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Title: Determination of salivary alpha-amylase and cortisol in psoriatic subjects undergoing the Trier Social Stress Test

Article Type: SI: Pharmacological Research

Keywords: Psoriasis; stress-related biomarkers; oral fluid; cortisol; salivary alpha-amylase.

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Abstract: The study was aimed to investigate the response in psoriatic patients of the two primary neuroendocrine systems, namely the Autonomic Nervous System and the Hypothalamic-Pituitary-Adrenal (HPA) axis, whose main biomarkers of activation are salivary □-amylase and cortisol, respectively. Reliable analytical procedures for the determination of salivary □-amylase enzymatic activity and cortisol concentration in oral fluid were developed. The non-invasive sampling that does not require trained personnel represents one of the main advantage of oral fluid analysis compared to the analysis of blood and its derivatives (e.g. plasma). Since oral fluid offers the advantage of a simple and stress free sample collection, it is particularly useful to quantify stress related molecules.
□-amylase activity was measured by a spectrometric method, whereas salivary cortisol by Reversed-Phase High-Performance Liquid Chromatography coupled to Electrospray Ionization Quadropole Time-of-Flight Mass Spectrometry. The effect of salivary pH and flow rate on salivary □-amylase activity and cortisol were also investigated.
Finally, both methods were preliminary tested on oral fluid samples of psoriatic volunteers underwent to a psychosocial stress task (the Trier Social Stress Test), as a first step of a more comprehensive study on the responsiveness of psoriatic subjects to stressors.

Dear Editor,

please find our study "**Determination of salivary α-amylase and cortisol in psoriatic subjects undergoing the Trier Social Stress Test**", describing reliable analytical procedures for the determination of salivary α-amylase enzymatic activity and cortisol concentration in oral fluid.

Psoriasis is a chronic and inflammatory skin disease (2-3% prevalence in the population) entailing a significant psychological distress and psychiatric morbidity, experiences of stigmatization and decreased quality of life. The emotional stress is considered to play an important role in the onset and exacerbation of the pathology, but it is at the same time a consequence of the pathology itself.

Salivary α-amylase enzymatic activity and cortisol represent the main biomarkers of activation of the two major biological systems involved in response to a stressor: the Autonomic Nervous System (ANS) and the Hypothalamic-Pituitary-Adrenal (HPA) axis. Oral fluid offers the possibility of non-invasive, low risk sampling which can be performed by untrained personnel. Oral fluid offers the advantage of a simple and stress free sample collection and for this reason it is particularly useful to quantify stress related molecules.

In this paper, we present analytical methods for the quantification of sAA and cortisol in oral fluid samples as a first step of a more comprehensive study on psoriatic patients. We optimized a spectrometric method for the determination of sAA activity though an enzyme specific assay, and developed a method based on High-Performance Liquid Chromatography coupled to Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry for the determination of cortisol concentration in human oral fluid samples. Both methods were tested on samples collected from psoriatic subjects during the Trier Social Stress Test (TSST). TSST is a social evaluative and mentally challenging task. We believe that the determination salivary α-amylase enzymatic activity and cortisol concentration in oral fluid would guarantee an improved investigation to assess how the two biological systems, ANS and HPA, are activated in response to a stressor in psoriatic subjects. Moreover, information were achieved on the role that stress-related biomarkers, such as cortisol and SAA, play in these mechanisms. On the basis of these premises, the quantification of salivary stress-related biomarkers whom are representative of the two main biological systems involved in responses to stressors could be also applied to evaluate the effectiveness of stress reduction strategies in the management of psoriasis.

For these reasons, we believe that the information contained in this paper might be useful to the scientific community, in particular to researches who work in the field of responsiveness to stressors, clinical and analytical chemistry.

On behalf of the authors, I declare that:

- ❒ Those named in the manuscript are the only authors of the paper;
- ❒ The authors have no conflict of interest of any nature;
- ❒ The study was approved from the local Ethics Committee and conducted in adherence to the Declaration of Helsinki.

I hope you enjoy reading the paper,

Yours sincerely

Francesca G. Bellagambi

Dear Editor,
we would like to express our gratitude for considering the manuscript Ms. Ref. No.: MICROC-D-16-00490, entitled "Determination of salivary alpha-amylase and cortisol in psoriatic subjects undergoing the Trier Social Stress Test" for publication in Microchemical Journal.
We would also thank all the Referees for the time spent improving our manuscript with their constructive and helpful comments.
The manuscript was carefully revised in accordance with the reviewers' comments and responses are reported below in purple. The manuscript, the references and the list of figure captions were modified. All changes in the manuscript are highlighted in track-changed mode.

Reviewers' comments:

Reviewer # 1: The aim of this paper is certainly promising and interesting, and sturdy enough from an analytical point of view. The English is good and the manuscript is generally clear well written.
Surely, the spectrophotometric method here presented for the determination of the sAA activity is not "revolutionary" or particularly attractive from a scientific point of view, however it is here presented for the first time and thoroughly described, maybe representing a reference methods for other researchers. Nevertheless, a possible flaw of this method can be represented by the lack of additional measurements involving the possible matrix effects or recovery. Similarly, no standard curves for quantification were obtained by the authors. Why? But since it is a method that involves the use of commercial reagents, it is possible to consider sufficient all the evaluation tests carried out by the authors to optimize the analytical procedure. Anyway, the studies on the stability of the samples add a rather significant value to the work.
Regarding the HPLC-ESI-Q-ToF for the determination of cortisol, this analytical method is "more suitable" for the publication on your journal. Most of the validation parameters (such as accuracy, precision, repeatability, limit of detection, limit of quantification, matrix effect, etc.) are reported. The stability both of standard solutions as well as of samples was also investigated. Maybe, the weakest part of the work is therefore represented by the determination of sAA and cortisol in a limited number of psoriatic subjects (as well as the lack of measurements on a control group). Even if at lines 1020-1022 the authors tried to avoid misunderstandings, this aspect can be more pronounced if the work is also presented as "an investigation on the relationship between stress and psoriasis to identify possible psoriatic patients who need a psychological treatment".

In conclusion, I consider the manuscript suitable for the publication in your Journal with minor revisions, that I invite the authors to make in order to increase its suitability for publication in Microchemical Journal.

For major and minor comments please see below.

General issues:

- Abstract:
- It should be rewritten highlighting the advantages of using non-invasive medium as saliva, as well as the insertion of the quality parameters of both methods at the end of the Abstract (LOD, LOQ, etc.).

Thanks to the reviewer for this important suggestion. The abstract was rewritten highlighting these aspects.

- Introduction:
- This section has been written as definition of psoriasis, oral fluid, cortisol and amylase in separate paragraphs.
- The introduction needs reduction and the author should start first by describing the disease (psoriasis), after introducing the oral fluid, and finally he has to describe amylase and cortisol.

Thanks to the reviewer for this helpful comment. The Introduction section was almost completely rewritten according to the reviewer's suggestion.

- Reference values of sAA in nominally healthy subjects is missing.

The authors apologize for this lack and the sentence "Level of sAA in nominally healthy subjects can vary from 50 to 1000 U/mL" was added in the revised manuscript.

- Result section:
- The quality of Figure 4 is too poor.

According to the reviewer's comment, we have replaced Fig. 4 in the revised manuscript. We also changed the relative caption, according to the new figure.

- The author introduces the Figure 5 but there is no interpretation of it.

The authors introduced Fig. 5 at line 978 of the unrevised version of the manuscript, and the interpretation of the Fig.5 was present from line 998 to line 1007.

Detailed comments:

Line 618: please change "this term is" with "this term was".

Done.

Line 623: why the sAA acronym is used for α-amylase? Maybe the author means salivary α-amylase.

Please see the revised version of the Introduction.

Line 627: whose?

In this case, "whose" is referred to sAA activity.

Line 630 and 631: HPA axis.

Done.

Line 633: please change "confirmed from Maruyama Y. et al." with "confirmed by Maruyama Y. et al."

Done.

Line 642: please change "Sympathetic-Adrenal-Medullary (SAM) system activity" with SAM system activity (the author has already mentioned this)

Done.

Lines 653-654: the sentence "Both blood and saliva cortisol levels seem to be related to physiological and psychological stress." is followed by 10 references. I suggest the author to rewrite it in a less cryptic way.

According to the reviewer's suggestion, the Introduction section was almost completely rewritten and this sentence was changed.

Line 659: please replace "expressed as" with "quantified with". Same line: what is the unit of measurement for PASI?

Done. We changed the sentence "The severity of disease is quantified by the estimation of the Psoriasis Area Severity Index (PASI)". The PASI is a dimensionless index.

Lines 659-660: please change "Aetiology is not fully understood yet" with "The aetiology of psoriasis is not…"

Done.

Line 752-753: "The interview was videotaped with a camera adjusted so that the subject's face and trunk were in full view " This sentence is not clear.

The sentence was rewritten as “The interview was recorded with a video camera framing the face and the trunk of the subject”.

Lines 905-906: he author stats : "Similar results were obtained for processed samples left at room temperature and analysed every hour for a time span of eight hours "Please add a figure to support this sentence.

The adjective “similar” was replaced with “same” as data practically overlapped the other data. After providing this information to the reader, we see no point in adding an additional identical plot.

Line 909: please comment your results on stability of sAA in OF samples.

This comment helped us to identify a typo in the caption of Figure 2, which was corrected. A comment was added concerning the stability of samples. Now the sentence is “Fig. 2 shows the variations of the sAA activity in POFS aliquots stored at 4 °C and at -80 °C for four weeks. In this period, the enzymatic activity decreased of about 15% in samples stored at 4 °C and remained almost stable in samples stored at -80 °C, as variations were within the experimental error This results is extremely useful for the performance of larger clinical studies.”.

Lines 931-932: please rewrite the sentence introducing a little bit the study to which reference is made.

Done. The sentence was changed to make the usefulness of that reference more clear to the reader.

Lines 954-955: please rewrite the sentence as "The LOD and LOQ values were calculated in accordance with IUPAC guidelines [56], and they resulted…". Moreover, I suggest to compare this results with literature.

The sentence was rephrased according to the reviewer suggestion (“LOD and LOQ values equal to 0.3 and 0.8 ngmL^{-1} were calculated in accordance with IUPAC guidelines [50]”). We found no other method in literature using ESI-Q-TOF for the determination of salivary cortisol, so a comparison would not be very meaningful in our view.

Line 965: mention the previous method was used here.

The method is the one described in the paper, the sentence was not clear due to a typo that was removed.

Reviewer #2: The study is addressed to investigate the response of two primary neuroendocrine systems in psoriatic patients by optimizing and developing methods for the determination, in their oral fluid samples, of α-amylase (sAA) activity and free fraction of cortisol respectively as main markers of activation.

Main Comments

The research is interesting and the work generally well performed even if a larger cohort of psoriatic patients should have been recruited.

Methods.

For the study both healthy volunteers and patients with psoriasis were enrolled but the two groups differ significantly for the number but above all for the age. It would have been better to use healthy subjects approximately of the same age of the patients to avoid possible related influence of this parameter on final results.

Authors are aware of the limitations in the clinical value of the results due to the low number of patients and insufficient matching between patients and control group. However, the main focus of the paper was to demonstrate that the analytical methods were capable to reliably assess tiny differences in cortisol concentration and salivary α-amylase activity in oral fluid samples. A more comprehensive clinical study has been planned and is going to be performed in the near future.

References

Too many references are reported. This list should be shortened.

The list was shortened as suggested from the reviewer.

Reviewer #3: The paper seeks to examine the possible association between psiosaris and stress through measurement of salivary a amylase and cortisol following TST testing of small cohort of patients. The study is well conceived, of a high standard and reports new data on the subject area. Publication is recommended after some minor editing/additions to text.

Comments

1. Abstract There is no mention of main/key findings of the research. A sentence or two should be added.

We thank the reviewer for his suggestion, the Abstract was improved accordingly.

2. L 652 Clarification "of cortisol" should be added after "reference concentration values"

Done.

3. L 674 ff It would be helpful to add a few additional sentences on the current state of the art of methods for determination of sAA and sCortisol.

The Introduction section was rewritten in the revised version of the manuscript. Additional sentences were added in relation to the current analytical techniques (and relative references) used to determine both analytes in oral fluid samples.

4. Results and Discussions. Was any correlation found between the sAA and sCortisol values for the patients?

sAA activity and cortisol levels were determined in OF samples collected from six psoriatic volunteers undergoing the TSST. Results were described in the Section 3.3. In particular, Fig. 5 shows how the salivary levels of both analytes changed in each subject before and after the TSST. A relatively large variability was observed in the individual responses, and the limited number of subjects do not provide the possibility to make a statistically reliable assessment of correlations. After this preliminary paper focused on the analytical methodology, a more in depth evaluation of the clinical part will be made once results of a study with a much larger number of patients will be available.

5. Although not in any way detracting from comprehension, the paper would benefit from some minor editing/correction of grammar.

The manuscript was revised by a native English speaker.

Overall very interesting and thorough study for which authors are to be congratulated.

Highlights

- Psoriatic subjects undergoing the Trier Social Stress Test.
- Determination of α-amylase activity and cortisol concentration in oral fluid.
- Monitoring of stressed subjects by α-amylase activity and cortisol level in oral fluid.

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Determination of salivary α-amylase and cortisol in psoriatic subjects undergoing the Trier Social Stress Test

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Abstract

The study was aimed to investigate the response in psoriatic patients of the two primary neuroendocrine systems, namely the Autonomic Nervous System and the Hypothalamic-Pituitary-Adrenal (HPA) axis, ~~in psoriatic patients~~ whose main biomarkers of activation are salivary α-amylase and cortisol, respectively. Reliable analytical procedures for the determination of salivary α-amylase enzymatic activity and cortisol concentration in oral fluid were developed. The non-invasive sampling that does not require trained personnel represents one of the main advantage of oral fluid analysis compared to the analysis of blood and its derivatives (e.g. plasma). Since oral fluid offers the advantage of a simple and stress free sample collection, it is particularly useful to quantify stress related molecules.

α-amylase activity was measured by a spectrometric method, whereas salivary cortisol by Reversed-Phase High-Performance Liquid Chromatography coupled to Electrospray Ionization Quadropole Time-of-Flight Mass Spectrometry. The effect of salivary pH and flow rate on salivary α-amylase activity and cortisol were also investigated.

Finally, both methods were preliminary tested on oral fluid samples of psoriatic volunteers underwent to a psychosocial stress task (the Trier Social Stress Test),~~.~~ as a first step of a more comprehensive study on the responsiveness of psoriatic subjects to stressors.

Keyword:

Psoriasis, stress-related biomarkers, oral fluid, cortisol, salivary α-amylase.

Highlights

- Psoriatic subjects undergoing the Trier Social Stress Test.
- Determination of α-amylase activity and cortisol concentration in oral fluid.
- Monitoring of stressed subjects by α-amylase activity and cortisol level in oral fluid.

1. Introduction

Psoriasis is a chronic and inflammatory skin disease (2-3% prevalence in the population) entailing a significant psychological distress and psychiatric morbidity, experiences of stigmatization and decreased quality of life [1]. Abnormally red and scaly patches of different size, from small and local to a complete body coverage, appear on patient's skin. The severity of disease is generally quantified by the estimation of the Psoriasis Area Severity Index (PASI), which ranges from 0 to 72. The aetiology of psoriasis is not fully understood yet, but multiple genetic and environmental factors seem to be involved. The emotional stress is considered to play an important role in the onset and exacerbation of the pathology [2,3], but it is at the same time a consequence of the pathology itself.

The term "stress" was coined by Hans Selye in 1936, who defined it as "the non-specific response of the body to any demand for change". Currently, this term is used to describe highly subjective phenomena (stressors) that can affect, mainly in an adverse way, both our mental and physical health. Generally, two major biological systems are activated in response to a stressor: the Autonomic Nervous System (ANS) and the Hypothalamic-Pituitary-Adrenal (HPA) axis, whose main biomarkers of activation are salivary α-amylase (sAA) in oral fluid (OF) [4] and cortisol in blood [5], respectively.

sAA is an enzyme produced in the oral mucosa from salivary glands (i.e. parotid, submandibular, and sublingual); sympathetic and parasympathetic nerves control the salivary secretions by releasing specific neurotransmitters at the nerve endings in these glands. sAA levels are not related to α-amylase levels in blood, which are derived from pancreatic secretion [6]. Level of sAA in nominally healthy subjects can vary from 50 to 1000 U/mL [7,8]. Since psychosocial stress induces the activation of sAA functions, this molecule is a prime candidate to monitor the autonomic [9,10] and the sympathetic-adrenal-medullary (SAM) system activity [11]. In 2004, Takai N. et al. investigated the effect of psychological stress on salivary cortisol and α-amylase levels in healthy young adults [12], concluding that the psychological stressor increased the sAA level, whose response and sensitivity to the stressor was higher compared to cortisol. They hypothesized that the major response to stress consists of a short latency catecholamine component, depending on the SAM system, and a slower glucocorticoid response, depending on HPA axis. According to this

view, the cortisol response in HPA axis is the final step of the normal stress response, and has a longer latency of secretion compared to sAA originated from the SAM system [13,14]. These results were confirmed by Maruyama Y. et al. [15], who evaluated the trends of sAA activity and salivary cortisol levels in 185 healthy volunteers with no history of psychiatric disorder undergoing a Trier Social Stress Test (TSST). A rapid variation of the sAA activity was observed with peak levels observed immediately after the test, whereas salivary cortisol showed a delayed response that continued to increase 20 min after the TSST.

The standard methods for the determination of sAA activity are specific for the evaluation of its kinetics. In general, they provide for the reaction of the enzyme with the appropriate chromogenic substrates, which are split to give intermediate products that absorb radiation at a specific wavelength. More AA is present in the saliva sample, more the substrate is cleaved into degradation products in a given period of time, the higher the absorbance measured at the wavelength of reference. Enzyme kinetic method [16], Enzyme-Linked Immuno Assays (ELISA) [17,18], and Radio Immuno Assays (RIA) [19] can be used for sAA activity quantification. However, colorimetric methods are most suited to use in quantitative sAA enzyme assays [20–22]. If either the substrate or one of the reaction products is coloured it may be measured directly in the photoelectric colorimeter. The possibility of using a colorimetric method for the quantification of the enzymatic activity offers the advantage of requiring lower costs and greater simplicity and speed of execution.

Cortisol is a steroid hormone, more specifically a glucocorticoid produced by the *zona fasciculata* of the adrenal gland, which marks the activation of the HPA axis. It has a peculiar circadian rhythm: plasmatic levels are minimal around midnight, start rising in the early morning to reach a peak 30-40 minutes after awakening [23], then slowly decrease during the day [24–26]. Plasmatic cortisol is predominantly bound to the corticosteroid-binding globulin (about 73%) and to albumin (about 24%). The biologically active free cortisol is about 3%, and it passively diffuses into OF via ultra-filtration [27]. Salivary cortisol correlates well with the free cortisol in blood [28,29]. In nominally healthy subjects, reference concentration values of cortisol are about 1-12 and 0.1-3 $ngmL^{-1}$ for samples collected in the morning and in the evening, respectively [30]. Both blood and salivary cortisol levels seem to be related to physiological and psychological stress because the activation of the HPA axis, with abnormal production and release of cortisol, was observed in various stressful situations [9, 31–34]. Currently, in addition to immunochemical methods, specific and sensitive techniques based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) would be developed for the determination of salivary cortisol to overcome the interferences due to cross-reaction of steroids or other substances [35,36].

Based on these background information, we hypothesized that monitoring the sAA and cortisol levels in OF during a stress test, namely TSST, could help to better understand the relationship between stress and psoriasis and to identify possible responders to a psychological treatment. The TSST is a social evaluative and mentally challenging task and was choose because it provokes the highest HPA axis stress response compared to other laboratory stressors [18]. The identification of the subset of stressed patients may be clinically important, because a psychological treatment may help these patients to reduce the pharmacological therapy.

Oral fluid offers the advantage of a simple and stress free sample collection [37,38], and for this reason it is particularly useful to quantify stress related molecules. The salivary cortisol concentration is not influenced by salivary flow rate and pH [39], but is not clear whether the same is true for the sAA activity [18,32, 40–42].

In this paper, we optimized a spectrometric method for the determination of sAA activity though an enzymatic specific assay, and we developed a method based on High-Performance Liquid Chromatography coupled to Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry (HPLC-ESI-Q-ToF) for the determination of the free fraction of cortisol in human OF samples. Moreover, the possible effect of salivary pH and flow rate on sAA activity was also evaluated since OF sampling is a critical step to obtain reliable data [43,44]. Both methods were used to analyze OF samples collected from psoriatic subjects during a TSST as a first step of a more comprehensive study on psoriatic patients. Moreover, the identification of molecules related to stress and the ability to determine them in non-invasive way opens up the possibility to develop, in future, complementary and cheap methods (i.e. artificial olfactory systems and sensors) for investigating the responsiveness to stressors in a larger cohort of stakeholders [45].

~~The term "stress" was coined by Hans Selye in 1936, who defined it as "the non-specific response of the body to any demand for change". Currently, this term is used to describe highly subjective phenomena (stressors) that can affect, mainly in an adverse way, both our mental and physical health. Generally, two major biological systems are activated in response to a stressor: the Autonomic Nervous System (ANS) and the Hypothalamic-Pituitary-Adrenal (HPA) axis, whose main biomarkers of activation are α-amylase (sAA) in oral fluid [1] and cortisol in blood [2], respectively. Salivary α-amylase levels are not related to α-amylase levels in blood, which are derived from pancreatic secretion [3]. In 2004, Takai N. et al. investigated the effect of psychological stress on salivary cortisol and α-amylase levels in healthy young adults [4], concluding that the psychological stressor increased the sAA level, whose response and sensitivity to the stressor was higher compared to cortisol. They hypothesized that the major response to stress~~

~~consists of a short latency catecholamine component, depending on the Sympathetic Adrenal Medullary (SAM) system, and a slower glucocorticoid response, depending on HPA. According to this view, the cortisol response in HPA is the final step of the normal stress response, and has a longer latency of secretion compared to sAA originated from the SAM system [5,6]. These results were confirmed from Maruyama Y. et al. [7], who evaluated the trends of sAA activity and salivary cortisol levels in 185 healthy volunteers with no history of psychiatric disorder undergoing a Trier Social Stress Test (TSST). A rapid variation of the sAA activity was observed with peak levels observed immediately after the test, whereas salivary cortisol showed a delayed response that continued to increase 20 min after the TSST.~~

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~~Psoriasis is a chronic and inflammatory skin disease (2-3% prevalence in the population) entailing a significant psychological distress and psychiatric morbidity, experiences of stigmatization and decreased quality of life [40]. Abnormally red and scaly patches of different size, from small and local to a complete body coverage, appear on patient's skin. The severity of disease is expressed as the Psoriasis Area Severity Index (PASI), which ranges from 0 to 72. Aetiology is not fully understood yet, but multiple genetic and environmental factors seem to be involved. The emotional stress is considered to play an important role in the onset and exacerbation of the pathology [41,42], but it is at the same time a consequence of the pathology itself.~~

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2. Materials and Methods

2.1 Statement of ethics and study subjects

This study was conducted according to the principles outlined in the Declaration of Helsinki on medical research involving human subjects. Nominally healthy volunteers and psoriatic subjects gave written informed consent prior to their participation. Thirty-five healthy volunteers (21 men and 14 women, 35 ± 13 years) and six psoriatic subjects (5 men and 1 woman, 60 ± 10 years) were enrolled. The oral fluid of healthy volunteers was needed for the development and optimization of the analytical methods.

2.2 Chemicals and Instrumentation

Hydrocortisone (purity≥98%), dichloromethane (purity ≥ 99.8 %), formic acid (purity ≈ 98%), and acetonitrile (purity ≥ 99.9%) were purchased from Sigma Aldrich (Italy). LC-MS grade water

was from Fluka (Italy). Sodium hydroxide pellets (purity > 97%) were supplied by Carlo Erba (Italy). Milli-Q water was produced by a Millipore Reagent Water System (USA).

Roll-shaped biocompatible synthetic swabs (*Salivette®* Cortisol) were purchased from Sarstedt (Germany). Regenerated cellulose syringe filters (0.2 μm) were purchased from Phenomenex (Italy). Pehanon narrow range (5.2 < pH < 6.8 and 6.0 < pH < 8.1) pH paper strips, with a resolution of 0.3 pH units, were purchased from Macherey Nagel (Germany). *Phadebas®* Alpha-Amylase Test was provided by Magle AB Life Sciences (Sweden). For sample incubation, a SW22 thermostatic water bath was purchased from Julabo (Italy). The UV-visible measurements were performed using a Lambda 25 Perkin Elmer UV-Visible spectrophotometer (USA) equipped with two quartz cuvettes with an optical path of 1 cm. HPLC-ESI-Q-ToF analyses were performed with a 1200 Infinity Agilent Technologies HPLC (USA) coupled to a 6530 Infinity Jet Stream ESI-Q-ToF (USA).

2.3 Oral fluid sampling procedures

All subjects refrained from smoking, physical exercise, food, alcoholic beverages, and soft drinks for at least 1 h prior testing.

2.3.1 Influence of the sampling protocol on the activity of sAA in oral fluid

Unstimulated and stimulated OF samples were preliminary collected from seven healthy volunteers (3 males and 4 females, age 35 ± 5 years) to investigate the possible effect of salivary flow rate and pH on the sAA enzymatic activity. Subjects placed the *Salivette®* swab in their mouth for 2 minutes to collect unstimulated OF samples. Stimulated OF samples were collected at different degrees of stimulation. Subjects received an input from a digital metronome (Real Metronome, Australia) to move the swab in their mouth at different frequencies (50, 100 and 150 min^{-1}). The sampling time (1–5 min) was optimized for each subject to keep sample volumes in the range 0.5–2 mL, enough for the analyses and not exceeding the sampling capacity (2.2 mL) of the swab. OF samples were recovered by centrifugation of the swabs at 5000 rpm for 5 min at room temperature (22 ± 2 °C). The sample volume was calculated from the weight difference of the swab before and after sampling assuming the OF density to be 1.0 gmL^{-1} [46]. The OF flow rate was estimated as the ratio of sample volume and sampling time. pH was measured immediately after sampling by two independent observers using narrow range pH paper strips. OF samples were stored at -80 °C when not immediately analysed.

2.3.2 Preparation of the oral fluid pooled sample

Stimulated OF samples were collected from 35 nominally healthy volunteers moving the swab in the oral cavity for 2 min at a self-selected pace. The OF samples were recovered by centrifugation of the swabs as described in the previous paragraph. The pooled OF sample (POFS) for the optimization of the analytical methods was prepared by mixing all collected samples, and dividing it into aliquots of about 0.3 mL, which were stored at -80 °C until analysis.

2.4 Oral fluid collection from psoriatic subjects undergoing TSST

Psoriatic subjects undergoing TSST (see 2.4.1) provided stimulated OF samples obtained by moving swabs in the oral cavity for 2 min at a self-selected pace. The OF samples were recovered by centrifugation of the swabs and treated according to the procedure described in the previous paragraphs. These samples were collected while each subject was waiting in the interview room (t_0), just before the TSST ($t_1 = t_0 + 10$ min), at the end of TSST ($t_2 = t_0 + 30$ min), and during a resting period in the interview room where the subject was left alone, at about 20 (t_3) and 40 (t_4) minutes after the end of the test. The TSST sessions were carried out from 9 A.M. to 1 P.M. and included the clinical evaluation of the PASI. Oral fluid samples were recovered according to the protocols described above and kept at -80 °C until analyses.

2.4.1 Stress interview and behavioural measurements

A psychosocial challenge consisting of a brief stress interview (speech task, 5 min) during which the patient sat in front of an interviewer in the presence of an audience was performed. In particular, each subject was asked to describe his distinctive personality features in a social context [~~7~~15].

The non-verbal behavior during the speech task was quantified by means of the Ethological Coding System for Interviews (ECSI) [~~52~~45]. The interview was recorded with a video camera framing the face and the trunk of the subject~~The interview was videotaped with a camera adjusted so that the subject's face and trunk were in full view~~. Subsequently, the behavioral assessment was carried out according to Troisi and colleagues [~~52–53~~46,47]. This version of the ECSI includes 37 different patterns, mostly facial expressions and hand movements. The behavioral patterns were grouped in seven behavioral categories, each reflecting a different aspect of the subject's emotional and social attitude, namely: (1) eye contact; (2) affiliation; (3) submission; (4) flight; (5) assertion; (6) gesture; (7) displacement.

2.5 Determination of sAA activity in oral fluid samples

2.5.1 Optimization of the Phadebas® Amylase test for the determination of sAA activity

Preliminary tests were carried out to evaluate a suitable dilution ratio for the determination of sAA activity in OF samples. For this purpose, eighteen solutions were obtained by diluting (v/v) three aliquots (10 μL) of a POFS with Milli-Q water at the following ratios: 1:500, 1:1000, 1:1500, 1:2000, 1:3000, and 1:4000. An aliquot (0.2 mL) of each solution was further diluted (20-fold) with Milli-Q water in a centrifuge tube before incubation in a water bath at 37 ± 1 °C. After 5 min, one reagent tablet was added into each tube and the solution was immediately vortex mixed for 10 seconds. The resulting solution was placed again in the water bath for a second incubation step of 15 minutes. After stopping the enzymatic reaction with 1 mL of 0.5 M NaOH and vortex mixing for 10 seconds, the sample was filtered at 0.2 μm, and the absorbance of the blue solution was measured at 620 nm.

A *blank* sample, consisting of 4.2 mL of Milli-Q water, was analysed as procedural blank every day during the measurements.

The enzymatic activity was determined from the standard curve provided by the manufacturer after subtracting the absorbance of the blank from the absorbance of each sample.

The intra-day and inter-day precisions of the optimized procedure, as relative standard deviation (RSD), were calculated from absorbance values measured in ten aliquots of an OF sample from a same subject and ten aliquots of the POFS for an evaluation at two different levels of sAA activity. Measurements were performed on both samples in a single day and on three consecutive days.

2.5.2 Stability of the sAA activity in oral fluid samples

The stability of the sAA activity was investigated by storing OF samples at different temperatures over different time spans. The first experiment aimed to verify the stability of samples kept in the laboratory during a working day, the other experiments to understand the best conditions for long term storage.

Ten aliquots (0.3 mL) of POFS were placed in test tubes and stored at room temperature (22 ± 2 °C). The content of the first five tubes was analysed every half hour, then the time interval was extended to 1 hour for the following four tubes and to 2 hours for the last one. Overall, a time span of eight hours was covered (0, 0.5, 1, 1.5, 2, 3, 4, 5, 6 and 8 hours). Three aliquots (10 μL) were taken from the tubes at each time, diluted 1:2000 with Milli-Q water and analysed according to the aforementioned optimized procedure (2.5.1).

Ten additional (0.3 mL) aliquots of POFS were diluted 1:2000 with Milli-Q water and immediately prepared for the analysis following the optimized procedure. After filtration at 0.2 μm, they were left at room temperature (22 ± 2 °C) and analysed at one hour interval from one another.

Two further sets of samples (Set 1 and Set 2), each consisting of twenty-seven aliquots (0.3 mL) of POFS, were placed in test tubes and stored in a refrigerator at 4 °C (Set 1) and -80 °C (Set 2), respectively. Every three days, three samples from both Set1 and Set2 were put at room temperature (22 ± 2 °C) for 30 min, and then diluted 1:2000 with Milli-Q water before the determination of the sAA activity according to the optimized procedure described in 2.5.1.

2.6 Determination of cortisol in oral fluid samples by HPLC-ESI-Q-ToF

2.6.1 Stock and working solutions

A stock solution at 1000 μgmL^{-1} of cortisol was prepared by dissolving the appropriate amount of the pure compound in acetonitrile. Working solutions at concentrations 0.1, 1, and 10 μgmL^{-1} were prepared in 10 mL glass flasks by diluting the stock solution 10000-, 1000- and 100-fold with LC-MS grade water. Appropriate volumes of the working solutions were used to prepare calibration solutions, to spike OF samples, as well as to fortify OF extracts in the concentration range of 1–100 $ngmL^{-1}$.

2.6.2 Mass spectrometry experimental conditions

The determination of cortisol was performed by high-resolution HPLC-ESI-Q-ToF-MS in order to eliminate any possible interfering species and ensure a high reliability of the analytical data. Obviously, for routine measurements the low cost HPLC-UV-Vis method can be used, once the analytical performances are tested and compared with the high-resolution method.

The following optimized operating conditions were used for the ESI interface: drying gas (N_2, purity > 98%) temperature 350 °C, drying gas flow rate 10 $Lmin^{-1}$, nebulizer gas pressure 35 psig, sheath gas temperature 375 °C, sheath gas flow rate 11 $Lmin^{-1}$, capillary voltage 3000 V, fragmentor 130 V, nozzle voltage 0 V, skimmer voltage 65 V, and octapole RF voltage 750 V. The declustering potential was set at 130 V and the voltage used for the collision induced dissociation (CID) for the MS/MS experiments at 25 V. The collision gas for the MS/MS analysis was nitrogen (purity 99.999%). The high-resolution MS and MS/MS acquisition range was set from 100 to 1000 m/z. Data were collected by a target MS/MS acquisition with an MS and MS/MS scan rate of 1.41 spectra/sec. The mass axis was calibrated daily using the HP0321 Agilent tuning mix (Agilent Technologies) in acetonitrile. The mass spectrometer control, the data acquisition and the data analysis were performed with the MassHunter® Workstation software (B.04.00). The data acquisition was performed in full scan mode to determine the precursor ion, by analysing standard solutions at a concentration of 10 $ngmL^{-1}$ and working in flow injection mode. The $[M+H]^+$ ion was determined at 363.21 m/z. Then, tandem mass spectra were acquired to optimize the parameters

related to the CID, detecting the following product ions: m/z 121.06, 309.18 and 327.19, working with a fixed collision energy of 25 V. The product ion 121.09 was selected during post-processing of the data for the quantitation of the analyte in extract ion (EI) mode.

2.6.3 Chromatographic separation

The chromatographic separation was achieved by a reversed phase column Zorbax Extend-C18 (50 x 2.1 mm, 1.8 µm; Agilent Technologies, USA) connected to an Eclipse Plus guard column (12.5 mm x 2.1 mm, 5 µm, Agilent Technologies, USA). The chromatographic conditions were optimized using ultrapure water with 0.1% (v/v) formic acid (A) and acetonitrile with 0.1% (v/v) formic acid (B). The LC conditions were the following: 15% B increased to 30% in 1 min and held for 9 min; column re-equilibration time: 5 min. The injection volume was 10 µL and the total run time was 15 min. The optimized column temperature and flow rate were 25 °C and 0.2 $mLmin^{-1}$, respectively. The auto-sampler operated at room temperature (22 ± 2°C).

2.6.4 Sample preparation

For the determination of cortisol, OF aliquots (0.2 mL) were added with 2 mL of CH_2Cl_2, mixed for 30 min using a rotary shaker, and centrifuged at 7000 rpm for 5 min. The organic phase was recovered in a conic glass tube, evaporated under a gentle stream of nitrogen, and then reconstituted in 100 µL of LC-MS grade water. The vials were weighed in all steps of extraction procedure to calculate the recovery of organic solvent.

2.6.5 Validation of the method

The method was validated by using POFS aliquots to determine the following parameters: selectivity, dynamic range, limits of detection and quantification (LOD and LOQ), repeatability and recovery. The quantitative evaluation of the matrix effect was performed by analysing three different sets of samples, i.e. calibrant solutions, spiked extracts and spiked samples, as proposed by Matuszewski et al. [~~54–55~~48,49].

The Huber statistical test excluded possible outliers while determining the validation parameters. The linearity of the method was verified by analysing (n = 3) calibration solutions, fortified extracts and spiked POFSs in the concentration range 0.5–100 $ngmL^{-1}$. The Mandel's fitting test was applied to fit a linear regression model and determine sensitivity.

The LOD and LOQ values were calculated in accordance with IUPAC guidelines [~~56~~50], as three and ten times the standard deviation (s_b) of the "low level spiked blank". The intra- and the inter-day precision were evaluated from ten replicate analyses of calibration solutions and spiked

POFS aliquots at three concentration levels (1, 10, 100 $ngmL^{-1}$) in the same day and three different days respectively, and expressed as relative standard deviation (RSD). The recovery of cortisol from OF was estimated at the same concentration levels by the standard addition method on POFS and LC–MS grade water aliquots (n = 6) spiked with known amounts of cortisol.

2.6.6 Assessment of calibration solutions and sample stability

The short and long term stabilities of both calibration solutions (1, 10, 100 $ngmL^{-1}$) and POFSs were assessed. Short term stability was evaluated by keeping 0.3 mL aliquots of both calibration solutions and POFSs spiked with 10 $ngmL^{-1}$ at room temperature and measuring the cortisol concentration every hour, for a time span of 8 hours. Similarly, spiked POFSs (10 $ngmL^{-1}$) were stored at 4 °C in the case of long term stability and analysed every five days over a period of four weeks.

3. Results and Discussion

3.1 Optimization of the sAA activity measurement in oral fluid

The Phadebas® colorimetric kit measures the sAA activity in biological fluids based on the hydrolysis of a water-insoluble, cross-linked, blue starch polymer into water-soluble blue starch fragments. The substrate is specific for sAA and doesn't cross-react with enzymes hydrolysing the non-reducing terminal residues of the starch, like β-Amylase and Invertase. Since the activity levels of sAA in OF are two-three order of magnitudes higher than in other biological fluids [~~57~~51], it was necessary to dilute the sample before the analysis.

Based on our results, the optimized procedure entails a dilution of the OF 1:2000 with Milli-Q water before sample preparation and a filtration at 0.2 μm before the spectrophotometric measurement.

The verification of the linearity of the analytical method was performed in the range of applicability indicated by the kit manufacturer by the analysis of POFS aliquots at different dilution ratios. Performing a dilution 1:500, 1:1000, or 1:1500, it was observed that the absorbance of the sample was out of scale (more than 2 AU), while diluting 1:2000, 1:3000, and 1:4000 the RSD, of the mean enzymatic sAA activity corrected for dilution factor, was 5%.

The intra-day and inter-day relative standard deviation (RSD) on ten aliquots of an OF sample from a same subject and on ten aliquots of the POFS resulted always better than 6% (Table 1).

Table 1

Intra- and inter-day precision of the determination of sAA activity both an OF sample from a same subject and ten aliquots of the POFS.

	Mean sAA activity [UmL^{-1}]	**Intra-day[a] RSD**	**Inter-day[b] RSD**
Single OF sample	170	3	5
POFS	400	5	6

[a] Calculated from ten replicates at each enzymatic activity level in the same day.

[b] Calculated from ten replicates at each enzymatic activity level value in three different days.

3.1.1 Stability of sAA activity in oral fluid samples

The results of the samples stored at room temperature suggest that the enzymatic activity of sAA remained stable over a period of at least 8 hours, since the observed variations were within the experimental error (Fig. 1). Similar results were obtained for processed samples left at room temperature and analysed every hour for a time span of eight hours.

Fig. 2 shows the variations of the sAA activity in POFS aliquots stored at 4 °C and at -80 °C for four weeks. In this period, the enzymatic activity decreased of about 15% in samples stored at 4 °C and remained almost stable in samples stored at -80 °C, as variations were within the experimental error~~.~~ This results is extremely useful for the performance of larger clinical studies.

3.1.2 Effects of salivary flow rate and pH on the evaluation of sAA enzymatic activity

For certain molecules and ions, the sampling procedure can modify the OF composition, and we identified salivary flow rate and pH as possible variables of interest for the present study. Salivary flow rate can change due to perceived stress [~~58~~52], depression [~~59~~53], age [~~60~~54], alcohol consumption [~~61~~55], exercise intensity [~~62~~56], as well as cancer and radiation treatment [~~63~~57]. In all these studies, fluctuations of the salivary flow rate correlate with fluctuations of the sAA activity, but it is unclear whether the fluctuations of the sAA activity result from the altered salivary flow rate or directly from stress factors [~~64~~58]. In 2006, Rohleder et al. concluded that sAA concentration is not affected by the OF flow rate [~~49~~18], whereas other authors reported the highest sAA activity in human OF at pH of 6.8 and 37 °C temperature [~~65~~59].

Unstimulated and stimulated OF samples at different stimulation levels (50, 100 and 150 min^{-1}) were collected from seven healthy volunteers to study the effect of flow rate and pH on the salivary sAA enzymatic activity. For the same subject, the data of samples gathered with a flow rate fairly constant during the observation period showed a maximum value of the sAA activity around pH = 6.6, confirming the results reported in literature [~~65~~59]. Fig. 3A shows the trend of sAA vs pH for a

nominally healthy volunteer who had an almost constant flow rate (1.3 ± 0.2 $mLmin^{-1}$) for the collected samples. In addition, a quite small change (about 10%) of sAA was observed in healthy subjects with constant pH and variable flow rate during sample gathering. Fig. 3B shows the trend of sAA vs flow rate for a healthy subject who had a constant pH 7.2 for the collected samples. Then, variations of sAA activity greater than about 20% can be considered statistically significant only at constant pH, and can be used for clinical purposes~~.~~, ~~As~~ as already reported in another studies on the influence of OF sampling procedures [43,44].~~shown in a previous work [50], stimulated OF samples are obtained at constant pH (about 7.2).~~

3.2 Optimized procedure for the determination of cortisol in oral fluid by HPLC-ESI-Q-ToF

Fig. 4A shows the comparison between the extract ion (EI) chromatogram of POFS, POFS spiked with 1 $ngmL^{-1}$ of cortisol, and a POFS spiked with 35 $ngmL^{-1}$ of cortisol, whereas Fig. 4B shows the representative tandem mass spectrum of the precursor ion of cortisol achieved applying a 25 V to the collision cell. The product ions with m/z 121.06 (quantifier), and 327.19 and 309.18 (qualifiers) showed the higher relative intensity and a good repeatability n = 5, RSD = 3). The determination of the analyte was performed by an internal calibration curve obtained by spiking POFS at five concentration levels (1, 5, 10, 50, and 100 $ngmL^{-1}$). The equation of the calibration curve for spiked samples, used to quantify of the analyte, was $y = 2.1\ 10^3\ x + 5.2\ 10^3$, correlation coefficient (R^2) of 0.9999.

3.2.1 Method validation

The calibration curves were linear in the range 0.5–100 $ngmL^{-1}$. The Mandel's fitting test confirmed the linearity of the calibration curves, both for calibration solutions and standard additions (fortified POFS extracts), with an R^2 of 0.9998 and of 0.996, respectively. The equation of the calibration curve for calibration solutions was $y = 1.0\ 10^3\ x + 0.5\ 10^3$, whereas the equation of the calibration curve for fortify POFS extracts was $y = 1.1\ 10^3\ x + 0.6\ 10^3$. The investigated range of concentrations was selected after determining the concentration of cortisol in the POFS (1 $ngmL^{-1}$).

The matrix effect was excluded by comparing the slopes of the calibration curves, at a confidence level of 95%.

LOD and LOQ values equal to 0.3 and 0.8 $ngmL^{-1}$ were calculated in accordance with IUPAC guidelines [50]~~The LOD and LOQ values, calculated in accordance with IUPAC guidelines [56], were 0.3 and 0.8 $ngmL^{-1}$, respectively~~. The cortisol recovery and the corresponding intra- and inter-day relative standard deviation (RSD) are reported in Table 2.

Table 2

Recovery, intra- and inter-day precision of the determination of cortisol both in calibration solutions and in spiked POFSs.

	Concentration [ngmL^{-1}]		Recovery	Intra-day[a]	Recovery	Inter-day[b]
	Expected	Measured	%	RSD	%	RSD
Calibration solutions	0.90	0.80	88	6	89	9
	9.6	9.1	95	5	96	6
	101	98	97	3	96	4
Spiked POFSs	0.80	0.70	86	7	87	10
	10.1	9.3	92	4	92	7
	99	95	96	3	95	7

[a] Calculated from ten replicates at each concentration value in the same day.

[b] Calculated from ten replicates at each concentration value in three different days.

3.2.2 Stability of cortisol in OF samples and calibration solutions

All the calibration solutions ~~where~~ were stable when stored for 8 hours at room temperature and 4 weeks at 4 °C, as in both cases the measured cortisol concentrations were within 5% of the initial value, which is consistent with inter-day precision. The cortisol concentration in POFS aliquots spiked with 10 ngmL^{-1} decreased of 10% when stored at room temperature up to 8 hours, and remained stable for four weeks at 4°C, since a decrease less than 2% was observed for all samples.

3.3 sAA activity and cortisol levels in oral fluid samples from psoriatic subjects undergoing the TSST

sAA activity and cortisol levels were determined in OF samples collected from psoriatic volunteers undergoing the TSST. All these subjects received systemic medications for psoriasis treatment. Subjects 3 and 5 showed a mild psoriasis, with a PASI of 11 and 8, respectively, at the time of the test, whereas symptoms were almost absent (Subjects 4 and 6) or absent in the other subjects (Subject 1 and 2).

Fig. 5 shows the variations of sAA activity and cortisol concentration in OF samples collected from the psoriatic subjects enrolled in the study. The analytical methods were suitable to measure the sAA activity and the cortisol concentration levels and to finely monitor their variations (full data are reported in Table 1S and Table 2S of supplementary information). Different patterns of response

to the test were observed, and non-verbal behaviors during the speech task were quantified by means of the ECSI, and the results are reported in Table 3.

Table 3

Non-verbal behavior categories assessed for each psoriatic subject with the ECSI during TSST.

Non-verbal categories	Psoriatic subjects					
	Subject 1	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**	**Subject 6**
Eye Contact	100	100	100	95	100	100
Flight	45	120	95	140	110	100
Submission	0	40	20	15	10	5
Affiliation	85	65	80	145	100	25
Gesture	100	55	60	25	25	50
Conflict	220	70	115	5	35	0
Assertion	30	70	70	10	55	0
Relaxation	10	10	20	105	55	15
Prosocial	85	105	100	160	110	30

Results of ethological analysis also indicated that we are not in agreement with the hormonal responses. Because some behavioral patterns exhibited during the stress interview (TSST) and grouped together in the categories reflect a higher stress perception, in our study conducted in psoriatic patients. In particular, the subject 1 and subject 3, which are characterized by a blunted neuroendocrine response as shown by both cortisol and sAA levels, they displayed a higher activation from non-verbal point of view, along with a higher score in Conflict/Displacement category. According to Troisi [~~52~~46], this behavioural category, largely described in animal models, gathers non-verbal behavioral patterns that appear in situations characterized by social tension and seem to reflect increased autonomic arousal. There is a large body of evidence showing that, both in non-human primates and human subjects, the occurrence of these behaviors is associated with quite heterogeneous social situations that have in common uncertainty and anxiety [~~52~~46]. For subject 1 the incongruence between hormonal (Fig. 5A) and behavioural assessment was also emphasized by a higher score in the gesture category, which is an index of a higher global psychomotor activity. Subjects 4 and 5 are characterized from neuroendocrine point of view by a hyperarousal with an increase of sAA activity (Fig. 5D and 5E), which corresponds to an autonomic activation. On the other hand, the subjects exhibited a higher score in relaxation category, which consists of behavioral patterns indicative of a low level of emotional arousal. On the other hand a reduced activation in

hormonal response was observed in subject 2 and subject 6 (Fig. 5B and 5F), characterized by a reduced activation in hormonal response, in agreement with a hypoactivity in non-verbal behavioural assessment. This is in accordance with our results that suggest how individual stress perception evaluated by non-verbal behaviour assessment is not necessary in agreement with the hormonal profile.

This hormonal-behavioral dissociation in psoriatic patients corroborates how the stress hormonal response is likely to be altered for HPA axis changes induced by the disease in this particular category of subjects, but it does not affect the behavioral field assessed through evaluation of innate non-verbal behavior.

4. Conclusions

~~An i~~Individual~~'s~~ reactions to ~~a particular~~ stressors, in our study represented by TSST, depends upon how stressful it is perceived. This individual response, both from the hormonal and behavioural point of view, suggested how psoriatic patients perceived stressful condition compared to hyper or hypoactivity of HPA axis. Clearly, a larger number of subjects and an independent evaluation of the stress levels is needed together with a carefully designed study to reach more firm conclusions and verify if psoriatics have a different response to TSST compared to healthy subjects. In a larger interventional study, psoriasis patients with self-reported, stress-responsive disease had higher levels of worry, more severe disease, lower baseline salivary cortisol levels and a blunted serum cortisol response to a social stressor, when compared to psoriasis patients with self-reported, non-stress responsive disease. Patients, who experienced persistently high daily stressors, had lower mean blood cortisol levels than those with lower levels of daily stressors [~~66~~60]. This is in accordance with our results that suggest how individual stress perception evaluated by non-verbal behaviour assessment is not in line with hormonal profile.

In conclusion, the proposed methods, even if applied to only a limited number of psoriatic volunteers, showed their potential in the study of not completely understood mechanisms of activation of the two biological systems, ANS and HPA, in response to a stressor. Moreover, information were achieved on the role that stress-related biomarkers, such as cortisol and sAA, play in these mechanisms. On the basis of these premises, the quantification of salivary stress-related biomarkers, which are representative of the two main biological systems involved in responses to stressors, could be also applied to evaluate the effectiveness of stress reduction strategies in the management of psoriasis.

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List of figure captions

Fig. 1. Stability of the sAA activity in OF samples stored at room temperature for 8 hours. Data are normalized to the first measured value, error bars represent the RSD on of the measurements performed in triplicate.

Fig. 2. Levels of sAA activity measured in OF samples stored at 4 °C (empty ~~full~~ square) and -80°C (~~empty~~ full square) over four weeks. Activity data normalized to the value obtained on the first day of analysis, error bars represent the RSD on of the measurements performed in triplicate.

Fig. 3. Effect of salivary pH and flow rate on sAA enzymatic activity: (A) at a constant flow rate; (B) at a constant pH.

Fig. 4. ~~(A)~~ Overlapping of EI chromatograms obtained for unspiked POFS (dotted line), a POFS sample spiked with ~~1~~ 5 ngmL^{-1} of cortisol (dashed line), and a POFS sample spiked with ~~35~~ 20 ngmL^{-1} of cortisol (continuous line).~~; (B) ESI-Q-ToF tandem mass spectrum of cortisol applying a 25 V to the collision cell.~~

Fig. 5. sAA activity and cortisol levels determined in OF samples collected from psoriatic patients during the experiment. The TSST started at t_1 and ended at t_2.

SUPPLEMENTARY INFORMATION

Table 1S

Salivary cortisol concentrations in OF samples collected from psoriatic subjects before and after a TSST.

	Salivary cortisol [ngmL^{-1}]				
Sampling time	**t_0**	**t_1**	**t_2**	**t_3**	**t_4**
Subject 1	1.9	0.9	1.3	2.6	3.0
Subject 2	2.0	2.4	2.9	3.4	12.0
Subject 3	2.1	1.9	2.2	1.7	1.9
Subject 4	4.3	3.0	1.7	1.8	1.4
Subject 5	0.7	0.7	0.6	0.5	0.8
Subject 6	2.4	2.5	2.0	2.3	1.8

Table 2S

sAA activity in OF samples collected from psoriatic subjects before and after a TSST.

	sAA activity [UmL^{-1}]				
Sampling time	**t_0**	**t_1**	**t_2**	**t_3**	**t_4**
Subject 1	228	228	262	268	326
Subject 2	471	406	640	608	662
Subject 3	262	257	257	290	380
Subject 4	341	518	699	874	718
Subject 5	769	1156	1424	655	817
Subject 6	256	225	310	431	581

Fig.1
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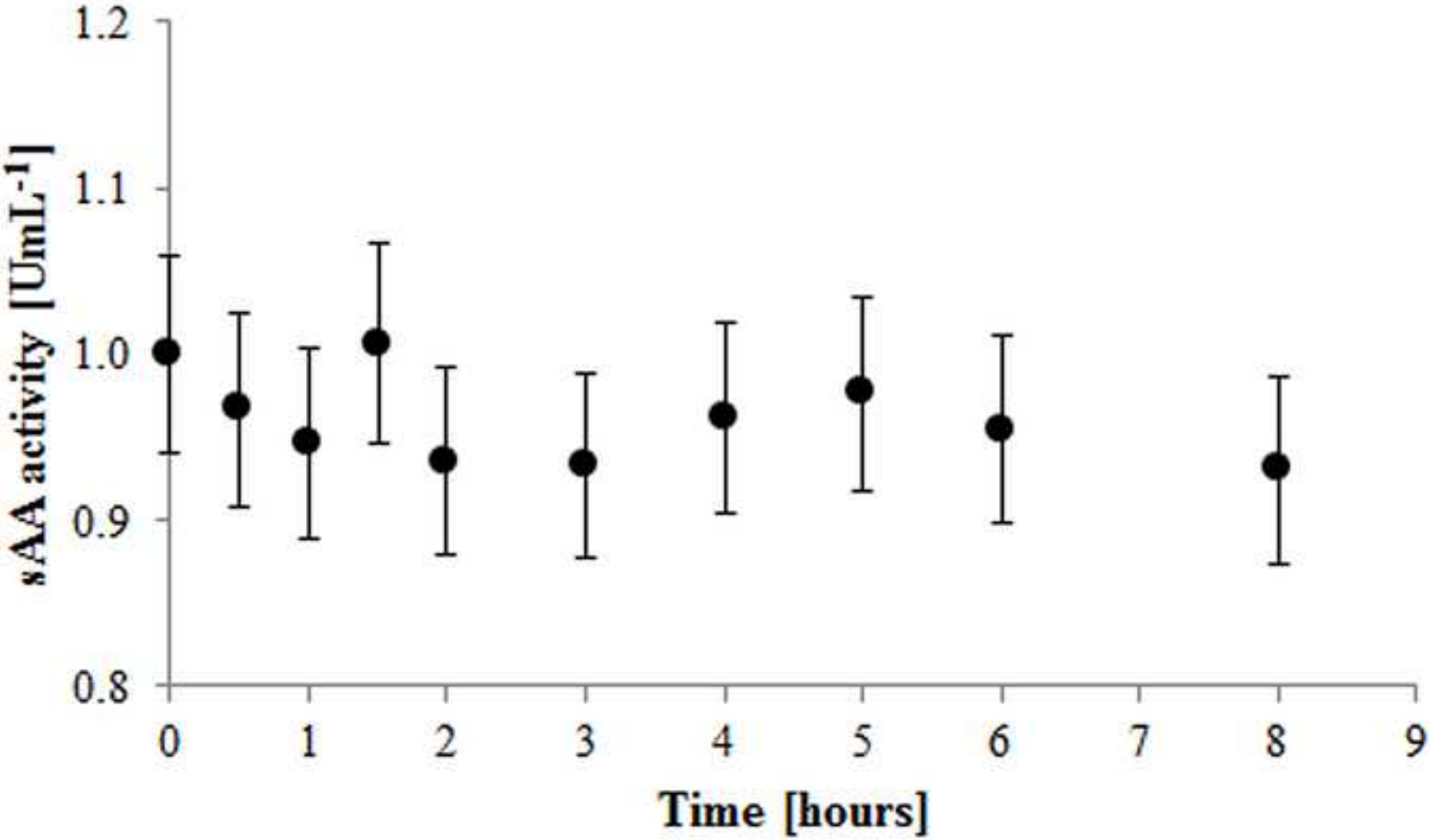

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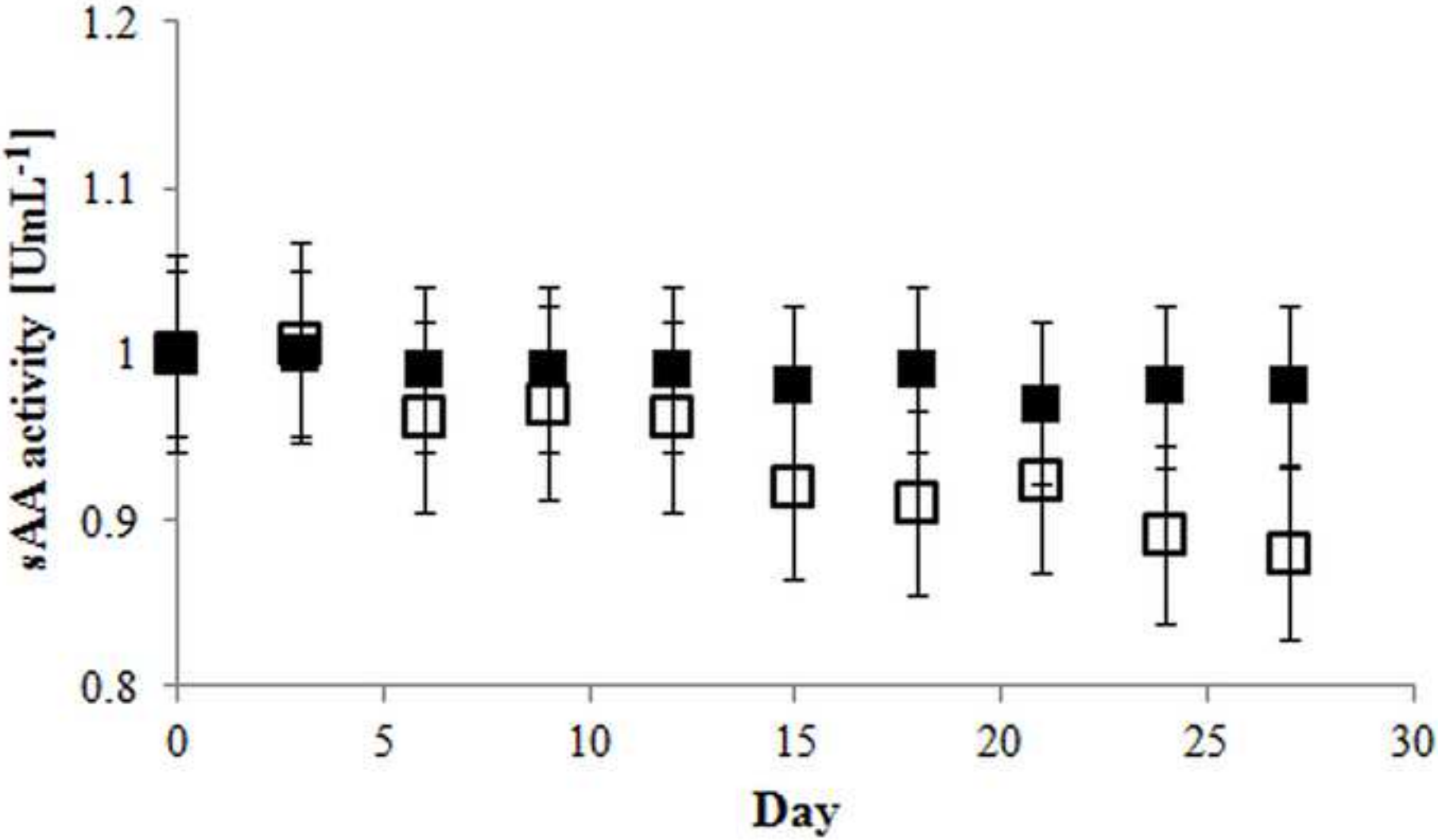

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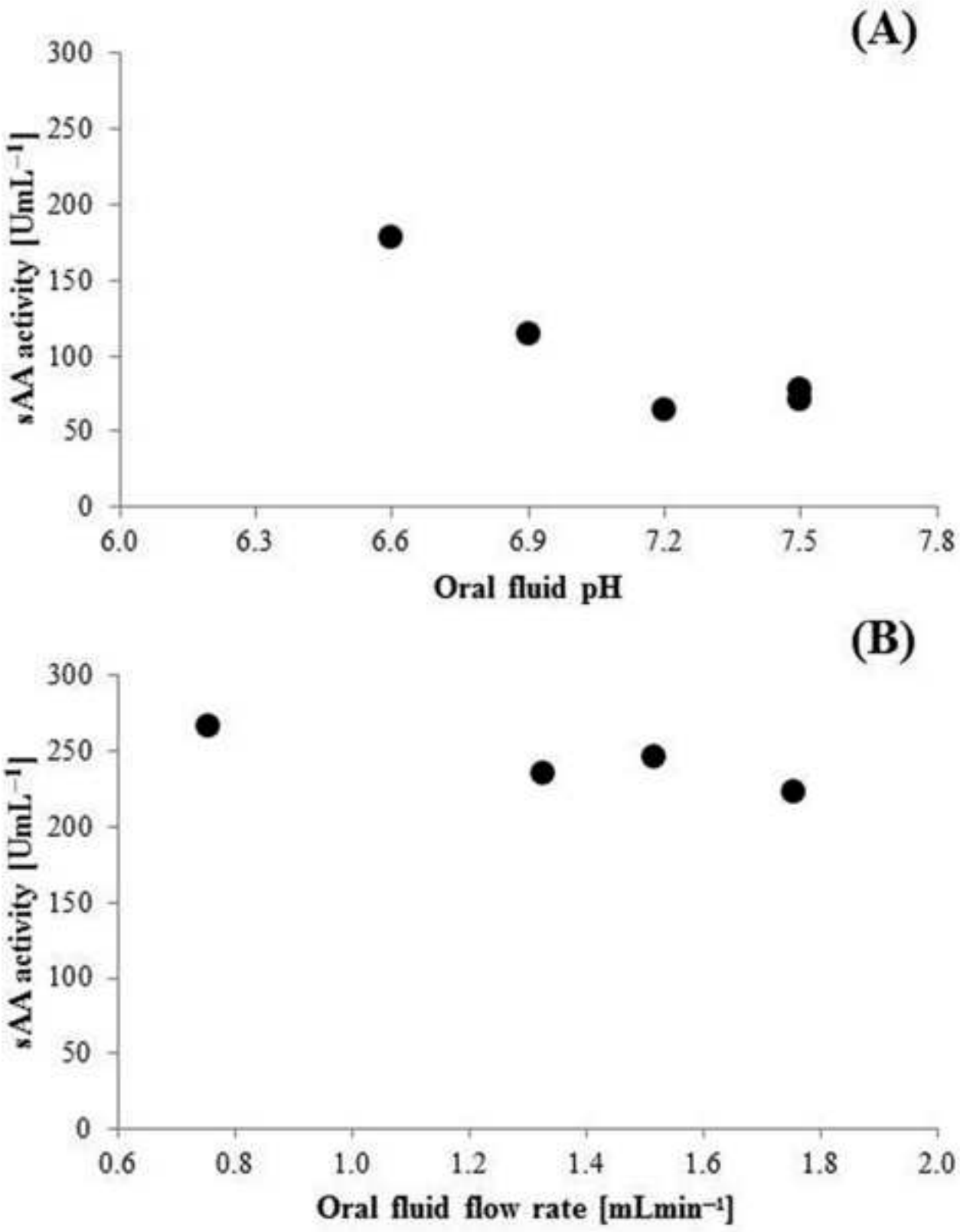

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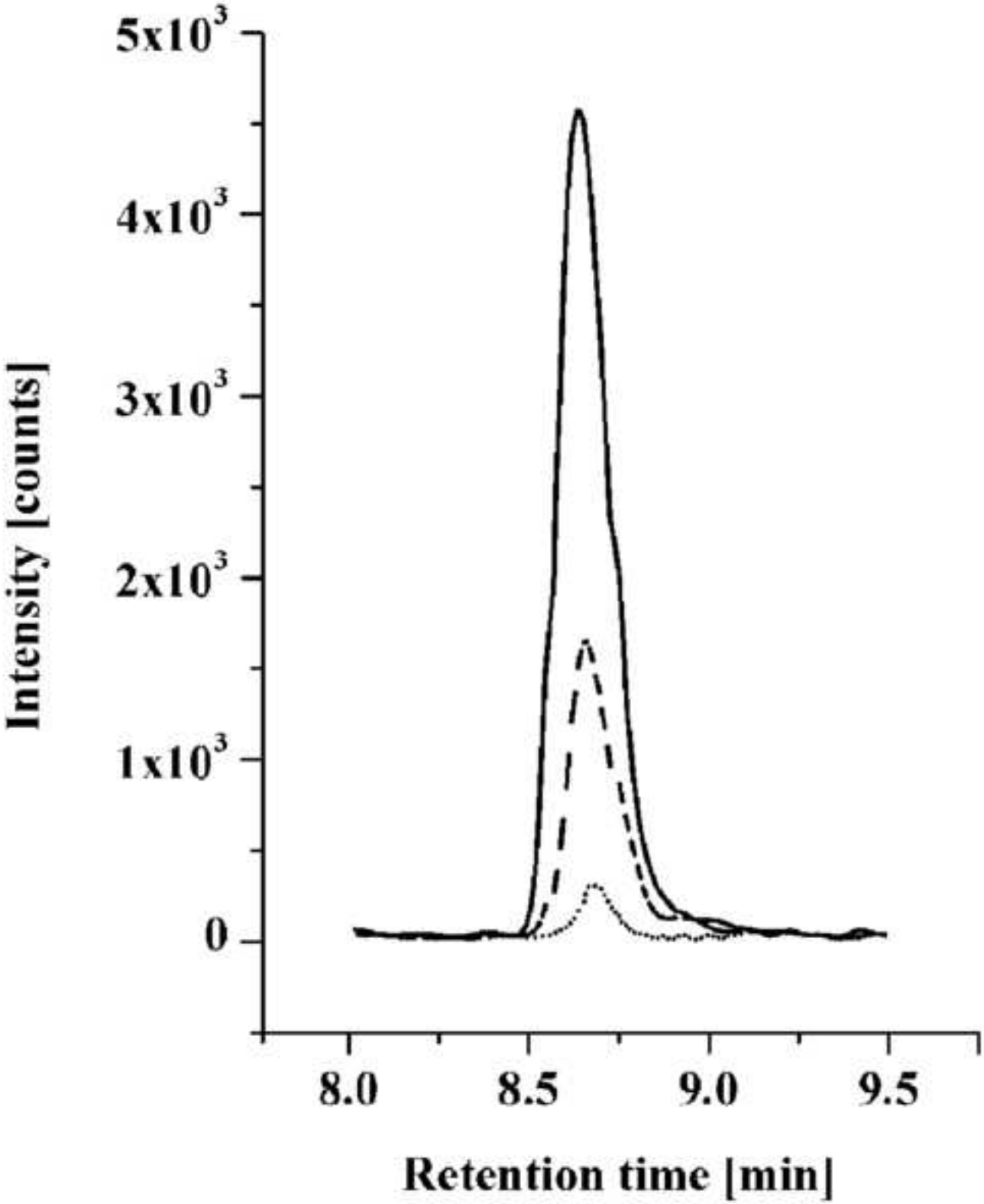

Fig.5

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