STRESS AND SALIVA :
BIOMARKER QUANTIFICATION AND METABONOMIC PROFILING

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01 BACKGROUND

01.FACTS

Did you know that 1st cause of plane crash remains human mistake ?

In 2015, 16 fatal aircraft accidents occasioned 560 deaths. Because pilots are actively engaged during each stage of a flight, there are numerous opportunities for this to go wrong, from failing to program the vital flight-management computer to miscalculating the required fuel uplift.

That's why stress and fatigue management remains a constant preoccupation in aeronautics in order to keep aerial people transportation **safe**.

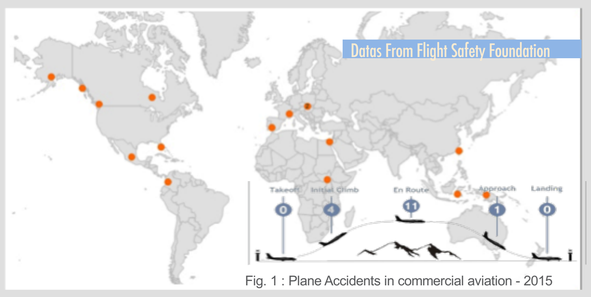


Fig. 1 : Plane Accidents in commercial aviation - 2015

02.BIOVOC PROJECT

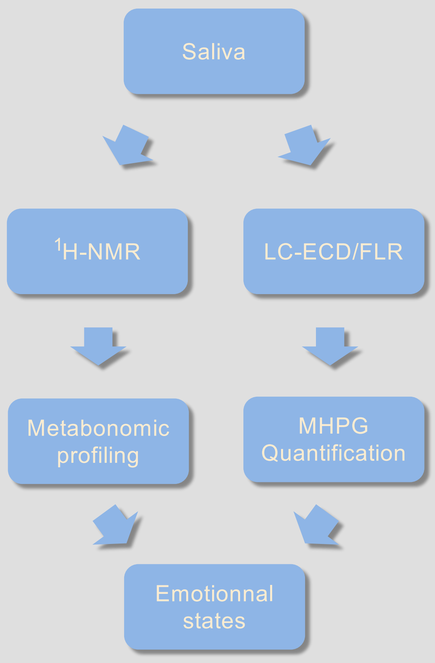
Biovoc project intends to objectify cognitive overload and mental fatigue of subjects using non-invasive methods. The goal of the project is to detect in **pilots' voice** recordings whether they are able to take control of the aircraft.

Our work aims on correlating physiological changes due to stress and emotions to voice signal modulations.

We chose **saliva** as a biofluid because its sampling is convenient and therefore limits bias due to experimental conditions.

03.INNOVATIVE STRATEGY

In this work, two strategies are proposed to assess physiological impact of emotional states. The first one involves a targeted analysis with the quantification of a biomarker named 3-methoxy-4-Hydroxyphénylglycol (**MHPG**). The second one uses a non-targeted analysis: **metabonomic profiling**. We applied our two strategies for a first set of assays in a driving simulator to evaluate the impact of **cognitive load and fatigue**. A second protocol concerns the assessment of psychosocial stress obtained from subjects performing the Trier Social Stress Test (TSST).



02 METHODS

01.BIOMARKER QUANTIFICATION

MHPG, the final metabolite Norepinephrine, is known to increase in **stressful situations**.[1,2] MHPG salivary concentration is correlated to blood concentration[3] which makes it a good candidate as a salivary biomarker of stress.

Its electroactive properties allow us to quantify it accurately with **electrochemical** methods such as amperometry.

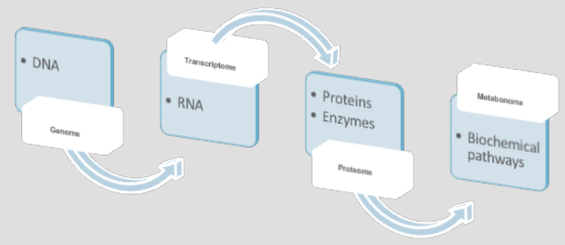
A **UPLC-ECD/FLR** method is under development for the quantification of salivary free MHPG in physiological range (10 - 15ng/mL).[4,5]

Separation	UPLC Acquity Waters
Detection	-Amperometry (Roxy reactor cell – Antec ©) -FLR Acquity detector
Column	Acquity CSH Phenyl-Hexyl (Waters) 1.7 µm x 2.1 x 100 mm – T° : 30° C.
Mobile phase	iso. 10 : 90 (v/v) ACN:H_20 15mM Ammonium acetate
Electrochemical conditions	Working potential +0.7V Magic Diamond working electrode Pd/H_2 reference
Analysis time	6 min
Flow rate	LC : 0.400 mL /min – ECD : 20µL/min

02.METABONOMIC PROFILING

The **metabnomic profile** depicts a view of all metabolites from a cell, a tissue, an organ or an organism. It relies on DNA expression but also on environment.

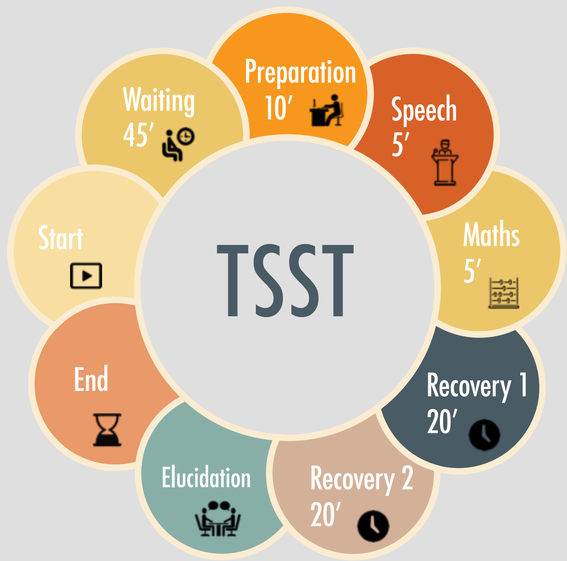
In order to assess the evolution of saliva metabolites when the subject is placed in a **stressful environment**, we decided to adapt the Trier social stress test[6] and observed the evolution of the metabonome at different moments of the test.



Saliva is prepared by microcentrifuge filtration and analyzed by ^{1}**H-NMR** 128 scans with NOESY-presat for water suppression[7]. Principal Component analysis (PCA) on SimcaP+ allows us to compare subjects[8].

The Trier Social Stress Test (TSST)

TSST was created in 1993 to induce psychosocial stress for research purposes. In this adaptation, we sample saliva at different moments along the tasks. Heart rate is monitored during a part of the test. The subject is asked to answer the STAI-Y (State-Trait Anxiety Inventory, a self assessment anxiety questionnaire) 3 times during the test. This will let us know how anxious the subject feels.



03 RESULTS

01.Method development

MHPG quantification

A calibration curve has been plotted with different aqueous standards of MHPG signal obtained by **ECD** and **FLR** detection. The results are comparable on both detection modes in terms of sensitivity in the 5-50 ng/mL linearity range with a **LOD** at **6,7** and **7,2** ng/mL for ECD and FLR respectively.

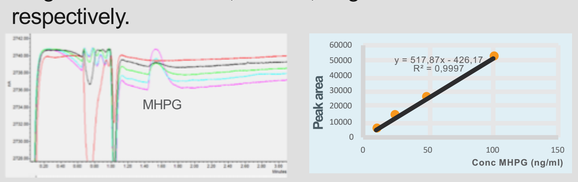


Fig. 2 : Overlay of chromatograms obtained on UPLC-ECD for aqueous standards of MHPG and calibration curve

Fig. 3 : Overlay of chromatograms obtained on UPLC-FLR for aqueous standards of MHPG and calibration curve

Sample preparation

Here we compared 2 SLE and 1 SPE method to extract and concentrate MHPG.

For all protocols : dilution of the saliva pooled and spiked samples with H_20 1:1 and elution with ethylacetate (EA).

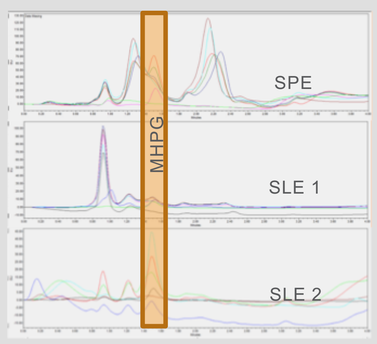


Fig. 4: Comparison of different sample preparation methods. Each figure represents the overlay of chromatograms obtained on UPLC-FLR for a growing concentration of MHPG

Preliminary results showed that the cleaner signal is obtained with SLE cartridge. SLE protocols will be further optimized to achieve a higher recovery and a better reproducibility.

02.TSST Study (on going)

17 subjects on 30 adult males took part to the TSST. Datas need to be completed for building a solid analysis of salivary metabonomes.

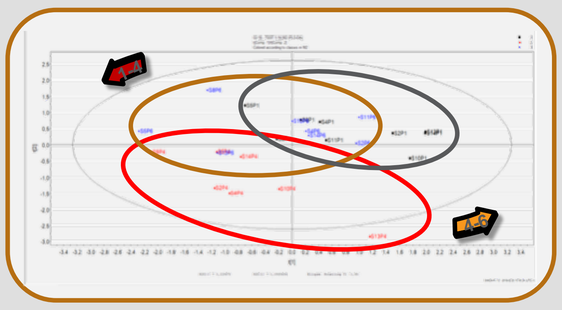


Fig. 5 : Score plots obtained after multivariate analysis performed on saliva samples 1H-NMR spectra. P1 is the sample collected at the beginning of the test, P4 right after stress tasks and P6 after the 2nd recovery period.

The scores plot presented below depicts a common behaviour for the three groups of sample with a back to normal trend at the end of the test.

This first assay let us think that there must be a metabonomic signature due to the stress task. More subjects are needed so as to build a more solid comparison of saliva samples.

From these first conclusions, we also explored the spectra to uncover which metabolites are responsible for the separation of samples.

	Metabolites signal intensity after stress tasks
More intense	Propionate
	Beta hydroxybutyrate
	Acetate
	Cystein
Less intense	Lactate
	Glucose
	methionin
	Choline
	Pyruvate

04 CONCLUSION & OUTLOOKS

01. PROJECT PROGRESS

The **multi-faceted approach** proposed in this project is very innovative in stress studies. Based on targeted biomarker quantification and non-targeted **saliva metabonomic profiling**, this on going study aims to build a better knowledge of the impact of stress and emotions on biochemical pathways.

A UPLC – ECD/FLR method has been developed for MHPG quantification. Specificity and sensitivity seem high enough for determination of salivary free MHPG in physiological range (10-15ng/mL)

The ongoing **TSST study** shows interesting preliminary results regarding the influence of psychosocial stress on salivary metabolic signature.

We identified some of the metabolites involved in the discrimination of subjects groups. A comprehensive study is being conducted to explore the mechanisms explaining this metabolic signature of stress.

02.NEXT STEPS

Final optimization of the LC and sample prep methods has to be achieved. Once settled, the method will be validated in accordance of the method of accuracy profiles.

The **TSST study** will be continued to assess the impact of environmental stress on salivary metabonomic profile.

03. FURTHER DEVELOPMENTS

To get a more detailed view of **physiological impact** of emotions, a **second** salivary **biomarker** will be targeted (salivary α-amylase or Cortisol for instance).

More subjects and controls must be included in the TSST study to raise the solidity of the comparison.

A new version of this protocol has been developped to take into account the speech signal variations. The results obtained on this parallel study will allow us to confirm the observations realized on this first work.

The methods will be thereafter used in a driving simulation context to control its efficiency before being adapted to plane simulator.

The final step of this project will consist of linking the voice signal changes to physiological expressions of emotions.

*Ready to take off ? Spit on the windscreen.

Method Validation → Driving Simulator Study → Plane Simulator Study

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