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Direct infusion Electrospray Mass Spectrometry as a new non-invasive tool for serum metabolomics in induced-stress subjects

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ABSTRACT – Background and Objectives: Nanotechnology is becoming a tool for the study of changes in the metabolome of patients in different states of disease. Analytical techniques such as Electrospray Mass Spectrometry, allow to find biomarkers by determination of metabolites. Nowadays, there is not an objective analytical approach for diagnosis of stress. Thus, the objectives of this pilot work are:

– Describing the development of a fast, direct and non-invasive analytical protocol, applied for the first time, to study the metabolomic profile of patients in different states through a disease.

– Testing the protocol in a pilot sample with non-stressed and stress-induced subjects.

Methods: High resolution direct infusion electrospray mass spectrometry has been used to analyse the metabolome of blood samples (0.3 ml) from six subjects.

Results: Data prove a clear discrimination between non-stressed and stressed states in the metabolome. Data showed different predominant metabolites in both states. Results allow objective characterization of the state of the patient.

Conclusions: Although this is a pilot study, the method was successful in discriminating different metabolites in non-stressed and stress-induced subjects.

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Introduction

The main aim of biochemical medicine is to understand the molecular biological mechanisms and being able to monitor them¹. These mechanisms can be studied by analysing the expressed genes and their corresponding proteins². However, our interest lies in knowing the differences in patients' biosynthetic cycle, which can help us to understand the different disease processes in subjects who present equivalent biological and environmental conditions³. The human cells produce small molecules known as metabolites. A metabolic profile is important to understand the human biology^{4,5}.

Metabolomics allows fast and simultaneous measurement of hundreds of metabolites of medical interest. Thus it is one of the tools to open up new prospects with great potential in the study of diseases^{6,7} such as coronary artery disease¹¹, in diagnostic protocols⁸, the search for biomarkers⁹ and the development of drugs¹⁰. The metabolomic analysis of cerebrospinal fluid has enabled us to predict the clinical outcome of subarachnoid haemorrhage¹². Metabolomic profiling in serum has also allowed us to classify patients with neurological diseases such as amyotrophic lateral sclerosis¹³.

Stress affects the biological functions causing biochemical instability and biological failure^{14,15}. Stressors can promote disturbances ranging from immune system dysfunction to psychiatric disorders^{16,17}, and cause neuropathological changes similar to those provoked by Alzheimer's disease¹⁸ or dementia¹⁹.

Stress is considered as a situation of biological and psychological activation triggered by the interaction of the individual with external agents that force his/her capacity of adaptation and survival²⁰. Stress is associated primarily with cortisol, the main stress hor-

mone which alters the body's functions^{21,23}. The biosynthesis of the cortisol cycle has been well-studied²⁴⁻²⁶. Cortisol has wide-ranging systemic effects on the organism: regulating insulin in blood²⁷, linked to the development of type II diabetes disease, to inflammatory processes (tumors) or immunological diseases²⁸, obesity, cardiovascular diseases, thyroid disorders, mental illness²⁹ and depression^{30,31}. The measurement of cortisol does not provide an overview of the biochemical profile, so individual specific metabolomic conditions in each patient cannot be known neither if there is a connection to any other pathology³². Likewise, tests carried out by psychologists to diagnose stress are highly subjective. They are composed of questions that the researched subject must understand and also differentiate and compare their emotional state. Besides, they also depend on the therapist's conclusions interpreting the answers and the morphological analysis of the patient³³.

Subsequently, stress has been considered, in this work, as an important factor to be studied under the metabolomic approach. The metabolome is the response of an organism to an alteration, and is a very useful tool for understanding the biological mechanisms of complex organisms. It is a step beyond the classical approach of biochemistry. This new approach applied to the study of stress is of importance since its method of diagnosis is limited. Nor are we aware of the behaviour of the metabolome in the early stages of any symptoms (stress) or what future links, there may be to any subsequent diseases occurring in the individual. This could allow us to set a specific diagnosis for each case and therefore initiate treatment prior to the development of the disease. Therefore, the identification of key biomarkers with a fast, simple and complete analysis is important for the early detection of certain diseases. In addition,

since stress is a multifactorial problem, it can be understood that it is unlikely that just a single biomarker (cortisol) is relevant to its possible diagnosis. A panel of biomarkers and their values are likely to be a more sensitive and specific approach to study the disease³⁴.

Current analytical methods such as NMR and mass spectrometry (MS) are often combined with chromatography, which requires several millilitres of sample and a specific clinical protocol³⁵. Chromatography gives good analytical results but, it has limitations when it comes to the yield and performance of the sample. There are attempts to apply new techniques to reduce analysis time and to increase the information obtained from each analysis^{36,37} the yield per sample and the reliability.

Recently, analytical science is searching for direct methods injecting samples directly into the mass spectrometer³⁸ improving reproducibility for the same non-target metabolites and, increasing responsiveness and yield³⁹.

The aim of this pilot study is to use a non-invasive and precise technique as direct infusion mass spectrometry, to find and characterize metabolic differences in two different biological situations of an individual: relaxed and stressed states.

Materials and methods

Participants and sampling

This study has been run with volunteer subjects from the University population. The subjects had an age range from 18 to 30 years (both gender). Before performing the sessions for induced-stress and relaxation, each volunteer read and completed a survey with a questionnaire which was focused on: on the volunteer's habits and life style, consumption of coffee, alcohol, psychotropic substances, and smoking habits. Questions in relation to chronic diseases or prescribed regular drug intake were included. Possible depressed patients with pre-existing physical or mental disorders, medication, and/or illicit drug use, were excluded from the group after this personal evaluation.

Individuals were adequately informed, and they gave their informed consent to participate in the study which was approved by the Ethics Committee at Clinical Hospital "Lozano Blesa" of the University of Zaragoza, Spain.

Demographic data of the samples that were selected are shown in Table 1.

Table 1
Demographic data of selected samples for the study.

Sample number	Age (years)	Weight (kg)	Height (cm)	Gender
Sample 1	20	67	171	Male
Sample 2	19	78	180	Male
Sample 3	19	77	185	Male
Sample 4	19	54	162	Female
Sample 5	22	93	182	Male
Sample 6	24	68	176	Male
Sample 7	21	61	170	Female
Sample 8	20	70	175	Male

Design of the relaxation and stress sessions

The design and preparation of the sessions were performed under the framework of the “ES3 project”⁴⁰. Data acquisition and sample preparation were done by the Zarademp team in collaboration with BSICoS I3A team. Each research subject underwent, a 35-minute relaxation session including audio and guided relaxation, in a space with dimmed lights while in a comfortable position. This state is considered in this work as basal. Stress was induced by a modification of the Trier Social Stress Test, which is widely used in stress research^{41,42}. The protocol follows the one described in reference⁴³.

The State Trait Anxiety Inventory (STAI)⁴⁴, Spanish version^{45,46}, measures both state anxiety and trait anxiety; The STAI has demonstrated adequate reliability (Cronbach’s alpha 0.90 to 0.94 for trait anxiety and state anxiety, respectively)^{45,46}.

The Perceived Stress Scale (EEP) is the most widely used psychological instrument for measuring the perception of stress. This Likert-type scale measures a person’s perception of potentially stressful events⁴⁷. The Spanish version⁴⁸ of the EEP demonstrated adequate reliability (internal consistency, alpha = 0.81, and test-re-test $r = 0.73$) validity (concurrent) and sensitivity. Additional data indicate adequate reliability (alpha = 0.82, test-retest, $r = 0.77$), validity and sensitivity of the short version of 10 items which is used in this study.

The stress sessions began after a prior 10-minute relaxation session. To stress the research subjects a protocol based on an emotional stress test was used. Biophysical parameters and biological sampling were carried out once the session had ended and were

recorded throughout the entire test. Biochemical parameters and blood pressure were taken at the end of each session (Table 2). The biological samples were stored in sterile, airtight compartments at -80°C until the time of analysis.

Reagents and samples

Blood samples for analysis were taken by pricking participants’ fingers. Approximately 0.3 ml was collected in a sterilized container tube with no chelating agent ethylene diamine tetraacetic acid (EDTA). The samples were immediately protected from light and stored at -80°C until analysis. No blood sample underwent any pre-treatment prior to mass spectrometry analysis.

Mass spectrometry (LC-MS) grade methanol and water for ESI analysis were used (Fisher). Formic acid (99% purity- Fluka) was applied as a protonate agent. 0.45 μm PVDF sterile filters 100 PC2 ROTH (Carl Roth GmbH.CoKG- TECNOCROMA) were used.

Sample preparation

Samples were thawed on ice and homogenized with vortex agitation. 100 μl of unclothed blood was taken and 100 μl of water was added and homogenized. 100 μl of the solution was collected and added to 300 μl of LC-MS grade methanol. The resulting solution was homogenized and left to stand for 30 minutes at -20°C . Subsequently, samples were centrifuged at 10,000 rpm for 10 minutes at 4°C and the supernatant filtered with a sterile PVDF 0.45 μm filter avoiding the dead volume. For positive mode detection, LC-MS grade methanol containing 0.1% formic acid (99% purity) was added to the sample, obtaining a sample dilution of 1:1000.

Table 2
Physiological measurements in the relaxation-stress sessions.

Biophysical variables	Location on the body	Measurement features
Skin temperature (ST)	Little finger of non-dominant hand and cheek	Continuous (250 Hz)
Skin conductance (SC)	First phalanx of ring and index fingers, non-dominant hand	Continuous (250 Hz)
Electrocardiogram (ECG)	(3 sources) thorax	Continuous (1 KHz)
Respiratory signal (RR)	Thorax	Continuous (250 Hz)
Blood pressure (BP)	Arm	Non-continuous (at the beginning and end of each session)
Wave pulse (PPG)	Middle finger of non-dominant hand	Continuous (250 Hz)
Electromyogram (EMG)	Left trapezium and orbicular muscles	Continuous (1 KHz)
Biochemical variables	Location	Measurement features
Prolactin	Blood	At the end of session (10:45)
Copeptin		
Glucose		
Cortisol	Saliva	10 minutes following relaxation (10:20) and at the end of the session (10:45)
a-amylase		

Instrumentation

Measurements were taken using a hybrid triple quadrupole/linear ion trap mass spectrometer 4000 QTRAP LC/MS/MS System (AB Sciex.) with an electrospray ionization (ESI) source interface for high-sensitivity, full-scan MS and MS/MS spectra with high selectivity. The system was operated using Analyst software version 1.5.2 (Build 5704) (AB Sciex) for data acquisition and pre-processing.

For MS/MS analysis collision-induced dissociation (CID) mode was used and was set to 30% to 50% normalized collision energy (CE) for selected molecular Mass/Charge peaks.

Data Processing and compound identification

Data was processed by using the Marker View 1.2 software (AB SCIEX) for statistical analysis, by picking and matching m/z across samples resulting into a two dimensional matrix of peaks and intensities after data normalization. m/z tolerance equalled to 50 ppm and presence of each feature in at least 3 samples. After performing a T -test, the subsequent principal component analysis Pareto (PCA) was performed. Metabolites with p -value lower than 0.05 were considered statistically significant. In order to find differences of biomarker concentration between stressed and relaxed groups Variable Importance in Projection (VIP) was used for pre-

dominant m/z selection in each group of data. R^2 and Q^2 values provided good quality of the resulting model.

Identification of significant compounds was made manually matching the experimental accurate mass and spectra with available information contained in metabolic databases. By means of MS/MS analysis, metabolite structure was confirmed by characteristic fragments described in literature or compared to standard compounds.

Results

Only the hydrophilic portion of the metabolome is presented in this paper, sample profiles were acquired for each research subject in relaxed (basal) and stress-induced condition (Figure 1). Each of these profiles showed a large number of signals, around 1500 m/z ⁴⁹. Each spectrum can be considered a metabolomic fingerprint of the subject in each state. The matrix effect on metabolomic profiles is a significant factor and is taken into account in the study. It was attenuated by optimizing the dilution factor, so the intensity of the m/z signal of each metabolite has a linear relationship with the concentration when dilutions are high^{50,51}.

In order to compare profiles of subjects in both states, two groups of m/z signal/intensity were established (X-B for basal-non-stressed state, X-E for induced-stress state). A chemometric partial least squares discriminant (PLS-DA) analysis was performed using the m/z signal/intensities of the samples from the two groups⁵². The models gave rise to a good classification outcome as shown in the score plots (Figure 2). These groups of results were evaluated statistically and the resulting models showed values in line with quality para-

eters R^2 and Q^2 (explained variance of approximately 99% and a predicted variance above 50%)⁵³. T -test analysis with p -values and data modelling using the PLS (Principal Least Squares) progression were carried out^{54,55}. A large number of signals could be studied in the discrimination of classes considering the Variable-Importance-in-Projection-(VIP) which was set at a minimum value of 2. Generally, VIP set at 1 can be considered important in a given model⁵⁶. With regard to the reproducibility of the method, this could be considered good with variation coefficients ($CV\%$) below 20% for the metabolites identified. Thus, subjectivity in the selection process is avoided⁵⁷.

A complex metabolite profile was obtained since there has not been any pre-treatment in the process of analysis. Around 1500 of MS peaks out of a sample were detected (Figure 1). All peaks were analyzed, evaluated and exported for statistical analysis. In order to differentiate between subjects in a basal and stress-induced state, the normalized data was classified into two sets (basal and stressed), and evaluated by T -test and partial least squares discriminant analysis-PCA (Figure 2).

Statistic results are shown in Table 3. Model built with the data, provided a good classification in two groups. Thus, by PCA we could confirm that the observed behavior is real and not random and study m/z peaks that can be specific for one group.

The more discriminant signals were selected according to Variable-Importance-in-the-Projection-(VIP) for later study by MS/MS and molecule identification (Table 3). After the data processing, we selected m/z values that presented a p -value which denoted a correct statistical hypothesis. Many low molecular weight metabolites presented alterations in stressed state samples as compared to basal state samples.

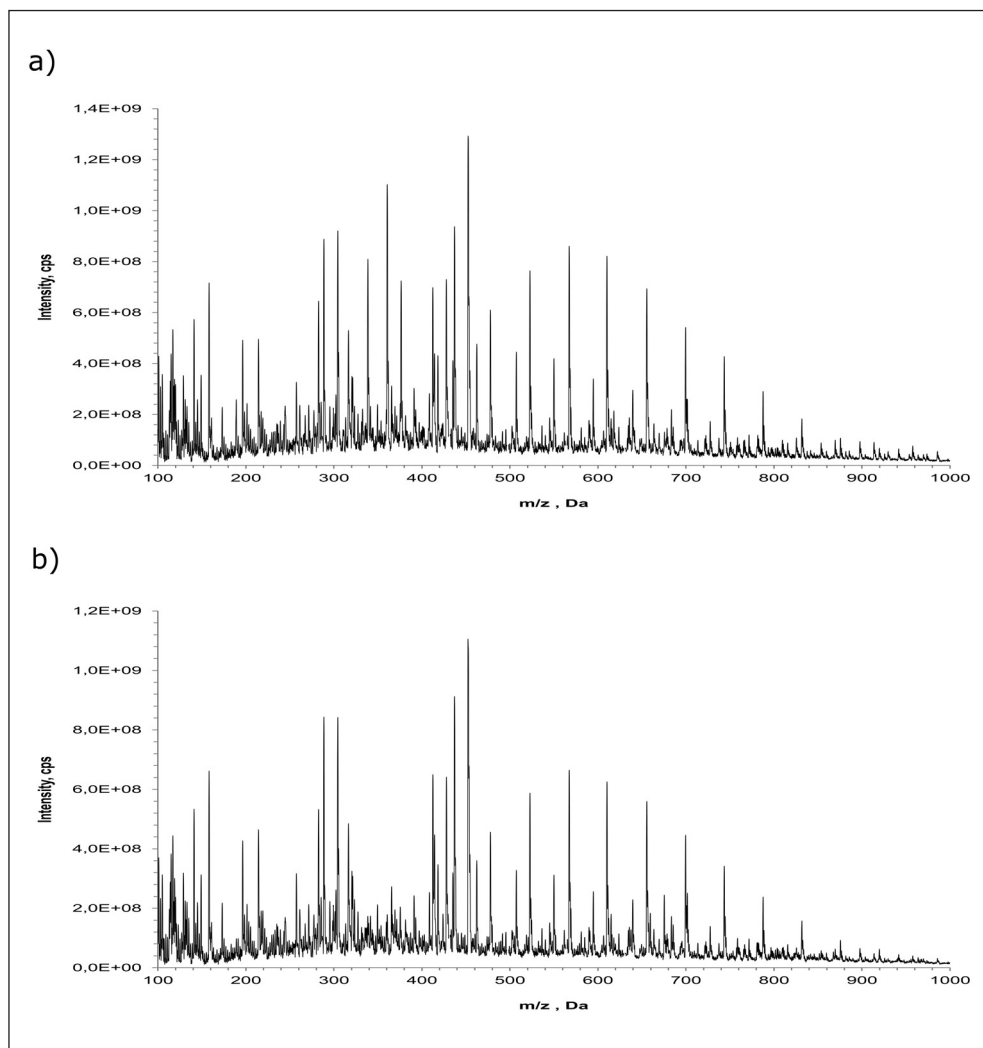


Figure 1. Example of full spectra metabolome profile of a subject detected for Basal (non-stressed) and stress-induced states.

(Range m/z (mass/charge): 100 to 1000). a) Basal state subject, b) stress-induced state subject. More than 1500 signals (m/z for possible compounds) were detected for each sample in each group of subjects.

In order to distinguish prevailing metabolites in both basal and stress groups, peaks with specific p -value and good CV were evaluated considering the VIP. Together with their VIP value, the PCA statistical analysis clarifies if the metabolite is of greater importance in each group (Table 3).

Examples of specific molecules for stressed subjects are the peak m/z 363.4653 $[M+H]^+$, (-7.26 Δ ppm) (Table 3) which was identified as Cortisol/Hydrocortisone and Estrone m/z 271.1706 $[M+H]^+$. Following this same procedure other compounds from steroid hormone metabolism were detected. Similarly,

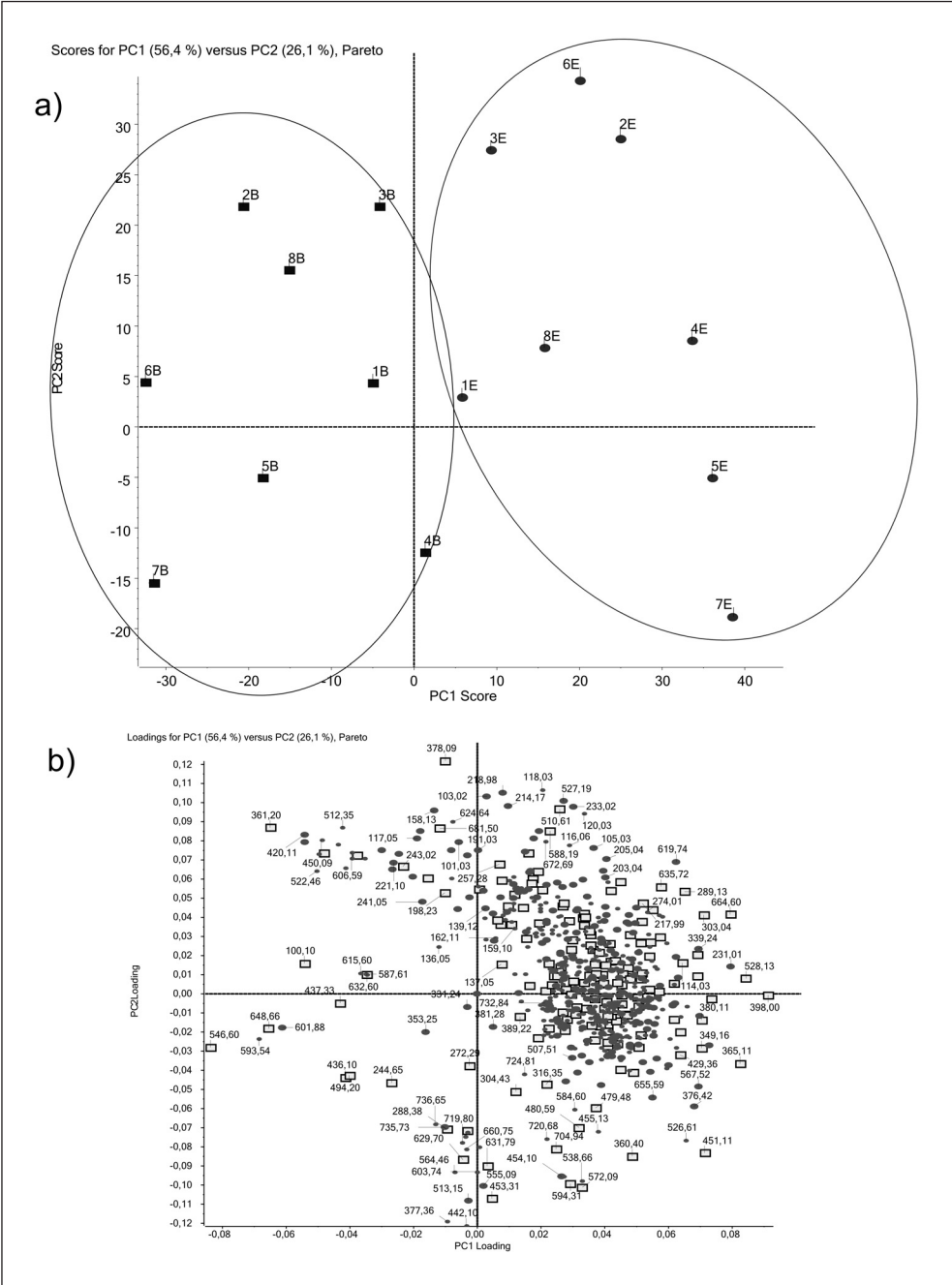


Figure 2. Diagrams of the statistical results of the whole MS (Mass Spectrometry) data found in both groups of subject states after using bioinformatics:

- a) Scores plots of Pareto PCA for basal state subject (squares), stress-induced state subjects (circles).
- b) Pareto loadings for PCA m/z (molecular mass/charge) peaks.

Table 3
Mass Spectrometry data showing only statistical significant differences between the stressed and the non-stressed state in the experimental individuals. Only the Predominant Metabolites for the Stress-Induced Group and for the Basal (non-stressed) Group are included in the table.

Predominant Metabolites in Stress-Induced Group	Formula	m/z [M+H] ⁺	Δm (ppm)	p -value	CV (%)	VIP
Cortisol/Hydrocortisone						
(11 β)- 11, 17, 21- trihydroxypregn- 4- ene- 3, 20- dione	C ₂₁ H ₃₀ O ₅	363.4653	-7.26	1.79·10 ⁻²	6.2	2.18
Aldosterone /						
11 β ,21-Dihydroxy-3,20-dioxopregn-4-en-18-al	C ₂₁ H ₂₈ O ₅	361.4485	1.77	2.58·10 ⁻³	7.6	2.09
Corticosterone /						
(11 β)- 11, 21- dihydroxypregn- 4- ene- 3, 20- dione	C ₂₁ H ₃₀ O ₄	347.2245	6.62	2.86·10 ⁻²	5.3	2.05
11-Deoxycorticosterone (DOC) /						
(1S,2R,10S,11S,14S,15S)-14-(2-hydroxyacetyl)-2,15-dimethyl tetrahydro [8.7.0.02,7.011,15] heptadec-6-en-5-one	C ₂₁ H ₃₀ O ₃	331.2253	-6.04	3.06·10 ⁻⁴	6.5	2.10
Progesterone (P4) / pregn-4-ene-3,20-dione						
	C ₂₁ H ₃₀ O ₂	315.2314	-3.17	4.10 10 ⁻²	9.7	2.68
Pregnenolone (P5) / 3 β -hydroxypregn-5-en-20-one						
	C ₂₁ H ₃₂ O ₂	317.2498	5.67	3.97·10 ⁻²	7.8	2.09
Cholesterol / (3 β)-cholest-5-en-3-ol						
	C ₂₇ H ₄₆ O	387.3598	-7.22	5.10·10 ⁻³	4.4	2.01
17 α -hydroxypregnenolone /						
3 β ,17-dihydroxypregn-5-en-20-one	C ₂₁ H ₃₂ O ₃	333.2403	-7.80	1.10·10 ⁻³	6.3	2.62
11-deoxycortisol / 17,21-Dihydroxypregn-4-ene-3,20-dione						
	C ₂₁ H ₃₀ O ₄	347.2257	10.08	2.14 10 ⁻²	7.3	2.09
17-deoxycortisol / 11 β ,21-dihydroprogesterone / (11 β)-11,						
21-dihydroxypregn-4-ene-3,20-dione	C ₂₁ H ₃₀ O ₄	347.2257	10.08	2.14·10 ⁻²	7.3	2.09

m/z (molecular mass/charge); [M+H]⁺ (ionization from protonation of molecular mass) Peak measured, p -value: function of the observed sample results (a statistic) that is used for testing a statistical hypothesis. Δm (ppm): mass error; CV: coefficient of variation; VIP: variable of importance in the projection. This data is key in the selection of specific metabolites for both basal state and induced-stress state subject group.

Table 3
Mass Spectrometry data showing only statistical significant differences between the stressed and the non-stressed state in the experimental individuals. Only the Predominant Metabolites for the Stress-Induced Group and for the Basal (non-stressed) Group are included in the table (continuation).

Predominant Metabolites in Stress-Induced Group	Formula	m/z [M+H] ⁺	Δm (ppm)	p - value	CV (%)	VIP
17 β -estradiol / (8R,9S,13S,14S,17S)-13-methyl-6,7,8,9,11,12,14,15,16, 17-decahydrocyclopenta[a]phenanthrene-3,17-diol	C ₁₈ H ₂₄ O ₂	273.1878	8.78	1.7·10 ⁻²	11.2	2.36
Estrone (E1) / (8R,9S,13S,14S)-3-hydroxy-13-methyl- 6,7,8,9,11,12,13,14,15,16- decahydrocyclopenta[a]phenanthren-17-one	C ₁₈ H ₂₂ O ₂	271.1706	2.95	8.0·10 ⁻³	12.3	3.01
Serine / 2-Amino-3-hydroxypropanoic acid	C ₃ H ₇ NO ₃	106.0514	9.42	3.20·10 ⁻³	7.2	2.41
Indole / 2,3-Benzopyrrole	C ₈ H ₇ N	118.0670	11.85	2.9·10 ⁻²	5.8	2.34
Phenylalanine / 2-Amino-3-phenylpropanoic acid	C ₉ H ₁₁ NO ₂	166.0858	-6.02	1.67·10 ⁻²	5.1	2.42
Dopamine / 3,4-dihydroxyphenethylamine	C ₈ H ₁₁ NO ₂	154.0857	-7.13	9.4·10 ⁻³	5.3	2.37
4-(2-Aminoethyl)benzene-1,2-diol	C ₈ H ₁₁ NO ₃	170.0826	5.29	2.4·10 ⁻²	5.8	2.27
Norepinephrine / 4,5- β -trihydroxy phenethylamine	C ₉ H ₁₃ NO ₃	184.0959	-7.60	8.10·10 ⁻³	6.0	2.35
Epinephrine / β ,3,4-trihydroxy-N-methylphenethylamine	Formula	m/z [M+H] ⁺	Δm (ppm)	p - value	CV (%)	VIP
Predominant Metabolites in Basal Group						
L-Tryptophan / (2S)-2-amino-3-(1H-indol-3-yl)propanoic acid	C ₁₁ H ₁₂ N ₂ O ₂	205.0967	-4.87	4.10·10 ⁻³	5.3	2.56
Serotonine / 5-Hydroxytryptamine	C ₁₀ H ₁₂ N ₂ O	177.1039	6.77	1.9·10 ⁻²	5.6	2.18
Melatonine / N-acetyl-5-methoxy tryptamine	C ₁₃ H ₁₆ N ₂ O ₂	233.1270	-8.57	3.0·10 ⁻²	6.8	2.41

m/z (molecular mass/charge); [M+H]⁺ (ionization from protonation of molecular mass) Peak measured, p -value: function of the observed sample results (a statistic) that is used for testing a statistical hypothesis. Δm (ppm): mass error; CV: coefficient of variation; VIP: variable of importance in the projection. This data is key in the selection of specific metabolites for both basal state and induced-stress state subject group.

statistical values expressed specificity for other metabolites in the metabolism of Serotonine (m/z 177.1039 [M+H]⁺), such as L-Tryptophan (m/z 205.0967 [M+H]⁺) or Melatonine (m/z 233.1270 [M+H]⁺), which were predominant in samples of basal state (Table 3).

MS/MS analysis was performed to confirm elemental composition of the peaks and identify the studied biomarkers (Table 4).

Discussion

This pilot study was designed to use direct infusion mass spectrometry as a non-invasive and precise technique to perform a metabolic-profiling screening and characterized metabolic differences in relaxed and stressed-induced subjects.

The comparison of the metabolomic composition profiles of the different states of the subject will form the basis of the search for biochemical data. The results show that the characterized metabolites by means of the new technique applied in this pilot work were different in the two compared states; relaxed and stressed-induced situations. The cortisol and their related metabolites are predominant in the stress state while serotonin and melatonin, among other metabolites, were found to be predominant in the relaxed state when comparing both groups of metabolic-profiling data.

Through studies in the past we know significantly more about the neurobiological correlations of stress⁵⁸. What we do not know is why these changes are associated with the development of disorders in some people but not in others. The importance of understanding the individual differences is key to the whole comprehension of stress.

Therefore, the possibility of developing a non-invasive, direct and fast method of analy-

sis, to assist the study of stress in an objective manner, can be considered a step forward both patient correct diagnosis and treatment. Moreover, given the risk that a patient will develop a neurological disease of greater medical significance, it would be very valuable to have, a prior basal and stressed state metabolomic profile to assist professionals treating said patient.

Since the complexity of the human metabolome is of a great magnitude, different complementary techniques for its study are being developed⁵⁹⁻⁶¹. The techniques most commonly used have been those based on NMR^{34,62-64}. However, it has its drawbacks since it cannot detect metabolites in very low concentrations, it requires blood samples in millilitres and a protocol that is slow and costly.

Therefore, the application of nanotechnology techniques in search of a non-invasive, fast and direct analytical method that could offer a patient's metabolomic-profile at a specific time is needed. In the method that we have developed the required sample amount is in microlitres (one drops of blood from the subject's fingers is enough). The sample does not undergo pre-treatment which might adulterate or remove biochemical data, so a more complete metabolomic fingerprint is expected.

Many authors in psychiatric or neurological disease research use metabolic-profiling associated to these diseases including LC/MS, GC/MS and EC/MS^{60,65,66}. However direct infusion mass spectrometry used in this work is completely unexplored.

This tool provides for the possibility of an analysis of multiple metabolites in a simple run, obtaining a metabolomic-fingerprint. Furthermore, the low-time-consuming step before MS allows for a faster analysis of samples, which reduces instrumental work and drift between analysis, increasing reproducibility and improving accuracy⁶⁷. This means an im-

Table 4
Confirmation of biochemical structure of specific found predominant metabolites in stress-induced and basal subjects.

Metabolites in Stress-Induced Group	<i>m/z</i> [M+H] ⁺	Formula [M+H] ⁺	RDBE.	MS/MS product ions <i>m/z</i>
Hydrocortisone	363.4653	C ₂₁ H ₃₁ O ₅	7	271(C ₁₇ H ₁₉ O ₃), 227(C ₁₇ H ₂₂), 61(C ₂ H ₅ O ₂)
Aldosterone	361.4485	C ₂₁ H ₂₉ O ₅	8	271(C ₁₇ H ₁₉ O ₃), 227(C ₁₇ H ₂₂)
Corticosterone	347.2245	C ₂₁ H ₃₁ O ₄	7	254(C ₁₇ H ₁₇ O ₂), 227(C ₁₇ H ₂₂), 61(C ₂ H ₅ O ₂)
11-Deoxycorticosterone	331.2253	C ₂₁ H ₃₁ O ₃	7	235(C ₁₇ H ₁₅ O), 227(C ₁₇ H ₂₂), 61(C ₂ H ₅ O ₂)
Progesterone (P4)	315.2314	C ₂₁ H ₃₁ O ₂	7	235(C ₁₇ H ₁₅ O), 227(C ₁₇ H ₂₂), 45(C ₂ H ₅ O)
Pregnenolone (P5)	317.2498	C ₂₁ H ₃₃ O ₂	6	235(C ₁₇ H ₁₅ O), 227(C ₁₇ H ₂₂), 45(C ₂ H ₅ O)
Cholesterol	387.3598	C ₂₇ H ₄₇ O	5	235(C ₁₇ H ₁₅ O), 227(C ₁₇ H ₂₂), 115(C ₈ H ₁₉), 57(C ₄ H ₉)
17 α -hydroxypregnenolone	333.2403	C ₂₁ H ₃₃ O ₃	6	235(C ₁₇ H ₁₅ O), 227(C ₁₇ H ₂₂), 45(C ₂ H ₅ O)
11-deoxycortisol	347.2257	C ₂₁ H ₃₁ O ₄	7	271(C ₁₇ H ₁₉ O ₃), 261(C ₁₇ H ₂₅ O ₂), 227(C ₁₇ H ₂₂), 61(C ₂ H ₅ O ₂)
17-deoxycortisol	347.2257	C ₂₁ H ₃₁ O ₄	7	271(C ₁₇ H ₁₉ O ₃), 261(C ₁₇ H ₂₅ O ₂), 227(C ₁₇ H ₂₂), 61(C ₂ H ₅ O ₂)
17 β -estradiol	273.1878	C ₁₈ H ₂₅ O ₂	7	243(C ₁₇ H ₂₃ O), 227(C ₁₇ H ₂₂)
Estrone (E1)	271.1706	C ₁₈ H ₂₃ O ₂	8	243(C ₁₇ H ₂₃ O), 227(C ₁₇ H ₂₂)
Serine	106.0514	C ₃ H ₈ NO ₃	1	91(C ₃ H ₇ O ₃), 90(C ₃ H ₈ O ₂ N), 61(C ₂ H ₅ O ₂)
Indole	118.0670	C ₈ H ₈ N	5.5	Base peak only
Phenylalanine	166.0858	C ₉ H ₁₂ NO ₂	5	90(C ₃ H ₈ O ₂ N), 77(C ₆ H ₅), 61(C ₂ H ₅ O ₂)
Dopamine	154.0857	C ₈ H ₁₂ NO ₂	4	109(C ₆ H ₅ O ₂)
Norepinephrine	170.0826	C ₈ H ₁₂ NO ₃	4	109(C ₆ H ₅ O ₂), 60(C ₂ H ₆ ON)
Epinephrine	184.0959	C ₉ H ₁₄ NO ₃	4	109(C ₆ H ₅ O ₂), 60(C ₂ H ₆ ON)
Metabolites in Basal Group	<i>m/z</i> [M+H] ⁺	Formula [M+H] ⁺	RDBE.(*)	MS/MS product ions <i>m/z</i>
L-Tryptophan	205.0967	C ₁₁ H ₁₃ N ₂ O ₂	6.5	118(C ₈ H ₈ N), 90(C ₃ H ₈ O ₂ N), 61(C ₂ H ₅ O ₂)
Serotonine	177.1039	C ₁₀ H ₁₃ N ₂ O	6	132(C ₈ H ₆ ON), 116(C ₈ H ₆ N), 44(C ₂ H ₆ N)
Melatonin	233.1270	C ₁₃ H ₁₇ N ₂ O ₂	7	132(C ₈ H ₆ ON), 116(C ₈ H ₆ N), 90(C ₄ H ₁₂ ON)

m/z (molecular mass/charge) [M+H]⁺ (ionization from protonation of molecular mass) Peak measured, **RDBE**: Ring double bond equivalent or degree of insaturation for elucidation of chemical structure, **MS/MS** (Tandem Mass Spectrometry) data, elucidation of fragmentation patterns for each *m/z* which confirms unequivocal structural and chemical characterization in all the cases.

provement in metabolomics applied to biological samples as previously described.

Since there are no published papers similar to this proposed-one, statistical models whose quality would be comparable or higher than other studies of psychiatric or neurological disorders have been used^{27,39}.

Data Information Management Systems (Bioinformatics), which has been already used in similar approaches in metabolomics applied to diseases^{10,11,12,27,39}, allowed a faster and more effective filtering and selection of data.

Mass spectrometry which will enable a more accurate measurement of mass and a better structural elucidation of the compounds to be studied using MS/MS was selected^{68,69}. Direct infusion mass spectrometry can be considered an effective tool in the study of metabolomic anomalies in stress research as proposed in this paper, since it has been successful in other diseases or pathologies^{70,71}. Direct injection analysis into the ionization source of the mass spectrometer without prior chromatographic separation is a technique commonly used with atmospheric pressure ionization techniques (ESI). It is a high-throughput approach already used in metabolic fingerprinting in crude fungal extracts⁵⁸ and yeast intracellular metabolomics⁷². There is not yet any study done in the study of stress. However, the results prove that the methodology applied is robust.

Since the predominant metabolites are different between the two studied states, this allows to objectively characterizing in which state is any of the subjects.

According to Cowen P.J.⁵⁸ and other authors⁷²⁻⁷⁴ our results confirm that serotonin and related metabolites decrease in stress situation. While during stress induction, glucocorticoid secretion increases as a response to stress^{58,73-76} becoming predominant.

Therefore, an objective tool, as the presented protocol, could provide important information to aid in this aim and, although the presented results emerged from a pilot trial, they seem promising.

Though the specificity and sensibility of Mass Spectrometry techniques for low-volumes and low-concentration samples have been already demonstrated, there is yet the need to address the problem of false negatives/positives when compared with the psychometric evaluation. Further research is currently being done to complete the evaluation of this technique.

Conclusion

The described method, based in this pilot study, is presented as a tool to identify biomarkers in stress-induced subjects. Direct Infusion Mass Spectrometry protocol in this work has been adapted to be applied for the first time in the classification stress-induced subjects. The results provide a preview of preliminary measurable outcomes of the initial objectives discriminating among stressed or relaxed subjects. It opens the possibility to compare metabolomic profiles. Furthermore, this methodology is a quick, direct and non-invasive method for patients. Nevertheless, further work needs to be done, such as, accurate quantification of compounds. On the other hand, a more exhaustive and complete research should be developed to be able to have a full screening of results among different types of patients and a comparative analysis between metabolomic/biological data and psychological diagnose. However, we consider that it can be foreseen many applications for the proposed protocol such as quick-quality-diagnose, pre-treatment of side effects, therapeutic target search etc., which can be of interest in the present-day analytical field.

Conflict of interest

The authors declare that they have no competing interests.

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