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Assessing emotional states :
Metabonomic profiling and quantification of a salivary biomarker

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**.

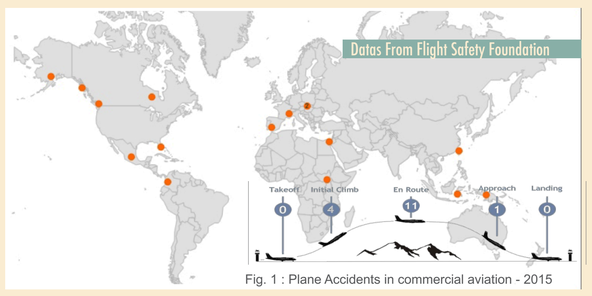


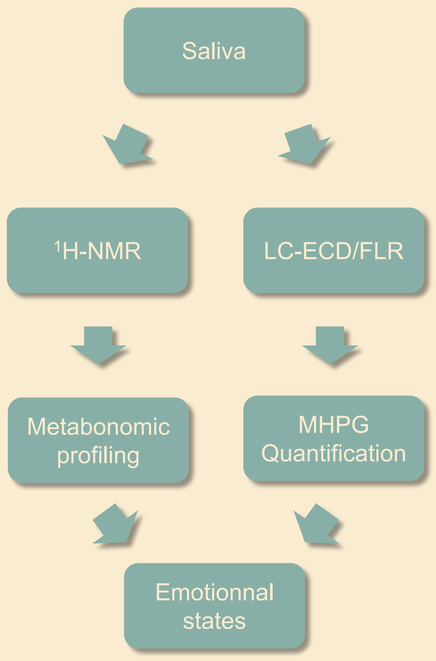
Fig. 1 : Plane Accidents in commercial aviation - 2015

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

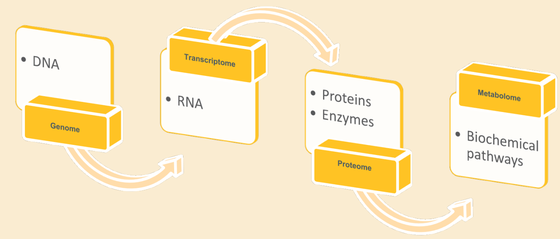
01.BIOMARKER QUANTIFICATION

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 : 50µL/min

02.METABONOMIC PROFILING

The **metabolome** 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 salivary metabolome when the subject is placed in a **stressful environment**, we decided to adapt the Trier social stress test and observed the evolution of the metabolome at different moments of the test.



Saliva is prepared by microcentrifuge filtration and analyzed by **^{1}H-NMR** 128 scans with NOESY-presat sequence to get rid of proteins signal. Principal Component analysis (PCA) on SimcaP+ allows us to compare subjects.

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.

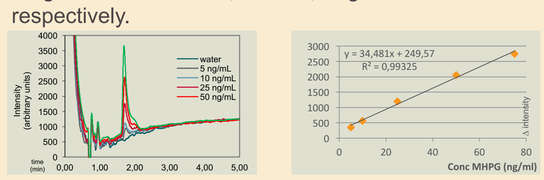


Fig. 2 : Overlay of chromatograms obtained on UPLC-ECD for aqueous standards of MHPG and calibration curve

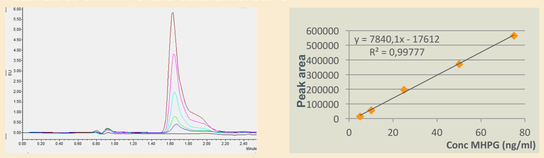


Fig. 3 : Overlay of chromatograms obtained on UPLC-FLR for aqueous standards of MHPG and calibration curve

Sample preparation
Saliva is a complex matrix that needs a pretreatment before injection. 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).

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.

SLE 1 : Isolute + Biotage © (Diateoms)	SLE 2 : Novum Luna Phenomenex © (Polymer)	SPE HLB Oasis Waters © (polymer)
Load 400µL sample	Load 1mL sample	1mL EA
Wait 5′	Wait 5′	1mL MeOH conditioning
900µL EA elution	5mL EA elution	1mL Water equilibrating
Wait 5′	Wait 5′	Load sample
900µL EA elution	5mL EA elution	H_20 Wash
Wait 5′	Wait 5′	1mL EA elution

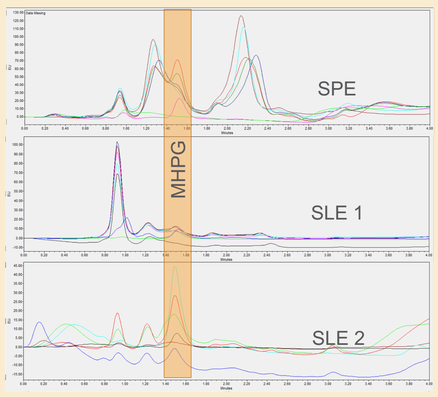


Fig. 4: Comparison of different sample preparation methods. Each figure represents the overlay of chromatograms obtained on UPLC-FLR for a growing concentration of MHPG

02.TSST Study (on going)

10 subjects on 30 adult males took part to the TSST. Datas need to be completed for building a solid analysis of salivary metabolomes.
A preliminary study was realized on student to assess the evolution of salivary metabolome related to an academic exam.

It's interesting to notice (*fig.5*) that we have been able to discriminate 2 groups using salivary metabolome :
-after academic exam
-resting condition
In fig. 6, are listed metabolites that could explain this group separation.

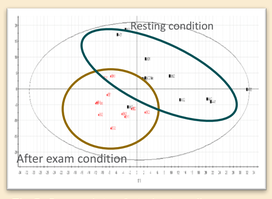


Fig.5: Score plots of students saliva Resting condition compared to after exam condition. We clearly see two different groups based on saliva metabolome

More intense	Alpha hydroxy n butyrate / n Butyrate
	Propionate
	Beta hydroxybutyrate
	Alphahydroxybutyrate / hydroxyvalérate
	Acetate
	Proline
	Valine / acetoacetate / alpha amino butyrate
	5 aminopentanoate
	Arabinose
Less intense	Lactate
	Glucose
	Allantoïne
	Urea
	3,4 dihydroxymandelate

Fig.6: Classification of salivary metabolites. After exam condition compared to resting condition

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 metabolomic 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)

A study is conducted to assess the impact of stress on salivary metabolome of male subjects.
We need to recruit more subjects to raise the robustness of the comparison.

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 metabolomic 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 DHEA for instance).

According to the results of TSST study, we will consider the use of sweat as biofluid for assessing the metabolome evolution.

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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