Reliability of the saliva self-sampling with and without supervision

Jana Jaklová Dytrtová[1,*], Radmila Dytrtová[2], Ishak Kovač[1], Michal Šteffl[1], Michal Jakl[3]

[1] Sport Sciences – Biomedical Department, Faculty of Physical Education and Sport, Charles University, Prague 6, Czech Republic

[2] Institute of Education and Communication, Czech University of Life Sciences Prague, Prague 5, Czech Republic

[3] Department of Agroenvironmental Chemistry and Plant Nutrition, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague – Suchdol, Czech Republic

* Corresponding author: dytrtova@ftvs.cuni.cz

ABSTRACT

One of the least invasive sampling methods suitable for self-sampling is saliva spitting. The aim of this study is to evaluate the suitability of saliva self-sampling for unsupervised testing. Two self-sampling strategies were compared on the basis of visual evaluation of samples, measurement of cortisol levels in samples and questionnaire survey. The saliva samples obtained by supervised self-sampling were found to be fully suitable for further analysis. In contrast, not all saliva samples obtained from unsupervised self-collection can be used: 13% non-compliance with the minimum required sample volume, 8% with some food/drink residues and 26% taken at the wrong day time. About 42% of the unsupervised probands made at least one significant error in the saliva self-collection procedure. These results indicate that the accuracy of the results based on the analysis of samples received from saliva self-sampling is limited. For clinical investigation, the presence of an inner standard (referring to the reliability of the sampling procedure) is required.

KEYWORDS

saliva sampling; cortisol responsibility; biosensors; hormones; immunoassays

DOI

10.14712/23366052.2023.1

INTRODUCTION

Based on many researches, saliva can be considered a valuable matrix for determining a number of physiological changes in the body associated with sports (training) activities and the study of physical and psychological stress in general. Saliva contains proteins including immunoglobulins, mucins and enzymes, hormones, biomarkers as well as pathogens (Roblegg et al., 2019). The saliva analysis can provide immunological, inflammatory, endocrine as well as metabolic information, it is also a valuable alternative for the determination of drugs and other illegal substances in elite sports (Gröschl, 2017; Thieme, 2012). Very interesting and beneficial is the potential in determining the amount of cortisol, testosterone and microRNA.

The fundamental question is how to determine the basic (daily) level of these biomarkers. It is suggested to use a morning saliva collection. However not all biomarkers have an eligible level in the morning because of its circadian variance (see below, Fig. 1). In general, the resulting inaccuracy can be caused by self-sampling performed after waking up, i.e. usually without (professional) supervision. It is generally believed that the saliva sampling can be performed by individuals without special training and without the need for special equipment or facilities (Bellagambi et al., 2020). If the sampling step is provided by self-sampling of individuals, it can significantly reduce the cost of the whole analysis. On the other hand, is the self-sampling really self-saving? Are there any pitfalls, which may decrease the reliability of the results?

Non-adherence to the protocol in terms of time can be addressed with electronic collection devices that record the exact time and date of sample collection. In the case study (Kudielka et al., 2003) that investigated the reliability of self-sampling of saliva samples based on physiological changes in daily cortisol levels, the authors came to the significant conclusion that a well-informed proband is much more reliable (compliant) than an uninformed proband for keeping set collection times. This phenomenon was demonstrated by the steep increase in cortisol levels between the collection immediately after waking up and after 30 minutes (cortisol awakening response (Fries et al., 2009). For the probands who did not keep the time for some reason, the increase was minimal. It means that cortisol can be used as a reliable biomarker of either the reliability of the proband or directly as an internal standard to determine the exact time of collection in self-collections.

In addition to the higher price, the need to obtain two samples, problems with freezing, storage and transport of samples for analysis, there is a fundamental problem with that basic daily cortisol level. Cortisol is a circadian hormone (Ljubijankic et al., 2008; Miller et al., 2016) and its level stabilizes (if the organism is not exposed to stress) in later hours (Fig. 1). Therefore, if it is necessary to obtain these "starting" values, it is necessary to find out whether the probands do not violate other parts of the prescribed protocol during self-sampling. The main factor is keeping a distance from eating and stress.

The first aim of this short study is to bring the information whether the individuals without prior training are able (compliant) to follow the written and video instructions (self-sampling protocol) and provide saliva self-sampling without significant mistakes. The second aim is to describe and highlight the most typical mistakes of saliva collection (sampling). The third aim is to quantify the overall mistakes in self-collected saliva sampling. We have focused on compliance with sufficient amount of the sample, avoidance of drinking or eating before sampling, and keeping the sampling

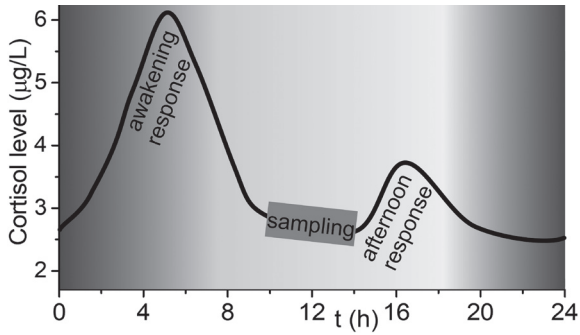

Figure 1 Daily course of cortisol levels with a marked baseline for sampling (adopted from Miller et al., 2016)

time. The timing of the sampling was checked by the amount of cortisol (Ljubijankic et al., 2008). Comprehension of the instructions was checked using a questionnaire.

MATERIALS AND METHODS

Saliva Self-Sampling

The test group in total number of 110 subjected volunteers (probands) was composed of men and women in the range of 21–54 years, either university students or graduates. Probands were randomly divided into two groups: probands providing saliva self-sampling under supervision (35) and probands providing saliva self-sampling without supervision (75). Both groups received the same instructions: 1) "do not eat or drink 30 minutes before sampling", 2) "the sampling has to be provided between 10 a.m. and 2 p.m." and 3) "the amount of saliva in the vial has to reach the red line at minimum" (Fig. 2).

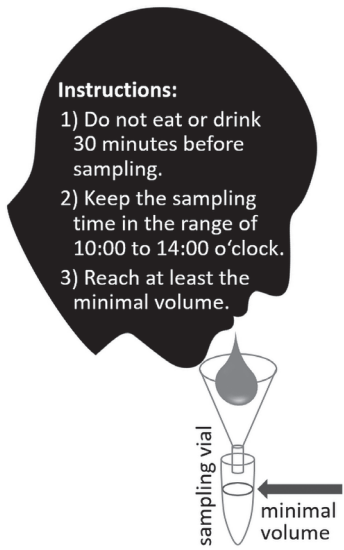

Figure 2 Instructions for saliva self-sampling

The instructions "how to self-collect the saliva" for both groups of probands were the same. Supervised group received the instructions from the supervisor directly. Unsupervised group received the instructions written and *via* video.

Control of the Self-Sampling Protocol

The probands (of both groups) received also the self-checking questionnaire (attached as supporting information – SI) containing questions for the checking the compliance with the self-sampling procedure:

1) The time of self-sampling: *"write the actual time"*.
2) Did I avoid drinking or eating before the self-sampling? Yes / No
3) Does the amount of saliva (without the froth) reach the red line? Yes / No
4) Was the saliva self-sampling stressful? Yes / No
5) Do you feel stressed today or recently? Yes / No

The saliva samples from the supervised group were collected and immediately frozen (–20 °C) to be stored until analysis. The probands from the unsupervised group were asked to freeze (in their home freezer) the samples immediately after sampling and deliver the samples to one of the three collection points within 5 days after the sampling.

Determination of Cortisol

All saliva samples were analysed for cortisol content in one day. The amount of cortisol was determined using enzyme-linked immunosorbent assay (ELISA) utilizing the 96-well microplate kit for salivary cortisol (Diametra Srl Unipersonale, Laboserv, Czech Republic) measured with a Microplate Photometer HiPo MPP-96 operated with specialized software QuantAssay. The cortisol content was determined using a calibration curve and the quality control was provided with inner control.

Cortisol was chosen to check whether the time of self-sampling was respected. Cortisol is a circadian hormone and its concentration in saliva (blood or urine) depends on the day time (Fig. 1) (Ljubijankic et al., 2008; Miller et al., 2016). For the probands, the time range 10 a.m. – 2 p.m., when the cortisol concentration reaches the daily minimum, was chosen. By this easy way, we can verify compliance with the sampling time. However, in some specific cases, the level of cortisol is not on its day plateau, e.g., people suffering from Cushing's syndrome (Raff, 2009), autism (Taylor & Corbett, 2014), people suffering from anxiety, fatigue (Powell et al., 2013), etc. have their specific diurnal trends. Therefore, in the (self-checking) questionnaire (SI), we ask about subjective (acute or long-term) feelings or if the proband suffers from a chronic or acute disease to exclude unsuitable samples.

RESULTS

Three parameters of the saliva samples were monitored: 1) appropriates of the sample amount, 2) absence/presence of food/drink residues in the samples, and 3) accuracy of the sampling time.

The evaluation of the first two parameters was evident. All probands declared a normal or low level of acute and long-term stress. None of the probands considered the saliva-sapling as stressful. None of the probands declare any chronic or acute disease.

In the first group (35 probands; saliva self-sampling with supervision – SSS) all the probands delivered their samples. The supervisor was present and picked up the samples. In the SSS group all the samples reached the required amount (the red line) and ere absence of food/drink residues. The average basal level of salivary cortisol was found 2.91 ± 1.36 μg/L, which corresponds with the basal level of day cortisol reached between 10 a.m. to 2 p.m.

In the second group (75 probands; saliva self-sampling without supervision – NSS), 13 of 75 (17%) probands did not deliver their sample to the sampling point. We expect that these 17% did not deliver their samples due to acute diseases, lack of time, or simply lost motivation to participate in the project. Therefore, in the second group, the number of probands decreased to 62. Of this number, 12.9% (8) of the samples from NSS group contained fewer saliva than required (and clearly marked), and 8.06% (5) of the samples were visibly contaminated with food/drink residues, 4.84% (3) samples had not contain enough sample for analysis together with food/drink residues. Samples containing food/drink residues and/or with insufficient amount of the sample (10) have not been analysed, it means that only 52 samples were analysed. urthermore, 25.8% of them did not follow the required sampling time (Table 1).

The average content of cortisol in the NSS group was 5.82 ± 4.94 μg/L. It was measured only in the samples, which were not contaminated with food/drink residues and contained enough sample for three replicates. Therefore the amount of analysed samples (probands) dropped to 36. The average value of basal cortisol found in the NSS group was much higher (5.82 ± 4.94 μg/L). This average basal level of cortisol in NSS is laden with great error in contrast to the average basal level of cortisol determined in SSS group. This error is caused by several very outliers. After eliminating these outliers in NSS, we get to the value of average basal cortisol 3.10 ± 1.52 μg/L, which is similar to the average basal level of cortisol found in SSS group (2.91 ± 1.36 μg/L). To reach the consent of baseline level of cortisol in both tested groups, 16 outliners (probands) had to be excluded. It means that only 36 samples from NSS group (75 probands at the beginning) were sampled appropriately.

Table 1 Summary of the frequency of non-compliance with the sampling protocol in the unsupervised group; CI stands for the confidence interval ($n = 62$, $p < 0.05$)

Protocol inaccuracy	% (95% CI)
Conditions not fulfilled correctly	41.9 (27.9–55.9)
Less amount of saliva	12.9 (7.2–18.6)*
Food/drink not avoided	8.1 (3.5–12.8)*
Sampling time not abided	25.8 (11.2–40.7)

* 4.84% of probands made a mistake in both parameters at the same time

DISCUSSION

The higher concentration of cortisol in the sample can be caused by the presence of disease and stress in general. However, probands did not declared any. Here it should be noted that for a truly accurate description of the proband, the questionnaire should contain a whole range of other personal information that could have an effect on the hormone level (Bhattarai et al., 2018). We also have to consider the possibility that the probands did not respond in accordance with the truth. However, a false answer should also be considered as the misuse of the sampling protocol. We have to also note that the time-checking system based on the determination of cortisol concentration cannot distinguish the samples taken in the evening from the samples taken between 10 a.m. and 2 p.m. In the evening, the level of cortisol drops to its second minimum (night minimum) (Fig. 1) (Miller et al., 2016).

In this study, the main objective was to estimate the ability of the large audience to follow easy instructions and follow exactly the simple self-sampling protocol independently. Among other things, there is a potential opportunity to record the resting cortisol level before sports performance. We proved that if the probands are under direct supervision, they can follow the sampling protocol exactly. However, such sampling might be in itself associated with certain discomfort and other (stressful) effects on the proband, therefore the supervising person has to minimize any discomfort of the probands, which might affect the sampling protocol of the basal cortisol level If the self-sampling process is provided without direct supervision (at home), 35.5% of probands have problems following the sampling protocol at least in one of the required items, 19.4% made two mistakes in the protocol, and one proband made three mistakes. Only 24 probands from 75 was able to deliver saliva sample, which was sampled properly. In a number of works, the authors also noted a fundamental difference in the required and actual sampling time in healthy probands (Bhattarai et al., 2018; Broderick et al., 2004; Kudielka et al., 2003). It is possible to use other or possibly more sophisticated methods, but the actual reasons and their simple solutions remain hidden. To prevent the unsupervised probands from making mistakes during the sampling procedure, we should understand the most probable reasons why the probands underestimate or do not comply with the sampling protocol. This theme opens the discussion in the field of behavioural psychology.

The instructions for saliva self-sampling were easy and well explained (required volume, required time, and avoidance of drinking/eating). Nevertheless, some significant part of the unsupervised probands had problems to follow them. We guess that the main reason why the probands omitted some of the sampling protocol requirements would be that they did not understand properly why it is important to keep them. They probably underestimated that some minimal volume for the analysis is required, that the presence of food/drink in the sample influences the results of the analysis, and that the sampling time is also important because it directly influences the content of cortisol.

From psychological, physiological and cognitive point of views, the non-follow-up of the sampling protocol in un-supervised group can be explained by:

1) **Haste** – probands had not enough time for saliva sampling during the day (from 10 a.m. to 2 p.m.) and they sampled saliva in different daytime (in the morning or more

probably in the evening) where the level of cortisol is not basal (Fig. 1). However, if the sample was sampled during the night, the cortisol level is at night minimum, which can hardly be distinguished from the day minimum. The presence of food/drink residues can also be caused by haste or insufficient time for sampling, in general.

2) **Saliva lack** – for some people it is not easy to spit. Moreover, the lack of saliva or dry mouth has also a physiological background – it is one of the physiological marks of stress (Bulthuis et al., 2018).
3) **Lack of understanding** – in some cases, the failure in the sampling procedure can be caused by bad/poor understanding of the instructions. It is known that many people pay little attention to reading the instructions or have a low level of comprehension of these instructions (Guthrie et al., 2004). To prevent this lack of reading we have prepared the video instructions. Nevertheless, there was no control that the probands saw the video, saw the whole video, and understood the instructions. However, the second part of the questionnaire (SI) provides feedback to the probands (and to us) that all the steps of the sampling protocol were exactly followed.
4) **Distrust** that strict adherence to the sampling procedure is really important. It is evident that a significant part of the probands do not trust that all the steps or requirements of the sampling protocol are important. In the questionnaire, all probands declared that they are unaware of any disruption of the sampling protocol – they respond to the questions "Did I avoid drinking or eating before the self-sampling?" and "Is the amount of saliva (without foam) reaching the red line?" positively – "yes", despite the fact that the true was evidently different for 24% or 16% of them, respectively. In addition, they filled in the self-sampling time in the required interval, although the actual sampling time was probably different for (at least) 16% of them. These facts can be alarming in broader perspective. Perhaps they were afraid to give the true answer as a confirmation of the sampling protocol failure, and their sample cannot be accepted for further analysis. Perhaps, expecting that it cannot be recognized, they did not follow the sampling protocol exactly. A fundamental difference in the behaviour of the probands is a comparison of healthy and seriously physically ill individuals, when the ill people try to follow the protocol thoroughly (Broderick et al., 2004).

It is also alarming that 13 probands from 75 (17% from the unsupervised group) did not delivered their sample at all. We expect that these people lost the motivation to participate in this experiment. There can be several reasons for not providing sampling or not delivering sample to our collection point. It could be the lack of effort to spend any time with sampling, sampling according to protocol, or delivering the sample. We have to notice that there was no penalization to quit the experiment in any point. The motivation for the probands to complete the sampling and deliver the sample to the collection point was the gained information about their level of cortisol and a gift per sample promised in advance.

CONCLUSION

Different results of the analysis of performed and submitted samples based on their comparison could be influenced by various reasons and factors. In particular, it was

a non-compliance with the saliva-self-sampling protocol (procedure); the absence of personal control (supervision) and direct visual instructions can have a significant effect.

All saliva samples collected from the supervised group of probands contained a sufficient amount of saliva, there was no presence of food or drink traces, and they were sampled in the required time. This result confirms that saliva self-sampling is easy and has a high predictive value if the self-sampling is supervised.

Approximately 35% of unsupervised probands, which delivered the sample, made at least one significant mistake and approximately 20% of unsupervised probands made at least two significant mistakes in the saliva-self-sampling procedure. The reasons, why some of the probands did not follow the instructions exactly, did not arise from the complexity of the procedure. The saliva-self-sampling procedure is very easy and understandable. On the other hand, a significant part of the probands tended to underestimate the significance of some steps of the sampling protocol. They probably did not believe the importance of these steps or did not believe that exact non-compliance with the sampling protocol would significantly affect the results of the analysis. This aspect has to be considered in the evaluation of reliability of all tests, which are based on some kind of self-sampling procedure. The self-sampling procedure has to contain a checking point – e.g. an inner standard, always present in correctly sampled samples.

Practice implications

This study has proven that approx. one third of adults have problems to follow the saliva-self-sampling procedure because of afore-mentioned reasons. This result is highly important and alarming in terms of rapid antigen self-testing strategies within epidemics as well as strategy of distance testing and collection of samples for basic research. The mistakes in self-sampling procedure without supervision can significantly affect the results of research. Researchers, physicians, or even politicians must be aware that the results of the test, which is provided in the self-testing regime, have limited informative value. Improper compliance with both separate steps: sampling protocol and analysis protocol fundamentally affects test results. In addition, it should be noted that the probands in our study had no conflict of interests in salivary cortisol results and their participation in the project was voluntary. Therefore, any conscious influence of the samples was excluded. However, people who are ordered to self-test for SARS-Cov2, for example, cannot be considered volunteers. The socio-psychological aspects play a role in epidemic-related testing. Many people may be concerned about the test result because it could affect their social position at work, with relatives, among friends. Employers should appeal to the personal (moral) responsibility of each employee while maintaining the quality and evidence of self-testing.

To provide the reliable results based on the unsupervised testing the presence of an inner standard referring to the compliance of the sampling procedure is required. The character of such inner standard has to be carefully chosen. Ideally, it is good to combine its determination with the detection method used for the main analyte; for RT-qPCR, it could advantageously be an RNA (gene) always present in saliva, for antigen testing it could be the presence of a hormone in saliva (e.g., cortisol).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at the publisher's website.

ACKNOWLEDGEMENTS

The work was supported by the Charles University grant Cooperatio no. 120 015.

REFERENCES

Bellagambi, F. G., Lomonaco, T., Salvo, P., Vivaldi, F., Hangouet, M., Ghimenti, S., Biagini, D., Di Francesco, F., Fuoco, R., & Errachid, A. (2020). Saliva sampling: Methods and devices. An overview [Review]. *Trac-Trends in Analytical Chemistry, 124*, Article 115781. https://doi.org/10.1016/j.trac.2019.115781.

Bhattarai, K. R., Kim, H. R., & Chae, H. J. (2018). Compliance with saliva collection protocol in healthy volunteers: Strategies for managing risk and errors [Review]. *International Journal of Medical Sciences*, *15*(8), 823–831. https://doi.org/10.7150/ijms.25146.

Broderick, J. E., Arnold, D., Kudielka, B. M., & Kirschbaum, C. (2004). Salivary cortisol sampling compliance: comparison of patients and healthy volunteers [Article]. *Psychoneuroendocrinology*, *29*(5), 636–650. https://doi.org/10.1016/s0306-4530(03)00093-3.

Bulthuis, M. S., Jager, D. H. J., & Brand, H. S. (2018). Relationship among perceived stress, xerostomia, and salivary flow rate in patients visiting a saliva clinic [Article]. *Clinical Oral Investigations*, *22*(9), 3121–3127. https://doi.org/10.1007/s00784-018-2393-2.

Fries, E., Dettenborn, L., & Kirschbaum, C. (2009). The cortisol awakening response (CAR): Facts and future directions [Review]. *International Journal of Psychophysiology*, *72*(1), 67–73. https://doi.org/10.1016/j.ijpsycho.2008.03.014.

Gröschl, M. (2017). Saliva: a reliable sample matrix in bioanalytics [Review]. *Bioanalysis*, *9*(8), 655–668. https://doi.org/10.4155/bio-2017-0010.

Guthrie, J. T., Wigfield, A., Barbosa, P., Perencevich, K. C., Taboada, A., Davis, M. H., Scafiddi, N. T., & Tonks, S. (2004). Increasing reading comprehension and engagement through concept-oriented reading instruction [Article]. *Journal of Educational Psychology*, *96*(3), 403–423. https://doi.org/10.1037/0022-0663.96.3.403.

Kudielka, B. M., Broderick, J. E., & Kirschbaum, C. (2003). Compliance with saliva sampling protocols: Electronic monitoring reveals invalid cortisol daytime profiles in noncompliant subjects [Article]. *Psychosomatic Medicine*, *65*(2), 313–319. https://doi.org/10.1097/01.Psy.0000058374.50240.Bf.

Ljubijankic, N., Popovic-Javoric, R., Sceta, S., Sapcanin, A., Tahirovic, I., & Sofic, E. (2008). Daily fluctuation of cortisol in the saliva and serum of healthy persons [Article]. *Bosnian Journal of Basic Medical Sciences*, *8*(2), 110–115. https://doi.org/10.17305/bjbms.2008.2962.

Miller, R., Stalder, T., Jarczok, M., Almeida, D. M., Badrick, E., Bartels, M., Boomsma, D. I., Coe, C. L., Dekker, M. C. J., Donzella, B., Fischer, J. E., Gunnar, M. R., Kumari, M., Lederbogen, F., Power, C., Ryff, C. D., Subramanian, S. V., Tiemeier, H., Watamura, S. E., & Kirschbaum, C. (2016). The CIRCORT database: Reference ranges and seasonal changes in diurnal salivary cortisol derived from a meta-dataset comprised of 15 field studies [Article]. *Psychoneuroendocrinology*, *73*, 16–23. https://doi.org/10.1016/j.psyneuen.2016.07.201.

Powell, D. J. H., Liossi, C., Moss-Morris, R., & Schlotz, W. (2013). Unstimulated cortisol secretory activity in everyday life and its relationship with fatigue and chronic fatigue syndrome: A systematic review and subset meta-analysis [Review]. *Psychoneuroendocrinology*, *38*(11), 2405–2422. https://doi.org/10.1016/j.psyneuen.2013.07.004.

Raff, H. (2009). Utility of salivary cortisol measurements in Cushing's syndrome and adrenal insufficiency [Review]. *Journal of Clinical Endocrinology & Metabolism*, *94*(10), 3647–3655. https://doi.org/10.1210/jc.2009-1166.

Roblegg, E., Coughran, A., & Sirjani, D. (2019). Saliva: An all-rounder of our body [Review]. *European Journal of Pharmaceutics and Biopharmaceutics*, *142*, 133–141. https://doi.org/10.1016/j.ejpb.2019.06.016.

Taylor, J. L., & Corbett, B. A. (2014). A review of rhythm and responsiveness of cortisol in individuals with autism spectrum disorders [Review]. *Psychoneuroendocrinology*, *49*, 207–228. https://doi.org/10.1016/j.psyneuen.2014.07.015.

Thieme, D. (2012). Potential and limitations of alternative specimens in doping control [Review]. *Bioanalysis*, *4*(13), 1613–1622. https://doi.org/10.4155/bio.12.150.