.

The volume recovery was excellent for the polyester and polyethylene Salivette and the Quantisal® at >95% when 2 ml and >90% when 0.7 ml were applied. The cotton wad had less volume recovery at only <80% when 2 ml and at <70% when 0.7 ml were applied.

The saliva had different consistency depending on the collection method. Whereas the native material is still sticky even after centrifugation, the absorption materials of Salivette® and Quantisal® seem to clean the matrix of the sticky and slimy components, probably mucopolysaccharides. However, some of the devices bring other, possibly contaminating, substances into the sample matrix. In particular, the cotton roll derived saliva shows a slight but apparent turbidity, and the sink deposit (Fig. 1A) contains more particles than when the polyester Salivette® is used. The clearest saliva was obtained by using either the polyester Salivette® or the Quantisal®.

3.3. Recovery of spiked analytes

3.3.1. Experiment 1: recovery after immediate sample preparation and influence of sample volume

None of the devices was optimal for all of the analytes. Therefore, we decided to provide a ranking of the top three devices showing the best analytical performance by recovery of the added amounts. The mean S.D. recovery for each analyte and device is compiled in Table 1.

Harmon et al. [31] described lower sample volumes of 0.25 ml affecting the recovery of salivary steroids. With the volumes tested in this study (0.7 and 2 ml), we could not observe significant influences on the recovery of the different analytes.

(a) The Salivette: The commonly used cotton version did not produce good results except for amylase and some drugs, being acceptable for phenobarbital, ethosuximid and busulfan (Table 1). Cotton was very poor for peptides with recoveries of <10% for IL-8, leptin, insulin, and acyl-ghrelin. The cotton Salivette performed worst of all systems in our test for steroid analysis (Androgens only 38.5–56.5% recovery).

The polyethylene version was originally approved by the manufacturer for salivary cortisol. In fact, this version performs well for salivary glucocorticoids (>90%), however, weaker for androgens (>75%). For 17OHP, it yielded relatively low, albeit consistent results of 77.0 ± 4.8. This Salivette yielded excellent recoveries in therapeutic drugs (>90%).

Moreover, some peptides and proteins (insulin, EGF, and amylase) could be measured in good accordance to the results of the reference samples, whereas others, such as IL-8 or acyl-ghrelin were recovered only at 23 and <10%, respectively.

The polyester Salivette® achieved perfect results for glucocorticoids and drugs within the acceptance range, but performed rather poorly for androgens (<80%). The polyester Salivette® was the only Sarstedt product to yield acceptable recoveries for the panel of small salivary peptides, but not for the amine melatonin ($59 \pm 6\%$). The very unstable acylated form of the gut peptide ghrelin could also be detected reliably when the polyester Salivette was used and the saliva was obtained immediately by centrifugation ($84 \pm 1\%$).

(b) The Quantisal®, originally designed and approved for salivary drug testing, was not in the top three systems for anticonvulsants (<90%), but performed acceptable for busulfan ($106 \pm 2\%$). This system yielded acceptable recoveries for the entire panel of steroids (>85%) and proteins/peptides (>85%). In fact, this combination makes it suitable for most endocrinological applications (Table 1).

(c) The Saliva-Collection-System® showed a good recovery in the majority of anticonvulsants (>85%) assayed chromatographically. However, the yellow dye in the collection liquid caused a false high background for ethosuximid. Consequently, the SCS® and our HPLC method cannot correlate in this specific case. Surprisingly low was the recovery of busulfan when collected with this device ($73 \pm 11\%$), which may be caused by disturbing effects of the collection liquid.

The SCS® performed successfully for amylase and s-IgA (>90%), whereby IL-8 and insulin were the only small peptides to be reliably analyzed in samples prepared with this collection method. The SCS® also performed acceptable for the amine melatonin ($92 \pm 11\%$). The analytical performance of this device for the other peptide immunoassays may be limited due to disturbances caused by the dye or the citric acid. For steroids, the recovery was in most cases below the 85% minimum of acceptance (Table 1).

3.3.2. Experiment 2: effect of longer exposure on the collection devices

In some parameters longer exposure on the collection devices had significant effects on recovery. Of course, these effects can only be evaluated with regard to the stability of the analytes in the simultaneously collected reference samples. A further decrease in recovery on the devices may then indicate adsorption effects.

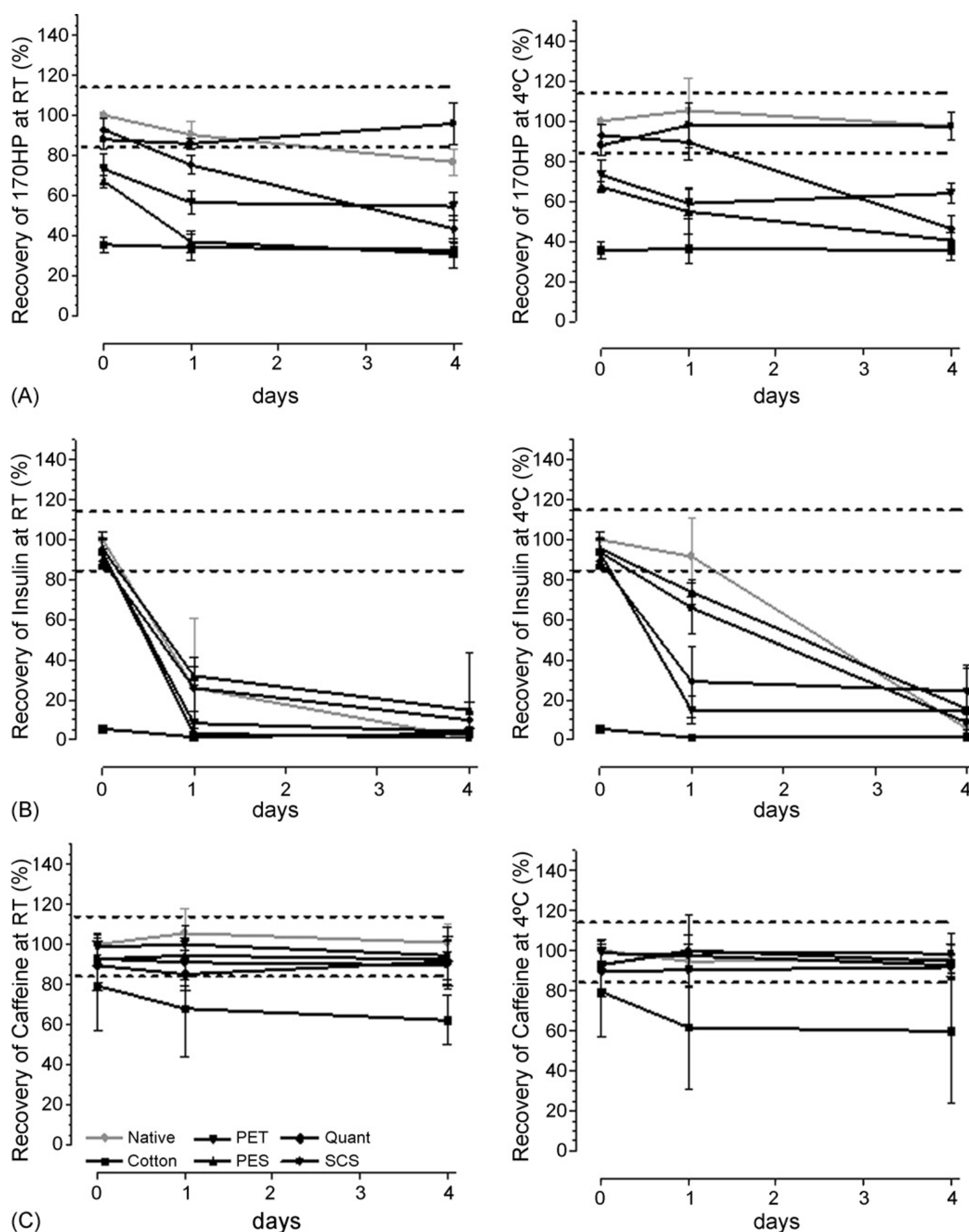

Fig. 2. Influence of storage conditions and exposure time on the recovery (%) of salivary analytes, examples shown for steroids (17OHP, A), peptides (insulin; B) and therapeutic drugs (caffeine C). Dotted lines represent the range of accepted recovery (100 ± 15%). The devices are as follows: (●) direct (native) saliva without collection device; (■:), cotton Salivette®; (▲:), polyester Salivette®; (▼:), polyethylene Salivette®; (⧫:), Quantisal®; (●), SCS®.

Salivary steroids remained stable in the reference samples for up to four days either cooled or at room temperature, with the exception of 17OHP being significantly lower (<85%; $p < 0.01$) when stored for four days at room temperature (Fig. 2A). The recovery after longer exposure on the Salivettes® and the Quantisal® decreased over time, depending on the temperature, in androgens and in 17OHP ($p < 0.001$), whereas the glucocorticoids were not affected by these exposure conditions. The recovery was significantly higher ($p < 0.05$) when the collection devices where kept under cooled conditions. Storage in the SCS® collection liquid did not yield any significant changes over four days.

Peptides and amines in saliva were less stable than steroids. Despite a good recovery of the spiked amounts in the immediately processed samples, none of the peptides investigated could be recovered within the acceptance range when stored either one or four days at room temperature. This decrease was highly significant in all peptides ($p < 0.001$). The results obtained from the collection devices were even worse (e.g., up to 70% lower in leptin) but as the stability was yet so poor in the reference samples, any storage at room temperature must clearly be avoided.

Storage at 4 °C yielded better results in the peptide recovery from the reference samples. After 1 day, leptin, EGF, IL-8, and insulin did not differ significantly from the immediately

processed samples. Melatonin was found slightly, but not significantly, higher (116 ± 17%). After four days, a significant reduction in the peptide/amine values (−70%, $p < 0.001$) could be observed with EGF declining by only 20%.

Leptin and IL-8 could be determined within the acceptance range for up to four days when the SCS® device was used; the polyester Salivette® was acceptable for at least one day at 4 °C. In the other devices, a significant decrease was found yet after 24 h exposure ($p < 0.001$). EGF could be measured from samples exposed to the polyester Salivette® for up to four days within the acceptance interval, whereas all other devices yielded a significantly reduced recovery of this cytokine. Insulin, which was stable for one day at 4 °C in the reference saliva, was found at significantly lower levels ($p < 0.001$) in the samples from any collection device (Fig. 2B). Finally, melatonin was equivalently stable when stored in the SCS® for up to one day at 4 °C when compared to the reference saliva, but none of the adsorbent devices yielded values within the acceptance range after one or four days exposure ($p < 0.001$).

Most of the therapeutic drugs were not significantly influenced by the storage conditions in the reference saliva. Lamotrigin, carbamazepine, and caffeine remained stable when stored up to four days either at room temperature or at 4 °C. Only theophylline, being stable for one day, decreased significantly ($p < 0.01$) till day four at both the temperatures. The exposure of the samples on the different devices led to significant decrease in lamotrigin using the SCS® and the polyester Salivette® after four days ($p < 0.01$), whereas the polyethylene Salivette® and the Quantisal® had no effect. Recovery of caffeine was not affected by any of the collection devices, except cotton, which yielded recoveries below 80% under all storage conditions (Fig. 2C). For carbamazepine, we found an effect of exposure time in the cotton ($p < 0.001$), the SCS® ($p < 0.001$), and the Quantisal® ($p < 0.01$).

3.4. Suitability of collection devices under in-vivo conditions

Significantly lower values of all steroids were found in the cotton Salivette® ($p < 0.001$) and the SCS® ($p < 0.05$) when compared to the native reference samples. The results obtained with the two synthetic fiber Salivettes® and the Quantisal® did not differ significantly from the reference samples.

Using the cotton Salivette®, salivary leptin, IL-8 or insulin were below the LOQ in most cases. For statistical analysis and graphical presentation the LOQ was then taken for these missing data (Fig. 3). In IL-8 and insulin (Fig. 3A) the results obtained for the synthetic fiber Salivettes®, Quantisal®, and SCS® did not differ significantly from the results measured from the native reference samples. Only leptin was found at lower concentrations when the polyethylene Salivette was used ($p < 0.05$, Fig. 3B).

Surprisingly, amylase activity gave higher results in the polyester Salivette ($p < 0.001$) in comparison to the reference samples and all other devices. In comparison, s-IgA was not affected by the method of sample saliva collection (Fig. 3C). Unfortunately no valid data could be obtained for ghrelin and melatonin under these in-vivo conditions, possibly because of the extremely short half-life of ghrelin [32] and the fact that in only a few midnight samples could melatonin be measured and all other samples were below the LOQ.

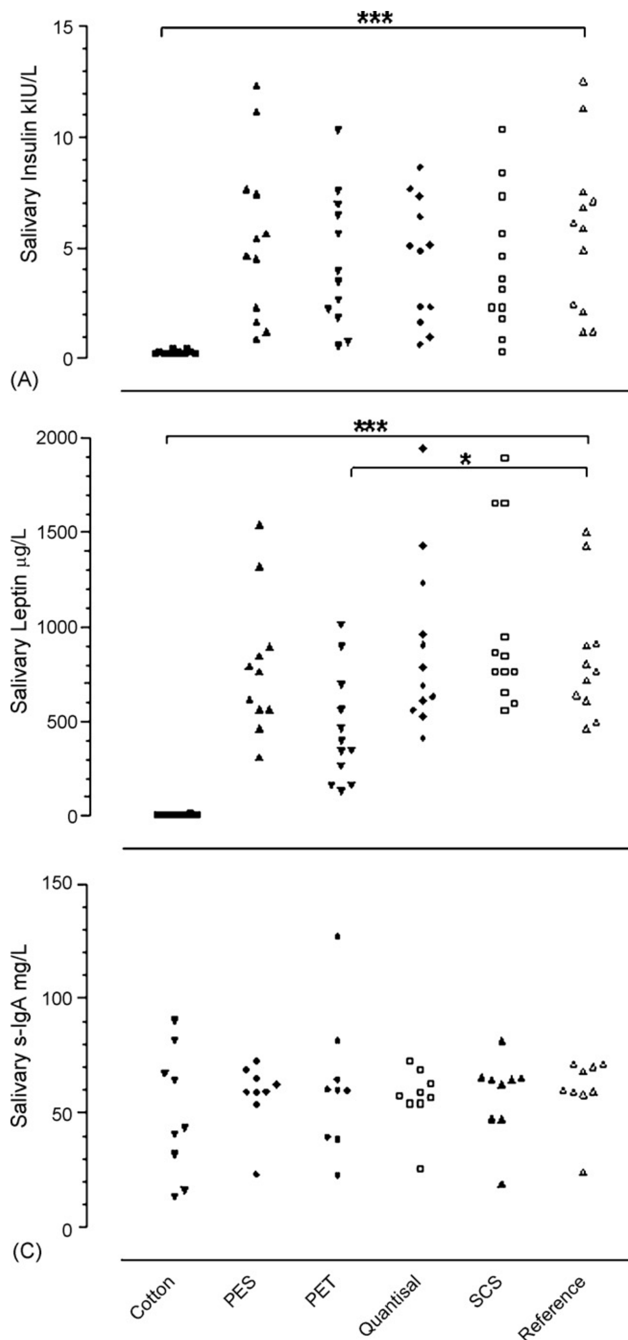

Fig. 3. Influence of the collection device on endogenous concentrations of salivary peptides and proteins, exemplary shown for insulin (kIU/l), the cytokine leptin (µg/l) and secretory IgA (mg/l). $^{***}p < 0.001$; $^{*}p < 0.05$. PES: polyester Salivette; PET: polyethylene Salivette.

4. Discussion

One main advantage of saliva is the non-invasive collection, most important in pediatrics, psychiatry or stress research. Another biochemical peculiarity is the lack of specific transport proteins in saliva (e.g. corticosteroid-binding-protein), allowing for the determination of free, physiological active concentra-

tions of steroids [33] or drugs [34] passively diffusing through the epithelia.

Consequently, the FDA keeps up with current developments [35], and supports the search for salivary diagnostic alternatives with extensive funding [36]. For example, most encouraging research is going on to develop point of care devices for the qualitative assessment of salivary biomarkers [37–39].

Meanwhile, analytical methods are available to meet specific demands in accuracy and sensitivity. However, there is still a backlog in the pre-analytical standardization of collection procedures for saliva, as was performed recently for the collection of oral cells for DNA analysis [40].

We wished to evaluate commercial collection devices, and to assess the reliability of these tools not only for steroids, as already shown before [41], but also for a panel of diagnostically important peptides and proteins and also several drugs measured in saliva for therapeutic drug monitoring, although the new polyethylene Salivette® is officially released only for the analysis of cortisol, as the Quantisal® is approved for salivary drug monitoring.

Each of the tested systems has its advantages and disadvantages regarding convenience of the collection procedure and analysis bias.

For therapeutic applications, it should be possible for patients to collect saliva at home under stress-free conditions without previous intensive training. This is, in fact, possible with the Salivette® or the Quantisal®, but Greiner's SCS® is more complicated and unsuitable for immediate use by untrained people. There are certain applications where the handling of the collection device may be as crucial as the analytical aspects, e.g. in pediatrics, psychiatry, forensic chemistry or sports medicine. However, what is obviously disadvantageous for patients may be irrelevant for participants of pharmaceutical or clinical studies who can undergo training with the specific devices in advance.

We consider the combination of sample collection and analyte preservation by stabilizers, as in the Quantisal® and the SCS® an important approach to avoid the problems of rapid degradation in some analytes during shipment [42–44]. However, in the current study, we found this preservation yet not sufficient to increase the stability of salivary peptides in particular. Consequently, these parameters still require a clinical environment for saliva collection in order to ensure rapid sample preparation and freezing.

With only few exceptions the cotton Salivette® did not yield acceptable recoveries. This finding was also confirmed in the samples obtained from the volunteers, where especially peptides could not be reliably measured from saliva collected with this device. So far, this was in accordance with previous publications analyzing the reliability of this device for steroids [45,46], peptides [47], and proteins [46]. The detrimental effect of cotton on analytical performance has been presumed to be due to cotton-derived substances distorting immunoassays by interfering with specific antibodies. We consider this not to be the only reason, since we used LC–MS/MS for steroid analysis and low recovery was still observed. However, the extremely bad performance with the peptides may be related to either extreme adsorption or disturbing cotton derived substances.

In contrast, the two synthetic fiber Salivettes® showed good recovery in the entire panel of therapeutic drugs and in glucocorticoids, which makes both devices suitable for most clinical routine applications. For these parameters acceptable results were still found when the samples remained on the synthetic fiber Salivettes® for up to four days. However, for other diagnostically relevant steroids, especially 17OHP used for screening and therapy monitoring in congenital adrenal hyperplasia [48], longer storage of the sample on these two Salivettes® cannot be recommended. Immediate sample processing by centrifugation and freezing is mandatory.

The Quantisal® was the best device when hormones were assessed and the sample was processed immediately, however, any storage of the sample on the device must be avoided. So here also an immediate centrifugation and freezing should be guaranteed to yield reliable results. We assume adsorption effects of the cellulose responsible for the decrease in steroid recovery, as these molecules appeared to be stable in the reference saliva.

Surprisingly, the Quantisal® was not as good as the synthetic Salivettes® for recovery of therapeutic drugs.

The SCS® was the only device allowing to measure steroids also after longer exposure equivalently to the reference samples. This seems to be less a stabilizing effect of the SCS® buffers than the lack of absorption, as observed in the solid-phase devices.

Especially for the use in hospitals, being subject of increasing cost pressure, the list prices per device may also be of interest. These were 0.4 € per piece of the Salivette®, 1.8 € per piece of the Quantisal® and 7.6 € per piece of the SCS® (manufacturer information).

In conclusion, there are still many improvements required to produce a collection device that combines ideal pre-analytic (recovery and stability) and utility for out-patient applications, where immediate sample processing is not guaranteed. For the immediate clinical use we can recommend both synthetic fiber Salivettes® for drugs and glucocorticoids, and the Quantisal® for peptides and for steroids.

Standard treatments as used for plasma or urine samples must also be applied for saliva: samples should be transported as fast as possible to the lab, cooling is strongly recommended and the addition of suitable preservatives would be most helpful. The use of pure saliva without using collection devices may still be suitable for specific research based investigations, but here too a standardization of collection (stimulated vs. non-stimulated flow) and treatment of the sample (preservatives/storage) must be kept in mind.

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