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Analysis of Skin Volatiles Using a PDMS Membrane and HS-SPME/GC-MS Methodology to Unveil Putative Biomarkers for Neurodegenerative Diseases

MASTER DISSERTATION

Beatriz Sousa de Andrade
MASTER IN APPLIED BIOCHEMISTRY

UNIVERSIDADE da MADEIRA
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CO-ORIENTATION
Jorge Augusto Machado Pereira

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Dissertação apresentada à UNIVERSIDADE DA MADEIRA para a obtenção do grau de Mestre em Bioquímica Aplicada, realizada sob a orientação científica do Professor Doutor José de Sousa Câmara e coorientação do Doutor Jorge Augusto Machado Pereira

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Analysis of skin volatiles using a membrane-SPME/GC-MS approach to unveil putative biomarkers for neurodegenerative diseases, Beatriz Andrade, Jorge Pereira, José Câmara, ***11th Encontro Nacional de Cromatografia;*** Costa da Caparica, Portugal (Dezembro 2019). https://11enc.eventos.chemistry.pt/images/programa.pdf

"The smell of dementia: identifying a signature in the human volatilome for the non-invasive diagnosis of neurodegenerative diseases", Jorge Pereira, Beatriz Andrade, José Câmara, ***NCD-CAPomics third meeting***; Pune, India (May 2019).

POSTER COMMUNICATIONS

"Volatilomics skinprinting as an untargeted approach to identify putative biomarkers of neurodegenerative diseases", Beatriz Andrade, Jorge Pereira, José Câmara**;** *XVIII Encontro Nacional da Sociedade Portuguesa de Química;* Porto, Portugal (June 2019).

RESUMO

As doenças neurodegenerativas (NDDs) são um grupo heterogéneo de perturbações caracterizadas pela degeneração progressiva da estrutura e função do sistema nervoso central ou periférico. De entre as NDDs, as doenças de Alzheimer e Parkinson são as mais prevalentes e a sua incidência está a crescer simultaneamente com a esperança média de vida.

Uma vez que ainda não existem biomarcadores (BMs) fiáveis para a maioria das NDDs, o diagnóstico depende essencialmente dos sintomas clínicos. Contudo, a neurodegeneração começa muito antes do paciente manifestar sintomas. Assim sendo, há um grande interesse no diagnóstico precoce das NDDs. Isto permitirá a antecipação do tratamento e a mitigação dos efeitos negativos da neurodegeneração. A caracterização da composição dos metabolitos orgânicos voláteis (VOMs) de biofluídos humanos está a ser explorada como uma ferramenta não-invasiva promissora para descobrir BMs para diagnóstico de doenças e infeções. Os perfis de VOMs fornecem informações importantes, particularmente acerca de alterações metabólicas relacionadas com o desenvolvimento de diferentes patologias, incluindo NDDs. Neste contexto, o objetivo deste trabalho foi o desenvolvimento de uma técnica utilizando membranas de polidimetilsiloxano (PDMS) e um método de microextração em fase sólida do *headspace* (HS-SPME) seguido de análise por cromatografia gasosa acoplada a espectrometria de massa (GC-MS) para a descoberta de possíveis BMs de NDDs. A técnica desenvolvida permitiu-nos identificar 82 VOMs – 67 no braço, 68 na orelha e 69 na nuca. Seria necessário, porém, analisarmos um maior número de amostras de forma a consolidar os resultados obtidos no grupo controlo antes de prosseguir com a amostragem de pacientes.

Este trabalho sugere assim que a análise dos perfis voláteis da pele poderá ser uma ótima ferramenta para a identificação de possíveis BMs voláteis para diferentes NDDs. Tais BMs teriam um grande potencial para serem facilmente integrados em dispositivos de teste rápido (POCT) a serem utilizados em ambientes clínicos.

PALAVRAS-CHAVE Pele, Voláteis, Biomarcadores, Neurodegeneração, Alzheimer, Parkinson

ABSTRACT

Neurodegenerative diseases (NDDs) are a heterogeneous group of disorders characterized by progressive degeneration of structure and function of the central or peripheral nervous system. Among NDDs, Alzheimer's and Parkinson's diseases are the most prevalent and their incidence is increasing concomitantly with average life expectancy.

While there aren't yet reliable biomarkers (BMs) for most NDDs, their diagnosis relies essentially on the clinical symptoms. However, neurodegeneration begins long before the patient experiences any symptoms. Therefore, there is an obvious interest in the early diagnosis of NDDs. This would allow the anticipation of the treatment and mitigation of the negative effects of neurodegeneration. The characterization of volatile organic metabolites (VOMs) composition of human biofluids is being explored as a promising and non-invasive tool to unveil BMs for the diagnosis of human diseases and infections. The VOMs profiles can provide important metabolic information, particularly about metabolic changes caused by different clinical conditions, including NDDs.

In this context, the aim of this work was to develop a technique using polidimetilsiloxane (PDMS) membranes and a method of headspace solid phase microextraction (HS-SPME) followed by gas chromatography coupled to mass spectrometry (GC-MS) analysis to discover possible BMs for NDDs. The developed method allowed us to identify 82 VOMs – 67 in the arm, 68 in the ear and 69 in the nape of control subjects. However, it would be necessary to analyse a larger group of subjects to improve the technique and obtain more robust data before introducing the NDDs patients sampling.

This work suggests that the analysis of volatomic profiles of the skin can be an important approach to identify potential volatile BMs for NDDs. Ultimately, this can be a seamless tool for the identification of putative volatile BMs of different NDDs with a great potential to integrate in point-of-care testing (POCT) devices for the clinical environment.

KEYWORDS Skin, Volatiles, Biomarkers, Neurodegeneration, Alzheimer, Parkinson

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ABBREVIATIONS

Abbreviation	Description
αS	α-synuclein
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
API-MS	Mass spectrometer with atmospheric pressure source
APP	Amyloid precursor protein
Aβ	Amyloid-β protein
BM	Biomarker
CAR	Carboxen
CBD	Corticobasal degeneration
CTE	Chronic traumatic encephalopathy
DLB	Dementia with Lewy bodies
DVB	Divinylbenzene
EI	Electron-impact
EGFR	Epidermal growth factor receptor
FTD	Frontotemporal dementia
GC-EAG	Gas chromatography electrophysiology
GC-IMS	Gas chromatography coupled to ion mobility spectrometry
GC-MS	Gas chromatography coupled to mass spectrometry
HD	Huntington's disease

HMDB	Human Metabolome Database
HS-SPME	Headspace solid phase microextraction
IMS	Ion-mobility spectrometry
IS	Internal standard
MRI	Magnetic resonance imaging
MSA	Multiple system atrophy
NIST	National Institute of Standards and Technology
NDDs	Neurodegenerative diseases
PARK2	Parkinson-2 protein
PD	Parkinson’s disease
PDMS	Polydimethylsiloxane
PET	Positron emission tomography
POCT	Point-of-care devices
PSEN1	Presenilin-1 protein
PSP	Progressive supranuclear palsy
PTR-MS	Proton Transfer reaction mass spectrometry
SESI	Secondary Electrospray Ionization
SIFT-MS	Selected ion flow tube mass spectrometry
SPECT	Single photon emission computerized tomography
SPME	Solid phase microextraction
VOMs	Volatile organic metabolites

PART 1.

INTRODUCTION

1. Neurodegenerative diseases (NDDs)

Neurodegenerative diseases (NDDs) are a heterogeneous group of diseases that are characterized by the progressive degeneration of the structure and function of the central or peripheral nervous system. Due to public health successes of the last century, longevity of the world's population is increasing. Since most NDDs are age-related, likelihood of developing NDDs grows with increasing age [1]. We must find means of preventing, delaying, slowing the progress or improving the symptoms of these diseases since they will result in huge human, social and economic costs and are among the greatest threats to the future of mankind [2].

NDDs can be characterized by a distinct clinical syndrome of neurological deficits, changes in behaviour, motor disorders and gradual functional decline, reinforced by an inexorable neuronal loss that is pathological for the age of the patient. NDDs comprise Alzheimer's disease (AD), Parkinson's disease (PD), frontotemporal dementia (FTD), dementia with Lewy bodies (DLB), chronic traumatic encephalopathy (CTE), multiple system atrophy (MSA), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), progressive ataxias and some rare degenerative conditions [2]. The phenotypes of these diseases differ, however, they show some similarities such as clinical phases – preclinical, mild and fully developed clinical manifestations – and proteins with altered physicochemical and neurotoxic properties [3]. AD and PD are the most prevalent NDDs. They can be characterized by three general phases: a period with asymptomatic changes in the brain; mild symptoms not meeting the criteria for the diagnosis of the full syndrome; and a final stage with characteristic manifestations meeting traditional diagnostic criteria. AD is an example of this progression [4].

There is an initial asymptomatic period when amyloid protein changes are demonstrable on amyloid imaging or with cerebrospinal fluid analysis, followed by early symptoms as episodic memory weakening and progression in biomarkers (BMs) to include atrophy on magnetic resonance imaging (MRI). In this period, there is no major impairment of daily activities and patients do not meet criteria for dementia. Lastly, cognitive function

worsens, and functional deficiency occurs, and the patient meets the criteria for AD dementia [5]. Most NDDs are thought to follow similar disease progression paths. The terminal phases of NDDs appear to be similar since patients are bereft of all function, paralyzed and incontinent, unaware of their surroundings or aspects of their own biography. They succumb to pneumonia, urinary tract infections, complications of decubitus ulcerations or age-related conditions such as stroke, cancer or heart disease [6].

1.1. Disease pathophysiology

1.1.1. Alzheimer's disease (AD)

AD is an age-dependent neurodegenerative disorder and the most common cause of dementia with aging. The early stages of the disease are characterized by short-term memory loss, however, as the disease progresses, the patients begin to develop difficulties in orientation, oral communication, and cognitive tasks. In some cases, they can also present language deficits, depression, aggression behaviour and psychosis. These last symptoms usually develop during the last stages of the disease and eventually the patients are incapable of taking care of themselves without caregivers [7].

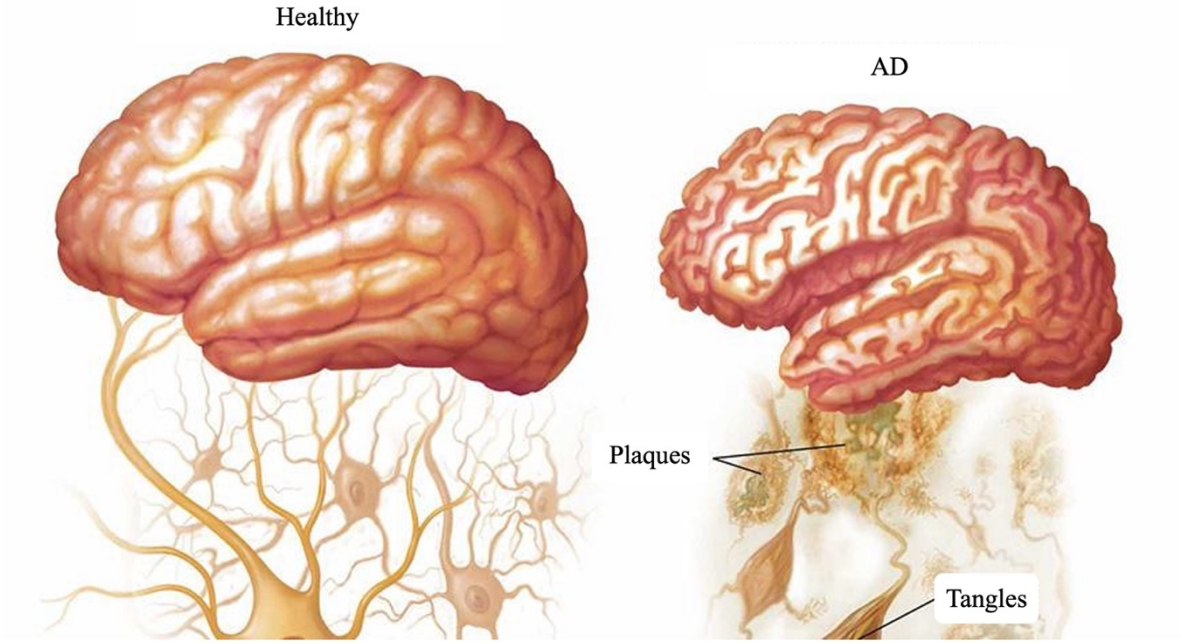


FIGURE 1. Healthy brain vs. Alzheimer's Brain
(Adapted from *The New Times-Rwanda's Leading Daily*)

The pattern of AD's progression is a consequence of the persistent loss of synapses and neurons in the regions of the brain that are responsible for memory, learning and cognition, constituted by the entorhinal cortex, hippocampus, frontal cortex and temporal lobes. (Figure 1) In 1907, two pathological features of AD were first described by Alois Alzheimer when he analysed brain sections of a 51-year-old woman who died after developing dementia [8]. These brain sections revealed extra-neuronal deposits called amyloid, as well as intraneuronal deposits, called neurofibrillary tangles in nerve cell bodies. In the '80s, the extra-neuronal deposits were found to be composed of a peptide called amyloid-β protein (Aβ) and the intra-neuronal deposits were determined to be composed of a protein called tau (t) [9,10]. Later, a third pathological feature was recognized as neuroinflammation, an apparent effect of over-activation of non-neuronal cells such as microglia and astrocytes [11].

1.1.2. Parkinson's disease (PD)

PD is a chronic progressive disease and has been considered the second most common NDD after AD since its identification and prevalence are estimated to double over the next decades [12]. PD is characterized by a significant reduction of the dopaminergic neurons in the substantia nigra together with physical signs such as rigidity of muscle on passive movement, instability in posture, resting tremor and akinesia [13]. (Figure 2, [14]) PD was, for the first time, distinguished from other causes of tremor by Dr James Parkinson in his famous 1817's paper 'An Essay on the Shaking Palsy' [15]. In that paper, Parkinson defined the disease as an 'involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellect being uninjured' [16].

PD is clinically characterized by a motor syndrome, however non-motor manifestations such as autonomic, psychiatric, and cognitive dysfunctions are becoming part of the wider clinical syndrome. Disorders that produce clinical parkinsonism share the feature of dopaminergic deficiency even if they have different molecular pathologies [17]. Neurons that degenerate in PD accumulate cytoplasmic inclusion bodies composed of α-synuclein protein (αS), referred to as Lewy bodies (LBs) [18].

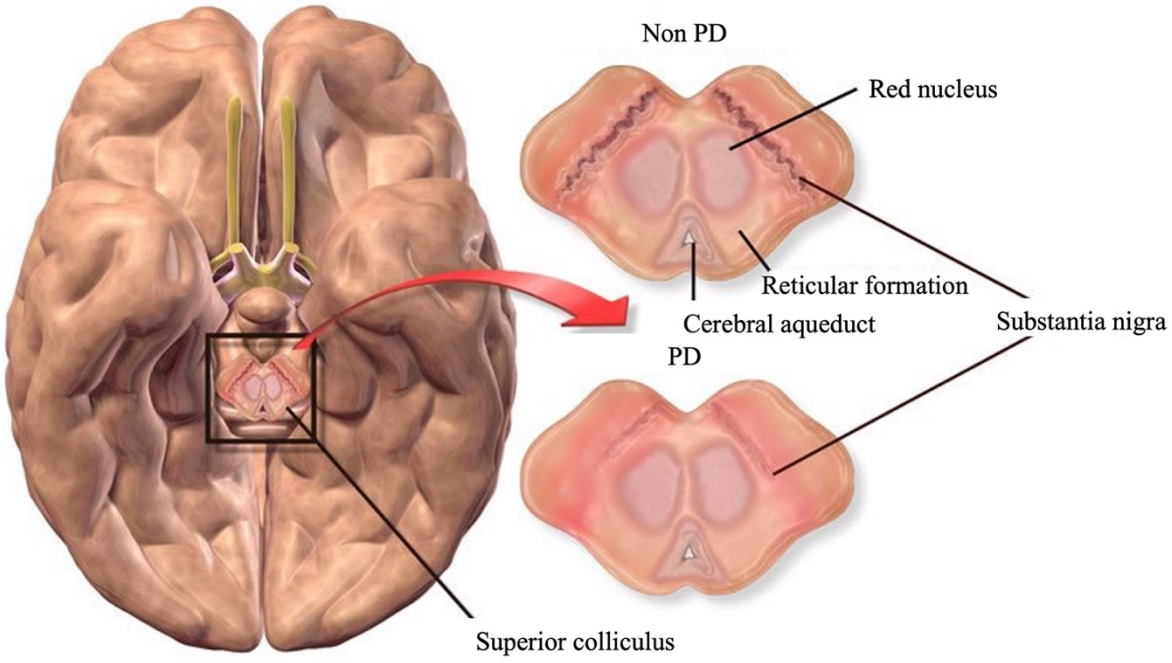


FIGURE 2. Healthy brain vs. Parkinson's Brain
(Adapted from *Diagnosis of Parkinson's Disease using Gait Dynamics and Images*)

Although LBs are the histologic brand of PD, they can be linked with a variety of other clinical conditions, such as dementia, psychosis, or dysautonomia. Besides, their distribution in the nervous system is more widespread than the substantia nigra [17].

1.2. Incidence and prevalence of main NDDs

It is common to associate memory loss and senility with the normal ageing process due to the high occurrence of dementia in the ageing population. NDDs such as AD interferes with the normal process of healthy ageing and alters its trajectory, radically shortening the individual's life expectancy. But worse than that, reduces the individual's productivity, weakens its cognitive faculties and deteriorates its quality of life, as well as from its family. Although the majority of AD cases are thought to be sporadic, there are some risk factors: age, family history, genetics (the apolipoprotein E4 allele), history of head trauma, female gender, vascular risk factors and environmental factors [19–22]. In the case of PD, we can link it to several polymorphisms, but likely, the majority of PD cases are not inherited but

related to environmental factors [23]. Amongst the many risk factors for neurodegeneration, the ageing process has by far the most impact. Therefore, it is vital to consider the basic mechanisms of ageing and their role in the beginning and progression of NDDs.

Evidence shows that all aged brains show distinctive modifications linked to NDDs, however it isn't concrete if these hallmarks represent products of brain ageing or are foreshadows for NDDs. As the brain ages, we find abnormal protein assemblies and inclusions bodies, however, in the brains with a disease, these lesions appear to be more region specific and cognitive changes with age are distinct from those observed in NDDs. This way, aged brains are more susceptible to NDDs in which the same injuries accumulate with the smaller ones already present in them. For this reason, normal aging, neurodegeneration and dementia can overlap [24].

As the elderly population increases, the financial load of age-related health conditions will rise, and effective preventive and therapeutic advances are crucially needed. It is estimated that 5.8 million Americans are living with AD in 2019. From those, 5.6 of them are estimated to be at least 65 years old and 81% of them are at least 75 years old. Overall, this means that 10% of Americans aged ≥65 years old have AD and this percentage increases with age: 3% of people aged 65-74; 17% of people aged 75-84; 32% of people aged ≥85 have AD [3]. Studies have suggested that approximately 491,000 people aged ≥65 years old will develop AD in the United States in 2020 [25]. However, these numbers maybe be much higher, as pointed by other studies [26]. Because of the increasing number of people age ≥65 years old, the annual number of cases of AD is projected to double by 2050 [27]. Worldwide, it is estimated that 50 million people have AD and this number can be up to 132 million by 2050 [28].

Although PD has lower numbers than AD, nearly 1 million Americans were living with PD in 2020, a number that is estimated to rise up to 1.2 million by 2030. Approximately 60000 are diagnosed in the US each year and more than 10 million people are living with PD worldwide. Incidence of PD increases with age, but an estimated 4% of people with PD are diagnosed before 50 years old [29]. Overall, NDDs constitute a severe economic burden across the world. The total estimated worldwide cost of dementia in 2015 was 818 billion dollars and this number is projected to rise to 2 trillion dollars by 2030 [28].

1.3. Current NDDs diagnosis and biomarkers

Currently, the diagnosis of NDDs is still made when motor symptoms occur, although the pathological events that trigger these diseases began decades before of the first signs of cognitive dysfunctions. It is therefore very difficult to identify the NDDs pathologic processes based on the clinical phenotype alone [30]. Unlike other types of prevalent diseases, NDDs like AD does not have any well-determined and reliable biological markers that can allow physicians to accurately predict the risk of developing AD. Doctors have been able to predict the trajectory of an individual presenting memory changes that are indicative of dementia with neuropsychological examinations, history taking, neurological examination, blood work and brain scans. In this way, they can rule out other disorders but they are not able to provide a definitive diagnosis [31]. Therefore, the discovery of biological markers or biomarkers (BMs) for the early diagnosis will affect the management of AD and PD in several aspects.

Finding BMs for NDDs will allow the detection of high-risk individuals even before symptoms develop and it will help to differentiate AD and PD from other clinical syndromes, therefore allowing a better prognostic. BMs will also help to determine the medical effectiveness of new disease-specific neuroprotective therapies and may indicate the biological transformations underlying the disease process, this way allowing the development of new therapeutic procedures [30]. Amongst the important challenges confronting the physicians is the improvement of diagnostic processes to: detect early symptoms, in a phase that intervention can have greater impact; provide an accurate measure of disease progression; stipulate evidence for the relevant biological activity of a trial drug; describe pathophysiological procedures responsible for the disease. BMs are cellular, biochemical or molecular alterations that are measurable in the biological media such as human tissues, cells or fluids [32]. Some widen the definition, including biological characteristics that can be measured objectively and valued as markers of pathological biological processes or even pharmacological responses to therapeutic intervention [33]. BMs for NDDs can be divided into genetic, neuroimaging (structural and functional) and biochemical, being that neuroimaging and biochemical BMs indicate the presence of the pathology, while genetic BMs are only considered for risk evaluation [30].

Often, several potential BMs are reported in the literature, but each of them must be evaluated for its specificity and sensitivity for a certain disease even if they are used to determine stages of a given disease [34]. Since higher specificity is usually achieved at the expense of reduced sensitivity, an appropriate compromise between both must be obtained. In cases in which an affordable, safe and effective therapy is available for a condition, a less specific test can be used even if it means that people who do not have the disease are given the treatment. However, if the treatment is expensive or has significant side effects, more specific tests are required. Regarding NDDs, BMs able to detect the pathology will certainly allow a more reliably diagnose than the traditional cognitive and neurological phenotypes that are currently used. This could be accomplished by the detection of altered levels of tau and amyloid proteins in the cerebrospinal fluid, by the use of structural MRI to identify disease-specific patterns of atrophy, imaging of metabolic or cerebrovascular flow using positron emission tomography (PET) and single-photon emission computerized tomography (SPECT) as well as PET imaging using ligands which indicate the presence of Aβ deposits [30]. However, these are expensive and in certain cases invasive approaches that are difficult to implement and disseminate in the different health care systems around the world.

To sum up, diagnostic markers, for example, high levels of tau protein in the cerebrospinal fluid, characteristic electroencephalography discharges and MRI indicators, are used extensively in the clinical environment, however, they are considered exclusionary markers, since they do not provide a concrete diagnosis, but exclude the diagnosis of some NDDs, in this case, they exclude the diagnosis of AD [35]. So, if researchers find a specific BM correlated with particular NDD, individuals who are at high risk of developing the disease could be placed on a disease-mitigation medication program before the onset of clinical symptoms [31].

2. Volatile organic metabolites (VOMs)

2.1. Definition and potential use in disease diagnosis

Volatiles and, in particular, VOMs, are everywhere and directly and indirectly affect the lives of many species in different ways. Recently, VOMs have received particular interest to obtain insights into physiological and pathophysiological processes and to exploit the knowledge of their concentration profiles in various body matrices for disease detection and therapeutic monitoring [36,37]. Considering that VOMs can be obtained readily and in a non-invasive way, they can be sampled often with little discomfort. However, in comparison with other markers (such as protein or nucleic acid), VOMs have less information on the total amount of volatiles produced by human cells and lack of understanding how their normal composition in breath is altered by stress, age, time of day, gender, activity, disease or transport to the location of detection. In most cases, the information about metabolic paths of VOMs production and degradation is absent, so it is vital to study the production of VOMs on the cellular dimension.

The major challenges for establishing VOMs analysis as diagnostic/monitoring tool are establishing standardized procedures for sampling and analysing of VOMs in different human biofluids, achieving unequivocal identification of VOMs, identifying the main causes for their variations in of diverse cell types or phenotypes of the same cell population, understanding the inter and intrapersonal variability and establishing the genotype-phenotype relationship for VOMs production [38].

2.2. Extraction and analysis of VOMs from human biofluids

VOMs are transported by diffusion through the air, performing numerous functions in the communication between insects and/or plants, for mating or even as flavours and fragrances [39]. These compounds, usually based on a hydrocarbon skeleton with oxygen, nitrogen and sulphur as the most common heteroatoms, have an almost infinite structural variety that determines the specific role these molecules have in nature. Biogenic VOMs are extremely selective for a particular target and this is an exceptionally important property,

resulting in very low detection limits of a given compound to its target species. This means that the receptor of the receiving species can selectively detect specific molecules at very low concentrations [39].

Volatiles are characterized by high vapour pressures, that facilitate their transport through the air and eventually reaching their biological targets. Biogenic VOMs are also characterized as being hydrophobic, a feature that can simplify their efficient evaporation from a water-based medium into the air [39]. The human VOMs can have different origins, for example, in exhaled breath, skin emanations, urine, faeces and saliva. In the case of exhaled breath, there are two basic approaches of sampling – direct (real-time) analysis and off-line analysis. In both analyses, it is very important to carefully choose sampling methods and usually, physiological parameters such as partial pressure of CO_2, temperature, flow and partial pressure of exhaled O_2 are used for sampling control [40]. For systemic metabolites, the use CO_2-controlled sampling is highly recommended to restrict the sampling process to the end phase. This allows the standardization of the sampling process and concentrates the target VOMs. For direct breath analysis, it is not required to preconcentrate or store samples, so this represents an interesting methodology for continuous monitoring of specific compounds, but often the equipment necessary for this analysis doesn't have enough promptness to recognize breath-to-breath resolved sampling or are too large to be adapted to the bedside [40].

There isn't a unique analytical method suitable to quantify the volatile composition of all biofluids, so researchers use the procedures and apparatus that are available and best tailored for each type of biofluid. The main method used for VOMs analysis has been Gas Chromatography coupled to mass spectrometry (GC-MS), however, other methods such as Proton Transfer reaction mass spectrometric (PTR-MS) [41], Selected ion flow tube mass spectrometry (SIFT-MS) [37], Ion-mobility spectrometry (IMS) [42], laser spectrometry [43], photoacoustic spectroscopy [44], and sensors and sensor arrays [37] have also been used for this purpose. Microextraction techniques have gained popularity for the pre-concentration of BMs and following laboratory analysis. Nevertheless, the efficiency of the extraction will depend on the physical and chemical properties of the adsorption procedures and applied materials. Furthermore, these techniques can suffer from carryover and poor

quantification of certain mixtures depending on the sample matrix [40]. In 2014, a compendium of all the VOMs emanating from the human healthy body was reported, for the first time [45]. This review reported 1849 VOMs: 874 compounds were identified in the exhaled breath, 279 compounds in urine, 504 compounds in skin emanations, 353 compounds in saliva, 130 compounds in blood samples, and 381 compounds in faeces. Among the 874 VOMs found in the exhaled breath, 22.8% have also been identified in faeces, 16.2% in saliva, 15.7% in urine, 11.9% in skin emanations and 8.0% in blood [40].

2.3. Skin volatile organic metabolites (VOMs) and neurodegenerative diseases (NDDs)

Odours produced by human skin have a great interest to researchers in many fields. Applications of skin VOMs can vary from the design of perfumes and deodorants, the ecology of blood-sucking insect vectors of human diseases, forensic studies and diagnostic tools. Biosynthetic pathways and the production of volatiles by plants have been investigated and reported in many studies [46,47]. However, in the case of mammalian VOMs, especially regarding humans, this is a more complex work and has been challenged with many technical complications [48,49]. Human odours are produced in minor quantities and they tend to have high variabilities due to environmental factors such as diet, disease, or even skin microbial composition, which can strongly affect the production of human body odours [50]. The use of fragranced products such as soap, shampoo, deodorants and perfumes typically result in the detection of exogenous compounds in the skin VOMs analysis, so volunteer subjects are often asked to avoid these products before the odour sampling.

The production of VOMs by the skin is mainly performed by the emissions of three types of glands: eccrine, sebaceous and apocrine glands. (Figure 3) The allocation of these gland types on the skin is reflected by the distinctive human scents produced by different body parts [51]. Eccrine glands, the most abundant and extensively distributed on the skin surface, are responsible for producing odourless sweat and are mainly concentrated on the hands and feet. Apocrine glands, found in the axillae and genital areas, are responsible for lipid, protein and steroid secretion. Finally, sebaceous glands, distributed all over the human body but mainly concentrated on the head, are responsible for lipid and sebum emission.

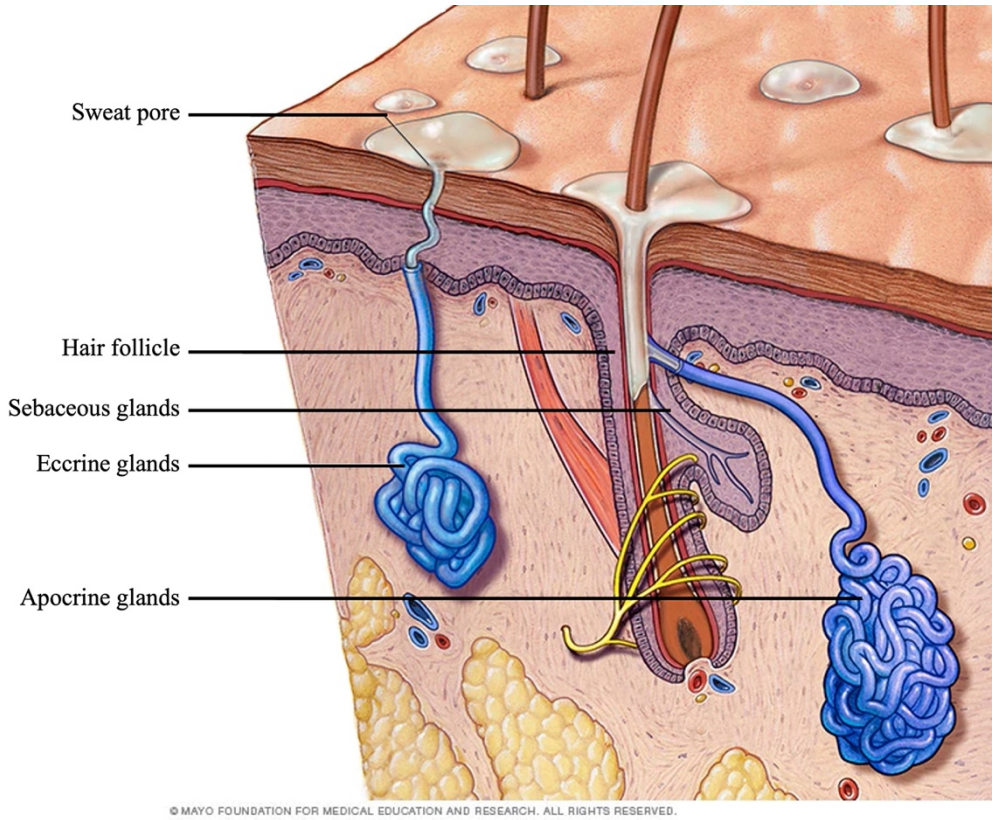


FIGURE 3. Glands responsible for the emission of skin VOMs
(Adapted from *Mayo Foundation for Medical Education and Research*)

Several studies have sampling skin VOMs using different sampling methods. As seen in Table 1, different sampling methods for VOMs have been applied over the years. In early studies, solvent extraction was a recurrent method. In this technique, a solvent is used to extract a sweat sample, often a pad/textile that was previously used to collect the compounds. One of the disadvantages of this method is that it extracts some compounds that may not be volatile at body temperature. Headspace adsorption onto various polymers has also been used for sampling of VOMs. In this method, skin volatiles are first collected on gauze or cotton pads and then the VOMs release by the pads are collected into adsorbed traps. However, this procedure has two main disadvantages: first, by using gauze or cotton as an intermediate, one can fail to transfer some metabolites; second, the intermediate sorbent must be analytically clean previously to the sampling [52].

TABLE 1. Previously reported methods for sampling and analysis of VOMs

Year	Body Part	Collection method	Extraction method	Analysis method	Study sample	VOMs identified	Ref.
1974	Axilla	Cotton pads	Solvent extraction	GC-MS	12 males	5α-androst-16-en-3α-ol	[53]
1990	Foot	Socks	Solvent extraction	GC-MS	10 males	Isovaleric acid Short-chain fatty acids	[54]
1996	Axilla	Cotton pads	Solvent extraction	GC-MS	6 females	36 metabolites	[55]
1996	Forehead/ Trunk	Sweat samples	Solvent extraction	GC-MS	14 adults	21 carboxylic acids	[56]
2005	Axilla	Sweat samples	Solvent extraction	GC-MS	9 individuals	4 metabolites	[57]
2005	Axilla	Gauze pads	HS-SPME	GC-MS	4 males 4 females	54 metabolites	[58]
2005	Hand/Arm	Direct sampling	HS-SPME	GC-MS	15 individuals	35 metabolites	[59]
2006	Axilla	Cotton pads	Solvent extraction	GC-MS	Unknown	28 carboxylic acids	[60]
2007	Axilla	Sweat samples	Solvent extraction	GC-MS	197 adults	44 metabolites	[61]
2008	Upper back/ Forearm	Sweat samples	SPME and Solvent extraction	GC-MS	13 males 12 females	100 metabolites	[62]
2008	Whole body	Dynamic HS	Solvent extraction	GC-EAG	9 adults	24 compounds	[63]
2008	Forearm/ Abdomen	PDMS pads	Thermal desorption	GC-MS	1 male	6 compounds	[64]
2009	Foot	Cotton strips	HS-SPME	GC-MS	6 individuals	9 fatty acids	[65]
2009	Hand	Direct sampling	SESI	API-MS	1 male	19 metabolites	[66]
2010	Hand	Gauze pads	SPME	GC-MS	5 males 5 females	37 metabolites	[67]
2011	Hand	Sorbent textiles	SPME	GC-MS	3 males 3 females	58 metabolites	[68]
2011	Hand	Gauze pads	SPME	GC-MS	2 males 2 females	5 main metabolites	[69]
2011	Axilla	Sweat samples	HS-SPME	GC-MS	2 males	44 metabolites	[70]
2011	Hand	Gauze pads	SPME	GC-MS	4 males, 4 females	6 main compounds	[71]
2012	Hand	Gauze pads	SPME	GC-MS	15 males 16 females	36 metabolites	[72]
2012	Umbilicus	Direct sampling	GC-IMS	GC-MS	6 males 1 female	8 metabolites	[73]
2017	Hand and forearm	Cellulose film/AC	Thermal desorption	GC-MS	8 individuals	52 metabolites/ 30 metabolites	[74]
2020	Arm/Chest	Polymer and PDMS sheets	Thermal desorption	GC-MS	636 individuals	6 compounds	[75]

These methods need the employment of a solvent to elute volatiles in the adsorbent phase, resulting in a diluted solution. In consequence of that, a step of concentration is needed before GC-MS analysis, so volatiles with low molecular weight can evaporate. More recently, a new approach has been used – solid-phase microextraction (SPME). SPME was originally developed for monitoring air pollutants, but it soon began to prove its efficacy for other applications [76]. SPME is a simple technique, sensitive, doesn't need the use of solvents, and allows the entrapping of volatiles on adsorbent-coated fibres, followed by direct thermal desorption into a GC injector. SPME can be performed by direct immersion (in which the fiber is introduced directly into the sample) and headspace (in which the fiber is introduced into the air above the sample). In the case of skin VOMs, direct immersion can be performed when we have a liquid sample such as sweat samples and headspace samples are usually performed after a direct collection step using textiles or gauze, for example. Although SPME has been frequently used following a preliminary sample collection step, some recent studies with contact SPME have shown similar results to those acquired with HS-SPME. One of the materials that has been used for in vivo collection of VOMs has been polydimethylsiloxane (PDMS), a method that has been considered to have higher sensibility, which has a great importance, since VOMs of the skin are often presented in low concentrations [77].

Since the composition of skin volatiles can be influenced by external factors, in some studies, volunteers have been asked to follow some instructions to diminish these interferences. In most cases, they are simply asked to avoid the use of deodorant, perfumes, and required to use fragrance-free soaps. The chemical profile of skin VOMs has shown a great variation, mostly due to the difference in sampling methodologies. More than 400 metabolites have been isolated and identified from human skin extracts, however, headspace collection studies – which analyse naturally body temperature occurring volatiles – have only detected up to 90 VOMs [52].

As already referred, there are three types of BMs for AD: genetic, neuroimaging and biochemical. However, for a BM to be clinically useful and efficient, a non-invasive detection in accessible peripheral tissues is certainly more interesting, hence the value of skin as a source of putative BMs.

The abnormal deposition of aggregates of misfolded proteins is a major hallmark of NDDs. Such aggregates introduce a series of intracellular perturbations that lead to abnormal function of the cells, including synaptic dysfunction and disruption of cellular organelles and the cytoskeleton. These events trigger inflammatory responses that ultimately lead to cell death, with simultaneous progressive damage in neuronal function [78,79].

One of the main advances that has been made in terms of diagnosis of NDDs is related with PD, where the presence of αS forms insoluble neurofibrillary tangles distributed through the body, forming Lewy neurites [80]. A connection between the brain and the skin was suggested after researchers found the expression of αS in the epidermis and skin. The expression of αS was found to be moderate in PD patients and null in healthy control subjects [81,82]. Since neural and epidermal human tissue have the same embryological origin in the ectoderm, it is predicted that both types of tissues can accumulate similar misfolded proteins related with neurodegeneration [82]. Some studies have also reported that hormonally aged human sebocytes have expressions of genes related to signalling pathways functioning in NDDs [81,83].

Skin tissue has also been linked with the expression of genes identified as genetic biomarkers for NDDs such as APP, PSEN1 and PARK2 [81]. Also, several types of NDDs have dermal manifestations, for example, PD patients frequently present seborrhoea and hyperhidrosis together with the regular motor manifestations of the disease [84]. This is due to the involvement of PSEN1 in the epidermal growth factor receptor (EGFR) regeneration and partial loss of PSEN1 may lead not only to the development of seborrhoea, but also keratoses and inflammatory skin conditions [85]. In 2013, a report demonstrated that αS aggregates were present in the peripheral nerve terminals of the epidermis of PD patients [86].

The presence of protein aggregates in the skin cells of AD patients has also been studied and demonstrated since Tau protein was detected in the majority of the studied patients [87]. A study of 2002 reported a reduced antioxidant defence capacity in skin fibroblasts of patients with familial AD when compared with control patients [88]. It was also reported that patients with AD also present altered cutaneous vascular function and not

only in the brain [89,90], and an expression of Aβ and tau proteins were also found in mastocytes – cells of the cognitive tissue related with the immune system [91–93].

Therefore, skin tissue constitutes a promising source in the search for BMs of NDDs, because brain and skin have a strong connection at a physiological and pathological level. This can be a seamless tool for the premature diagnosis of NDDs, allowing a faster intervention in terms of treatment. Moreover, there is a great potential for integration into point-of-care testing (POCT) devices for the clinical environment.

PART 2.

EXPERIMENTAL

1. Extraction and analysis conditions

1.1. Materials and reagents

PDMS membranes were obtained from three different suppliers: *SolSep BV* (SSBV; Apeldoorn, Netherlands), *Interstate Specialty Products* (IST; Sutton, MA, USA) and *Shielding Solutions* (SS; Essex, England). The internal standards – 2-heptanol and 3-octanol – were obtained from *Sigma Aldrich* (St. Louis, MO, USA), 4-methyl-2-pentanol was obtained from *Acros Organics* (Geel, Belgium), 2-propanol was obtained from *Fisher Scientific* (Hampton, NH, USA), n-butyl acetate and 2-heptanone were obtained from *Acros Organics* (Geel, Belgium), hexanal and D-limonene were obtained from *Merck Schuchardt* (Hohenbrunn, Germany). The SPME fibre DVB/CAR/PDMS was obtained from *Supelco* (Bellefonte, PA, USA), the 2510 ultrasonic bath from *Branson* (Urdorf, Zurich, Switzerland), the Clarus 590 gas chromatographer and Clarus SQ 8 S mass spectrometer from *Perkin Elmer* (Waltham, MA, USA).

1.2. HS-SPME conditions

The HS-SPME extraction was performed using experimental conditions previously optimized in the laboratory for the analysis of the volatomic composition of saliva and urine samples [94,95], with the exception of the DVB/CAR/PDMS that was used instead of CAR/PDMS.

In this step, 30 µl of three internal standards with a 30 ppm concentration was added: 2-heptanol, 3-octanol and 4-methyl-2-pentanol. The extraction was performed in a water bath at 50°C for about 1h (Figure 4).

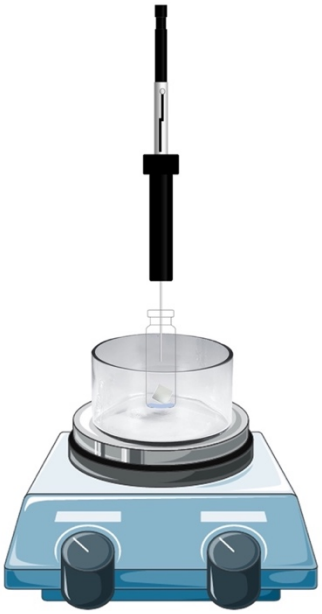


FIGURE 4. Experimental layout of the HS-SPME

1.3. GC-MS conditions

The GC-MS analysis was hold in a *Perkin Elmer* apparatus constituted by a Clarus 590 gas chromatographer and a Clarus SQ 8 S mass spectrometer (Figure 5). The GC-MS was equipped with a BP-fused silica column (60 m × 0.25 mm × 0.25 μm) from *SGE* (Dortmund, Germany).

FIGURE 5. Clarus 590 and Clarus SQ 8 S GC-MS equipment configuration used in this work

All the analysis had a 7 min period of injection and were performed under a constant He flow of 1ml/min. The total running time was 80.83 minutes.

The mass spectrometer operated with electron-impact (EI) ionization source, with a constant ionization energy of 70 eV. The data acquisition was obtained in the scanning mode with a mass range of 35-200 mz. The chromatograms and respective data were stored and analysed using the *TurboMass* software for GC-MS. The VOMs were identified by comparison of the mass spectres obtained for each compound with the mass spectres of the *National Institute of Standards and Technology* (NIST) 14 database. All the samples were analysed in duplicates.

2. Optimization of the sampling methodology

2.1. PDMS membrane conditioning

Five types of PDMS membranes from three different suppliers were tested for this work: a one-sided membrane from *SolSep BV* (SSBV, Netherlands), a two-sided membrane from *Interstate Specialty Products* (IST, U.S.A.) and three two-sided membranes from *Shielding Solutions* (SS, U.K.) with different measures of thickness: 0.5 mm, 1 mm and 2.4 mm. (Figure 6)

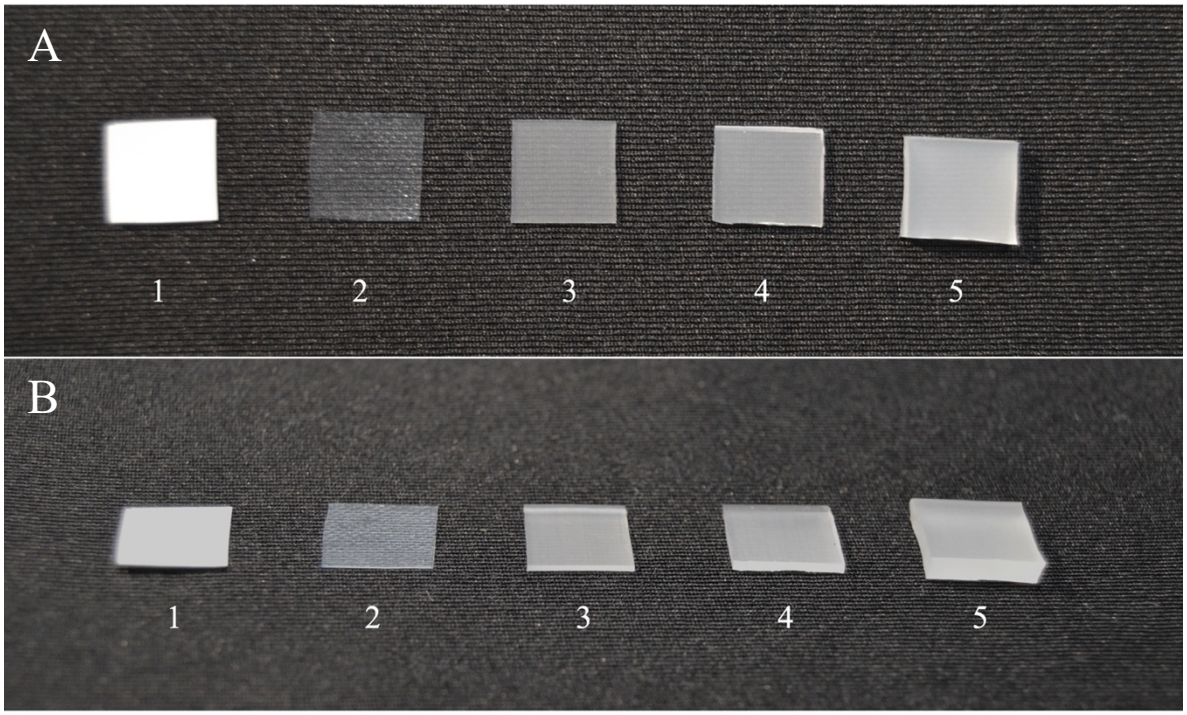


FIGURE 6. PDMS membranes used in this work
Legend: A–top view, B–side view, 1-SSBV; 2-ISP; 3-SS 0.5mm; 4-SS 1mm; 5-SS 2.4mm.

Using these membranes (1x1 cm), a membrane conditioning procedure was optimized, based on the instructions from one of the suppliers, SSBV. Accordingly, the experimental layout for membrane conditioning consisted of putting one PDMS membrane and 2 ml of 2-propanol in a 20 ml vial in an ultrasound (US) bath for 1, 2 and 3h. Then the membrane was transferred to a Petri dish and placed it in a drying oven at 65°C overnight. For the thinner SSBV, ISP and SS membranes (0.5 mm) 1h and 2h of US bath were assayed and for the thicker SS (1mm and 2.4mm) 1h, 2h and 3h of US. The conditioned membranes were stored in individual vials till being used for skin VOMs extraction. This was followed by a HS-SPME and analysed by GC-MS (Figure 7).

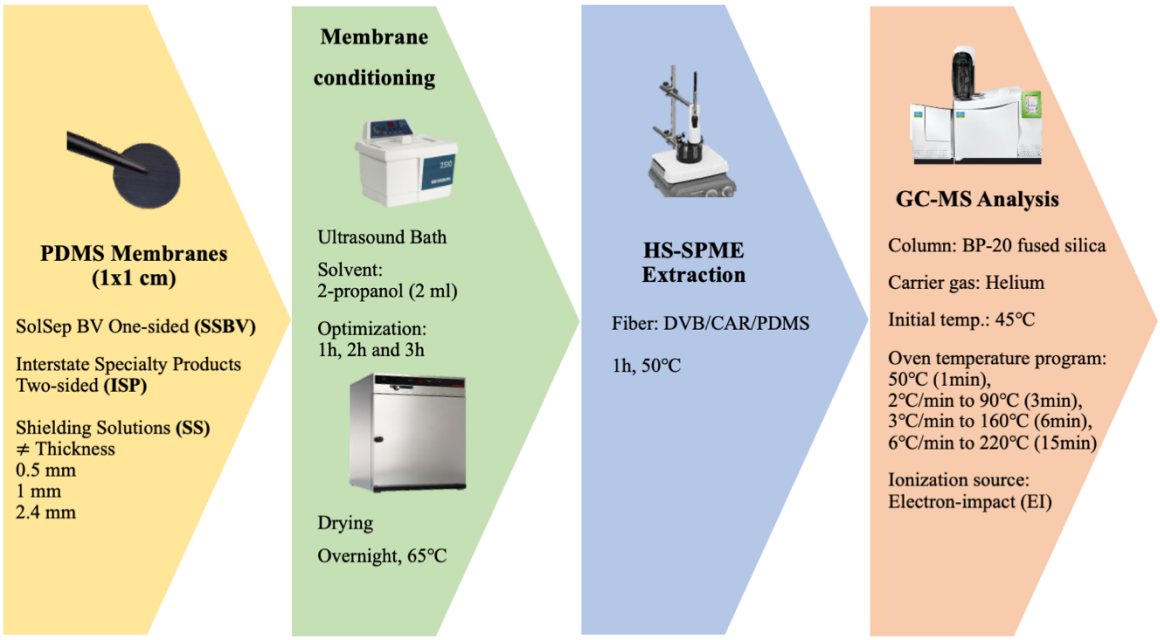


FIGURE 7. Experimental layout for membrane conditioning optimization

2.2. In vitro assays

After the optimization of the conditioning conditions for the PDMS membranes, an evaluation and selection of the best membrane for skin VOMs sampling was necessary. For that effect, a representative aqueous mix containing VOMs previously identified in the skin was prepared with 2-propanol, n-butyl acetate , hexanal , 2-heptanone and D-limonene – with similar concentrations to those detected on the skin (Table 2) [96]. This skin VOMs mix was used to evaluate the PDMS membranes capacity for skin VOMs sampling. The different types of PDMS membranes (described in the previous section) previously conditioned were incubated in a petri dish containing 100 µl of the skin VOMs mix (the PDMS membrane was placed above the mix solution, covered the dish and placed it in a drying oven at 37°C for 1h). Then PDMS membranes containing the trapped skin VOMs were then extracted by HS-SPME and analysed by GC-MS using experimental conditions previously optimized (described in section 1.2. and 1.3., Figure 8).

TABLE 2. MIX composition

Mix components	Concentration (ppb)
2-propanol	88
N-butyl acetate	889
Hexanal	8
2-heptanone	8
D-limonene	8

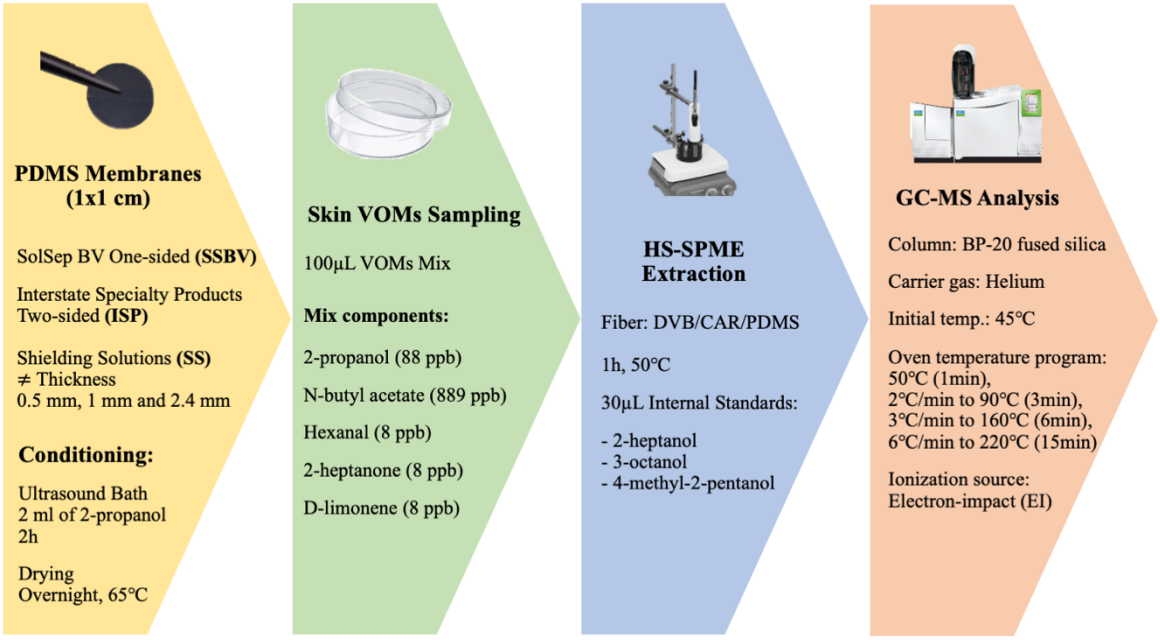


FIGURE 8. Experimental layout for *in vitro* assays

2.3. In vivo assays

Once the best PDMS membrane for skin VOMs sampling were selected, the next step was the determination of the best time of membrane exposure and body location for the sampling procedure. To avoid interferent VOMs from the materials used for sampling being extracted, the gauze was also previously conditioned with ethanol for 1 h and dried overnight at 65°C, and a hypoallergenic adhesive was also used. Previously to the *in vivo* assays, blank assays were performed to determine the eventual presence of contaminants derived from the gauze and the hypoallergenic adhesive. To determine the best sampling time, preliminary assays involved 30 min, 1h, 2h and 3h sampling in the arm were realized. Regarding the appropriate body location for sampling, three body locations were assayed – arm, behind the ear and nape (Figure 9) – that are easily accessible and allow a comfortable sampling for the subjects recruited.

FIGURE 9. VOMs sampling procedure (arm, behind the ear, nape)

A control group composed of 5 females and 3 males (Table 3) was recruited for this study performed according to the experimental layout shown in Figure 10.

TABLE 3. Control group description

Control	Gender	Age
1	Female	21
2	Male	34
3	Female	32
4	Female	31
5	Female	24
6	Male	24
7	Female	29
8	Male	30

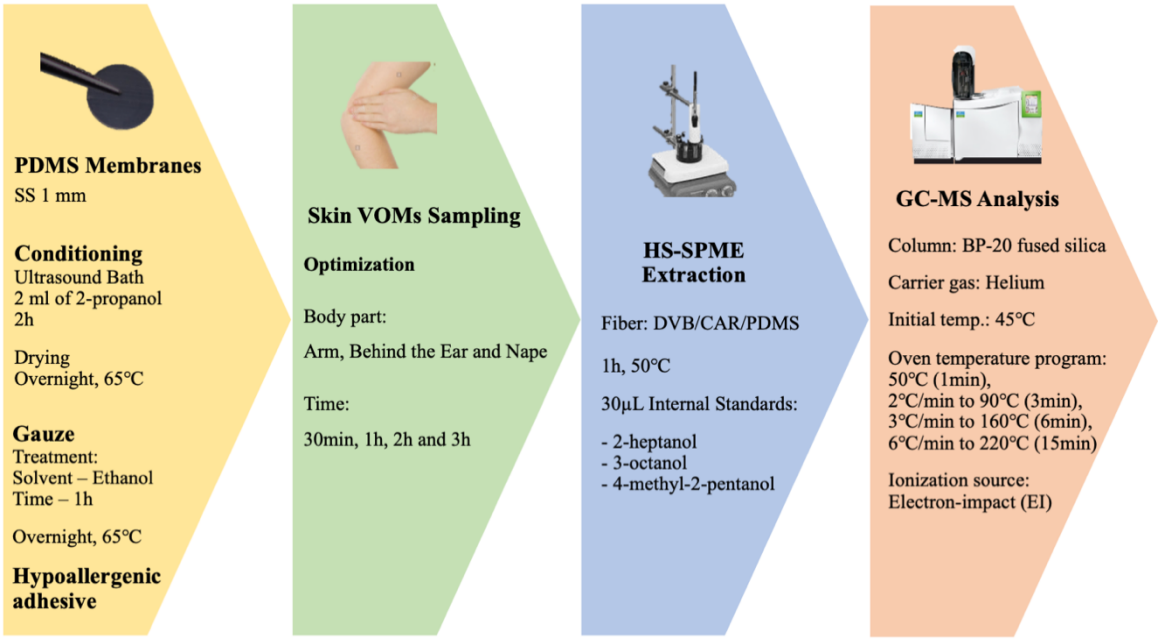


FIGURE 10. Experimental layout for *in vivo* assays

Following the preliminary *in vivo* assays, our goal was to move on to assays with patients affected with NDDs. However, the COVID-19 pandemic outbreak and consequent contingency measures did not allow this final interventional study. For obvious reasons, it would be impossible to respect most of those measures while doing skin VOMs sampling in NDDs patients, namely the social distance requirements and the access to the health care facilities where the patients are being assisted.

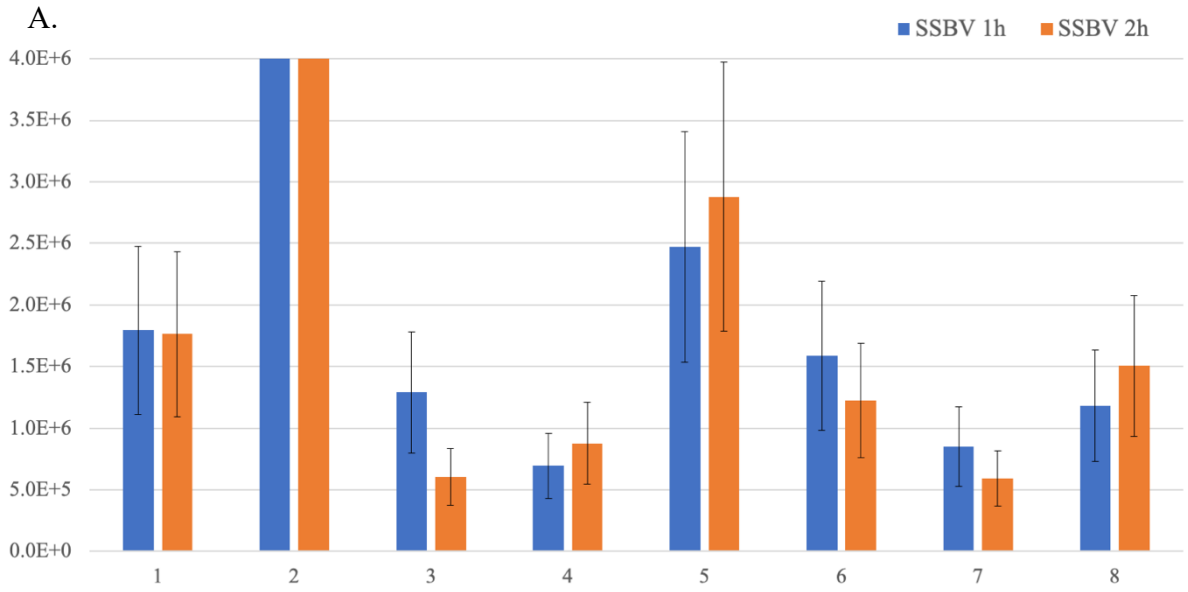
PART 3.

RESULTS AND DISCUSSION

1. Optimization of the sampling methodology

1.1. PDMS membrane conditioning

The raw PDMS membrane sheets were cut into 1cm^2 sections for sampling and analysed by HS-SPME/GC-MS. A considerable number of contaminants were observed in the raw PDMS membranes (Figures 11 and 12). Thus, membranes were conditioned to avoid such contaminants *carry over*. To define the best clean up procedure, membranes were subjected to 1h and 2h of US in isopropanol (Figures 11A, 11B, and 12A) and it was noted that, for the contaminants identified, a 2h-US proved to be more efficient, since most of the peak compounds decreased its intensity and, in the case of ISP and SS 0.5mm membranes, some contaminants weren't even detected after the 2h treatment. For the SS 1mm and SS 2.4mm, since they had higher thickness, 3h US was also assayed (Figures 12B and 12C). However, the 3h period is not more effective than 2h, because despite some contaminants were not detected in higher ultrasound bath time, other's peaks were more intense. Therefore, 2h of US in isopropanol was selected as the appropriate membrane condition procedure.



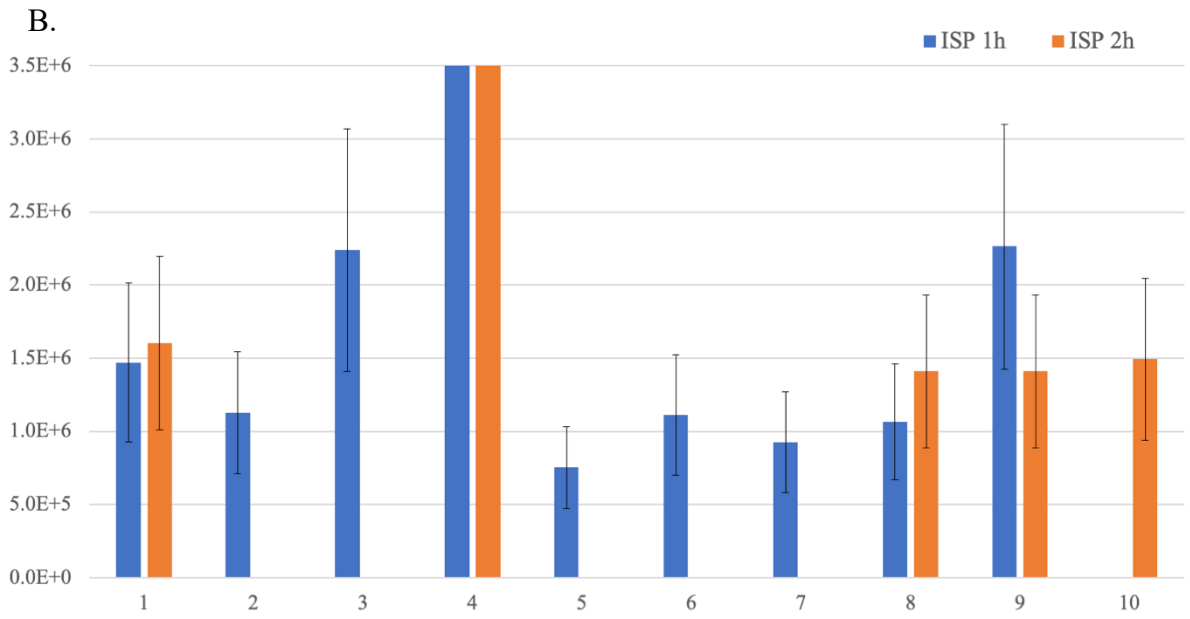
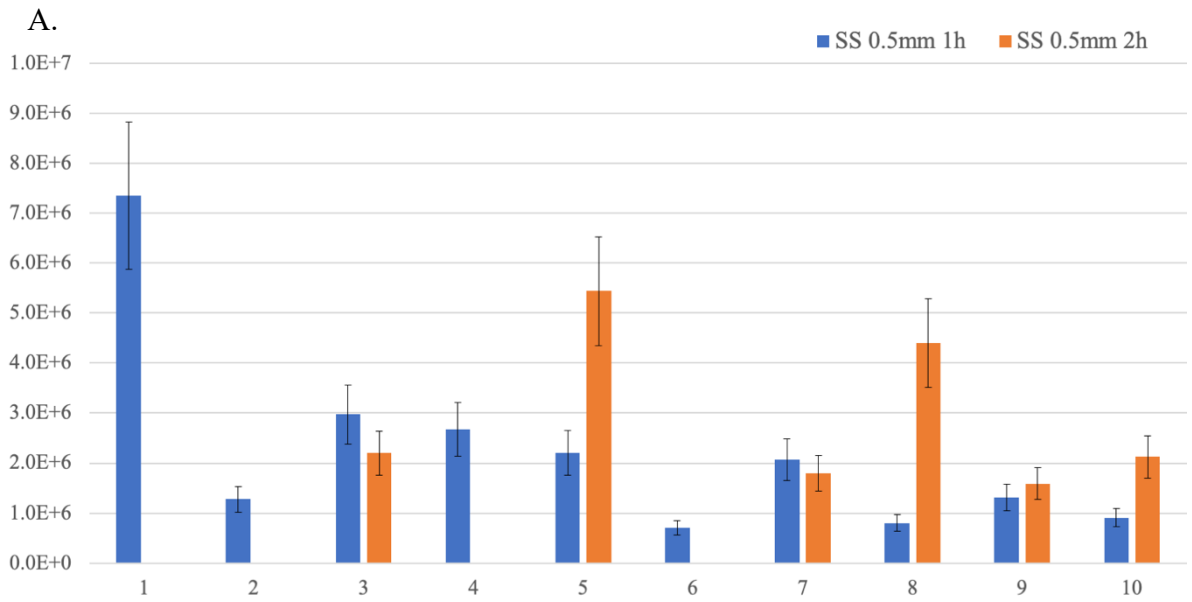


FIGURE 11. SSBV (A) and ISP (B) membrane conditioning
(raw data available in Appendix 1 and 2)

Legend A: 1 – TMS derivative - Oxalic acid; 2 – 2-ethyl-1-hexanol; 3 – 2,6-dimethyl-6-trifluoroacetoxyoctane; 4 – O-decyl-hydroxylamine; 5 – 2-(2-butoxyethoxy)ethanol; 6 – Phenol; 7 – 7-methyl-6-tridecene; 8 – 2,3-dichlorophenylester-6-fluor-2-trifluormethyl benzoic acid.
Legend B: 1 – n-propyl heptyl ether; 2 – 2,5-di-tert-butyl-3,4-bis(trifluoromethyl)-thiolane-3,4-dicarbonitrile; 3 – 2,6-dimethyl-6-trifluoroacetoxyoctane; 4 – 2-ethyl-1-hexanol; 5 – O-decyl-hydroxylamine; 6 – 6-ethyl-2-methyl-decane; 7 –Benzaldehyde; 8 – 2,6-dimethyl-6-trifluoroacetoxyoctane; 9 – 2-(2-butoxyethoxy)-ethanol; 10 – Phenol.



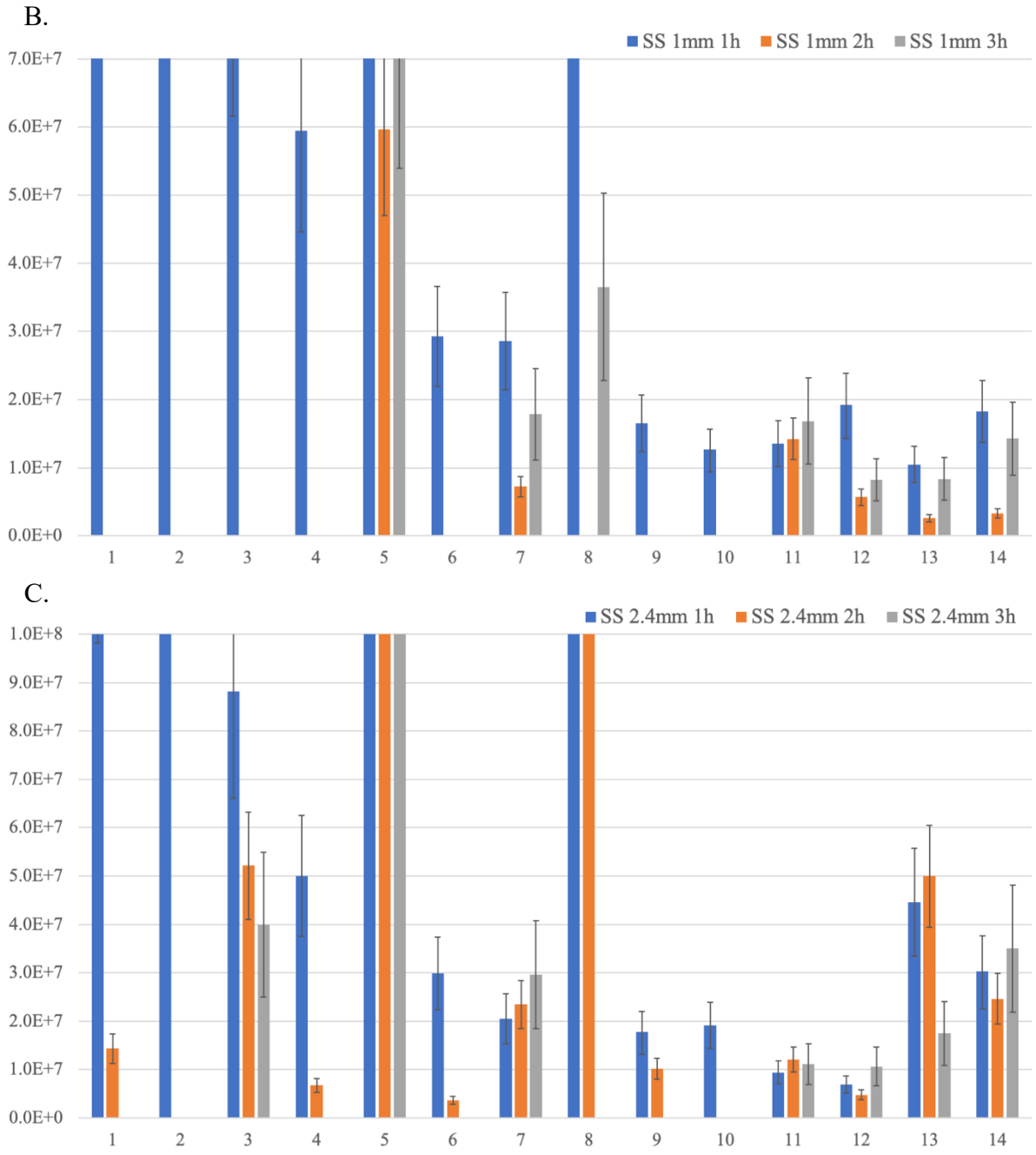


FIGURE 12. SS 0.5mm (A), SS 1mm (B) and SS 2.4mm (C) membrane conditioning (raw data available in Appendix 3, 4 and 5)

Legend A.: 1 – 1-methoxy-2-propanol; 2 – 1,3-dioxolane-2-methanol; 3 – TMS derivative - Oxalic acid; 4 – 1-(1-methylethyl)-2-nonyl-cyclopropane; 5 – 2-ethyl-1-hexanol; 6 – TMS derivative - Oxalic acid; 7 – E-14-hexadecenal; 8 – 2-(2-butoxyethoxy)-ethanol; 9 – 1,1-dimethylethoxybenzene; 10 – 2,3-dichlorophenylester-6-fluor-2-trifluormethyl benzoic acid.
Legend B. and C.: 1 – 2-propanol; 2 – Ethanol; 3 – 1,1,3,3,5,5-hexamethylsiloxane; 4 – Trimethylsilanol; 5 – Thiobis(methylene)bis(thiomethyl)silane; 6 – 1,3-diisopropoxy-1,3-dimethyl-1,3-disilaciclobutane; 7 – 1-dodecene; 8 – TMS derivative - Oxalic acid; 9 – 1,1,3,3,5,5-hexamethyltrisiloxane; 10 – 4-trifluoroacetoxytetradecane; 11 – 1-tetradecene; 12 – 2-ethyl-1-hexanol; 13 – TMS derivative - Ethylene glycol; 14 – Naphthalene.

1.2. In vitro assays

Following the optimization of the conditioning conditions for the PDMS membranes, the best membrane for skin VOMs sampling was assayed between the three vendors and thickness available (described in section 2.1 – Part 2). The two-sided SSBV membrane proved to be more effective for 2-propanol and hexanal extraction, however, it wasn't able to extract D-limonene (Figure 13). Moreover, it was also the membrane that retrieved higher standard deviations. In turn, the ISP membrane allowed the best extraction efficiency for 2-heptanone, though with higher standard deviations and, similarly to the SSBV, D-limonene was not detected in this condition. Regarding the SS membranes (0.5-, 1-, and 2-mm thickness), lower standard deviations were obtained and the SS 1mm thickness proved to be a better fit. Overall, the SS1 mm membrane showed better results with more compounds detected, reduced standard deviations and larger areas for the majority of the compounds (Figure 14).

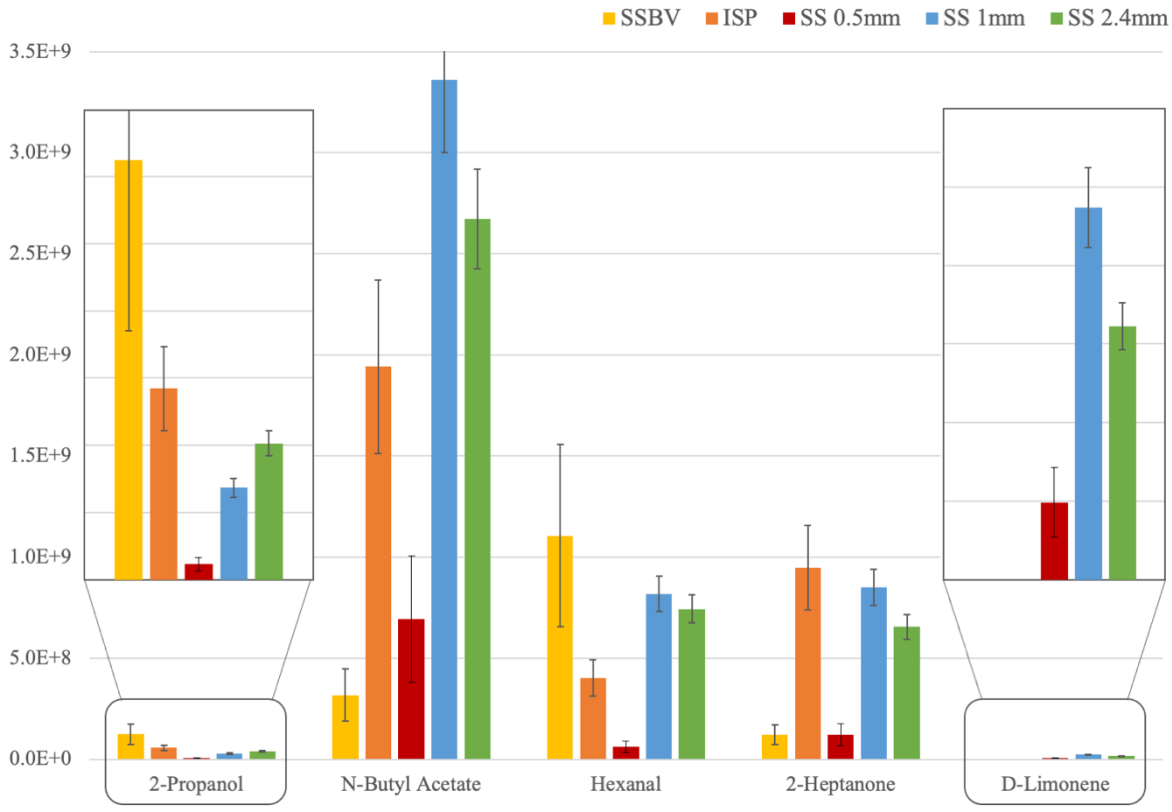


FIGURE 13. In vitro assays for membrane selection
(raw data available in Appendix 6)

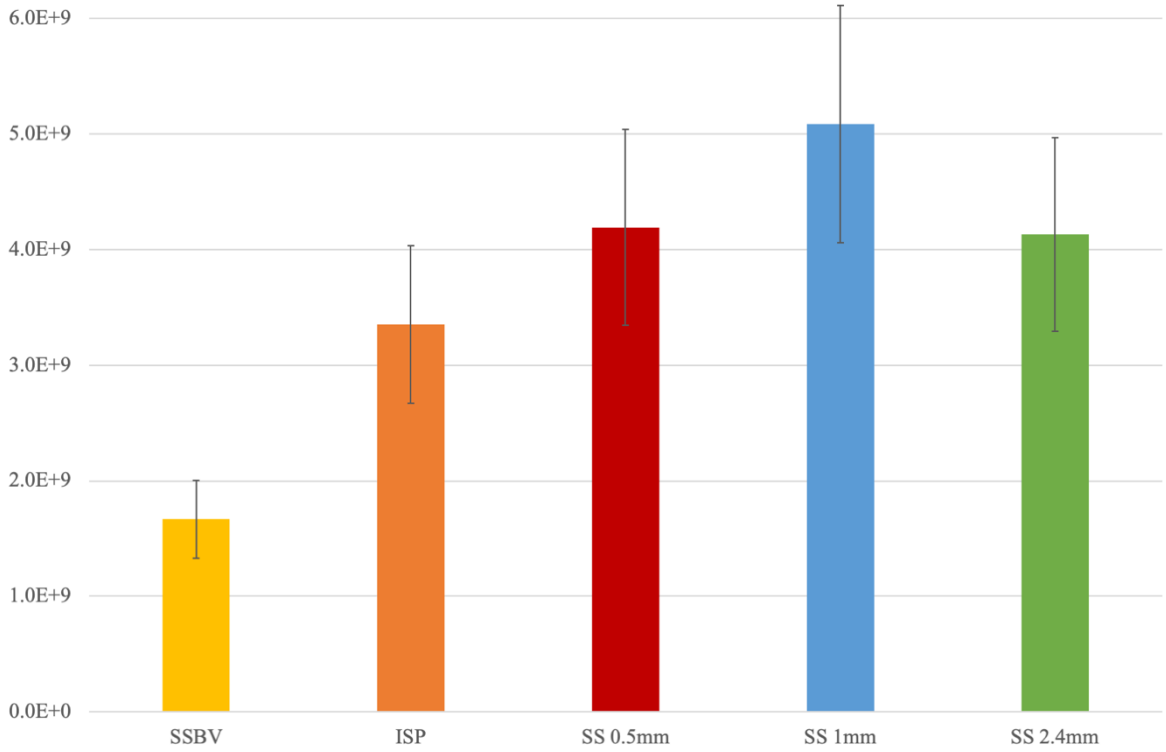


FIGURE 14. In vitro assays total areas
(raw data available in Appendix 6)

1.3. In vivo assays

To obtain the best experimental conditions for skin VOMs sampling, both the sampling place and exposure or sampling time were assayed. The best extraction time for skin VOMs sampling was assayed in the arm during 30 min, 1, 2 and 3h. (Figure 15). The 30 min-sampling is not very efficient, since it was the one with less VOMs detected, although with higher peaks for some compounds (1-butanol, 1-dodecene, 2-butoxyethanol and naphthalene – Figure 15). Higher areas were obtained with 1 h of sampling for many of the VOMs identified, as well as with 2h and 3h for many others (Figure 15). Taking in consideration the clinical condition of the patients with NDDs that were planned to enrol in this study, 1h of sampling seems the most appropriate sampling time, mitigating eventual disturbances for these patients.

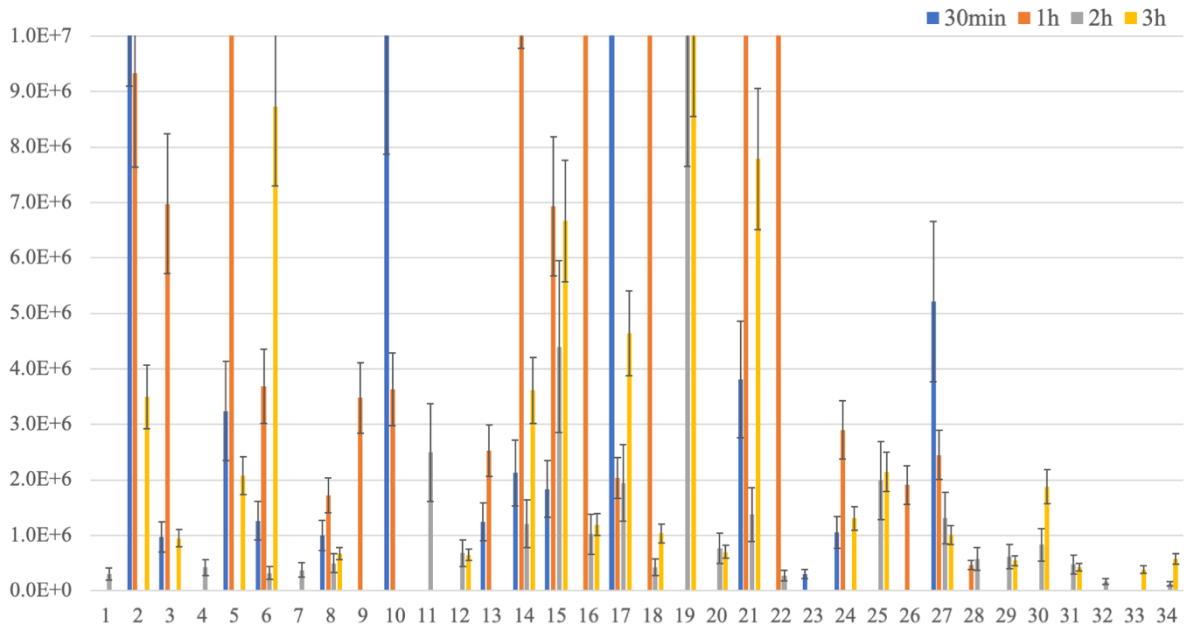


FIGURE 15. In vivo assays for best sampling time
(raw data available in Appendix 7)

Legend: 1 – Hydrazine carboxamide; 2 – 1-butanol; 3 – 3-heptanone; 4 – n-ethyl-n-methyl-1-propanaminemethyl; 5 – 2-ethylhexanal; 6 – D-limonene; 7 – 1H-tetrazol-5-amine; 8 – 6-ethyl-2-methyl-decane; 9 – 3,5-octadien-2-ol; 10 – 1-dodecene; 11 – 2,6-dimethyl-6-trifluoroacetoxyoctane; 12 – 5-methyl-1-phenyl-1-hexanone; 13 – n-octyl phenyl ketone; 14 – Octanal; 15 – 6-methyl-5-hepten-2-one; 16 – 4-azidoheptane; 17 – 2-butoxyethanol; 18 – 3,7-dimethyl-3-octanol; 19 – 1-tridecene; 20 – 7-methyl-6-tridecene; 21 – Benzaldehyde; 22 – Linalool; 23 – (S)-3,4-dimethylpentanol; 24 – Acetophenone; 25 – 1-methyl-1-(2-methylpropyl)-2-nonyl-cyclopropane; 26 – Phenylmethyl ester acetic acid; 27 – Naphthalene; 28 – 3-isopropylbenzaldehyde; 29 – 6,10-dimethyl-(E)-5,9-undecadien-2-one; 30 – Octanoic acid; 31 – 1-methoxyethanol benzoate; 32 – Butylphenyl ester carbonic acid; 33 – Tetramethyl-phosphorodiamidous fluoride; 34 – n-methyl-1-octadecanamine.

With the optimization steps that were already done, an experimental methodology was defined for the extraction and analysis of skin VOMs (Figure 16).

After the choice of 1h of skin sampling, a control group composed of 8 individuals was assayed in the arm, behind the ear and nape as possible sampling locations. Figure 17 shows a typical chromatogram of the ear and the arm.

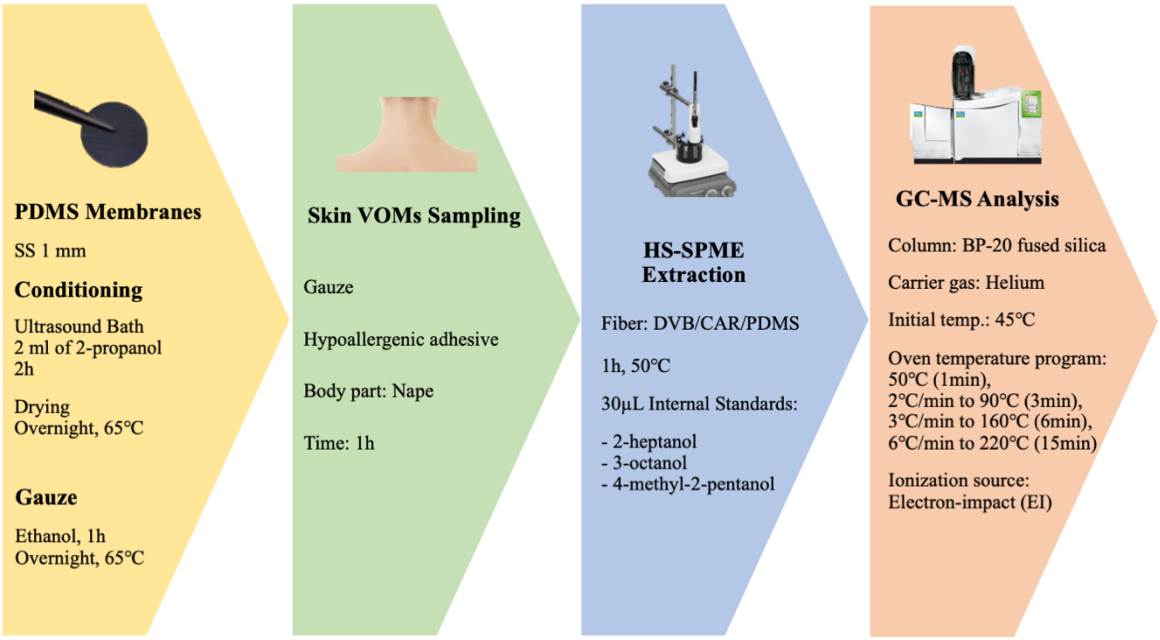


FIGURE 16. Optimized experimental methodology

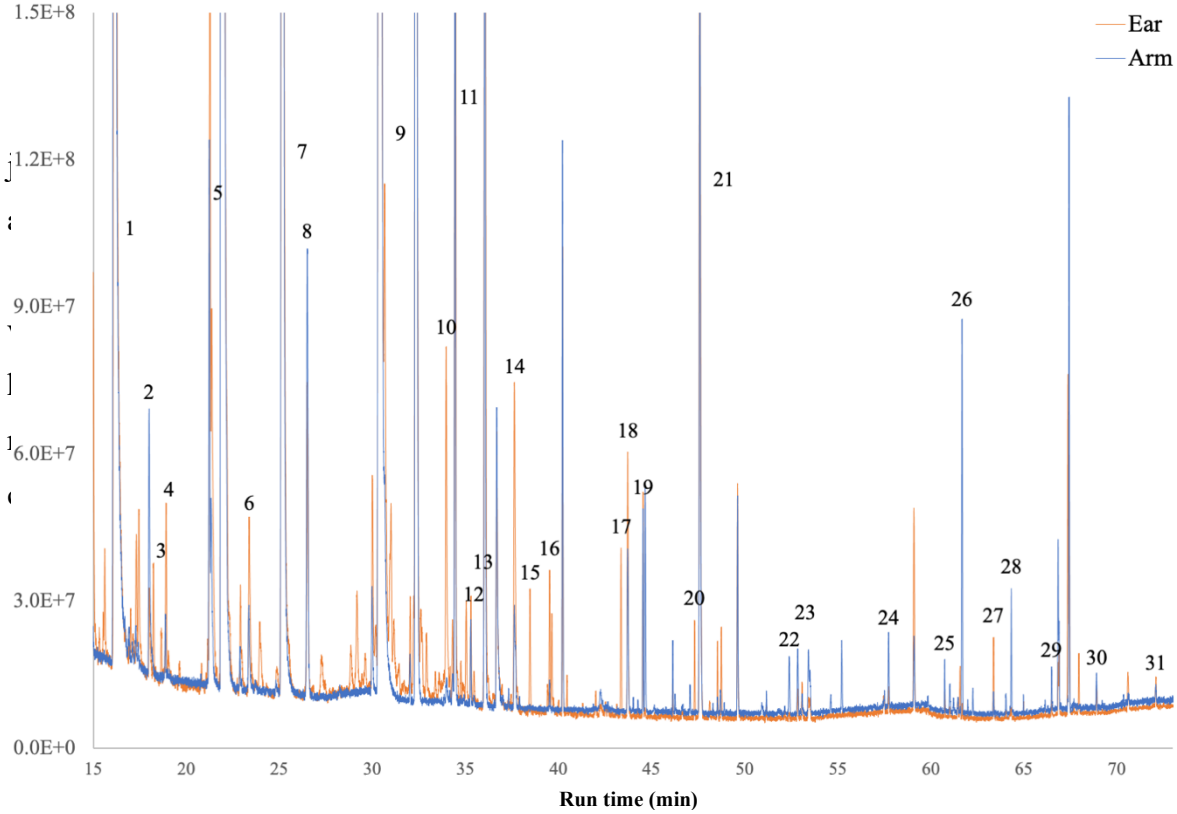


FIGURE 17. Typical chromatogram
(All peaks identification available in Appendix 8)

Major peaks identification: 5 – 1-dodecene; 8 – 6-methyl-5-hepten-2-one; 10 – 2,2,4-trimethyl-1-pentanol; 11 – 1-tridecene; 13 – 7-methyl-6-tridecene; 21 – Naphthalene; 26 – 2-methylbutyl acetate.

At this point of the experimental work, different problems in the procedure arose, justifying repetitions of the same control with several weeks of difference. The results show a media of the duplicates for each repetition.

As can be observed in Figure 18, the results for the same control reveal a high variability since there are many differences between the total areas of the replicas. The same happened with the compounds identified, since the number of VOMs identified in the two repetitions also shown a big inconsistency. (Figure 19). Nevertheless, most of the identified compounds belong to alcohols, hydrocarbons and cyclic hydrocarbons families.

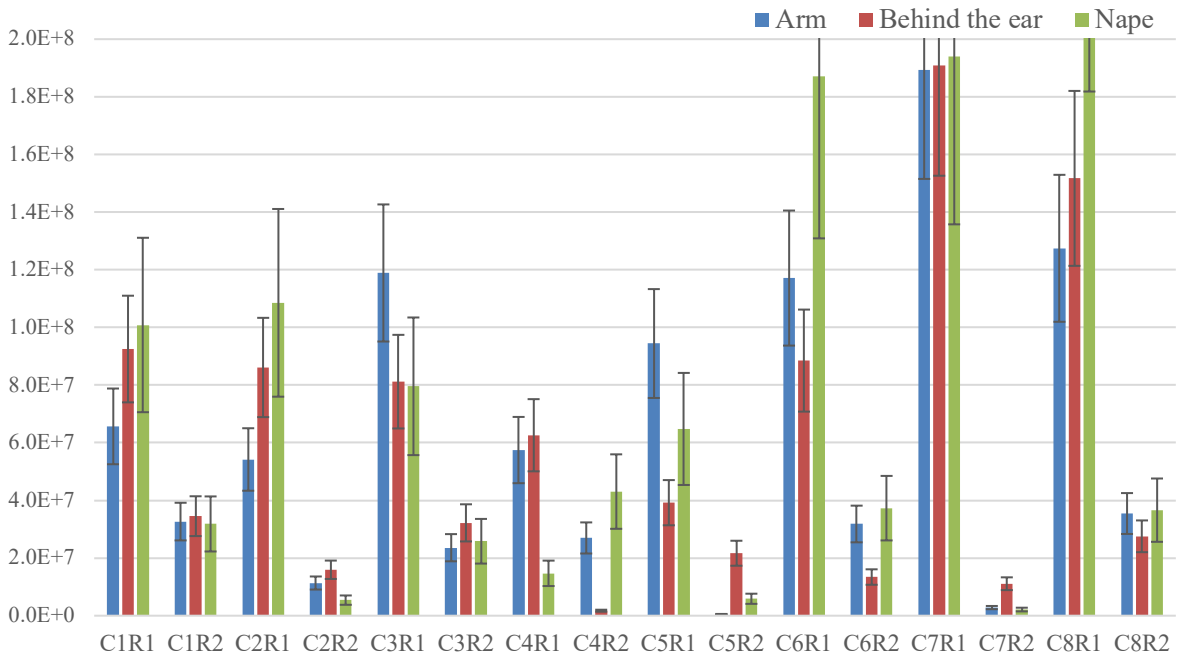


FIGURE 18. Control group total areas
(raw data available in Appendix 9; CxRx – Control x, Repetition x)

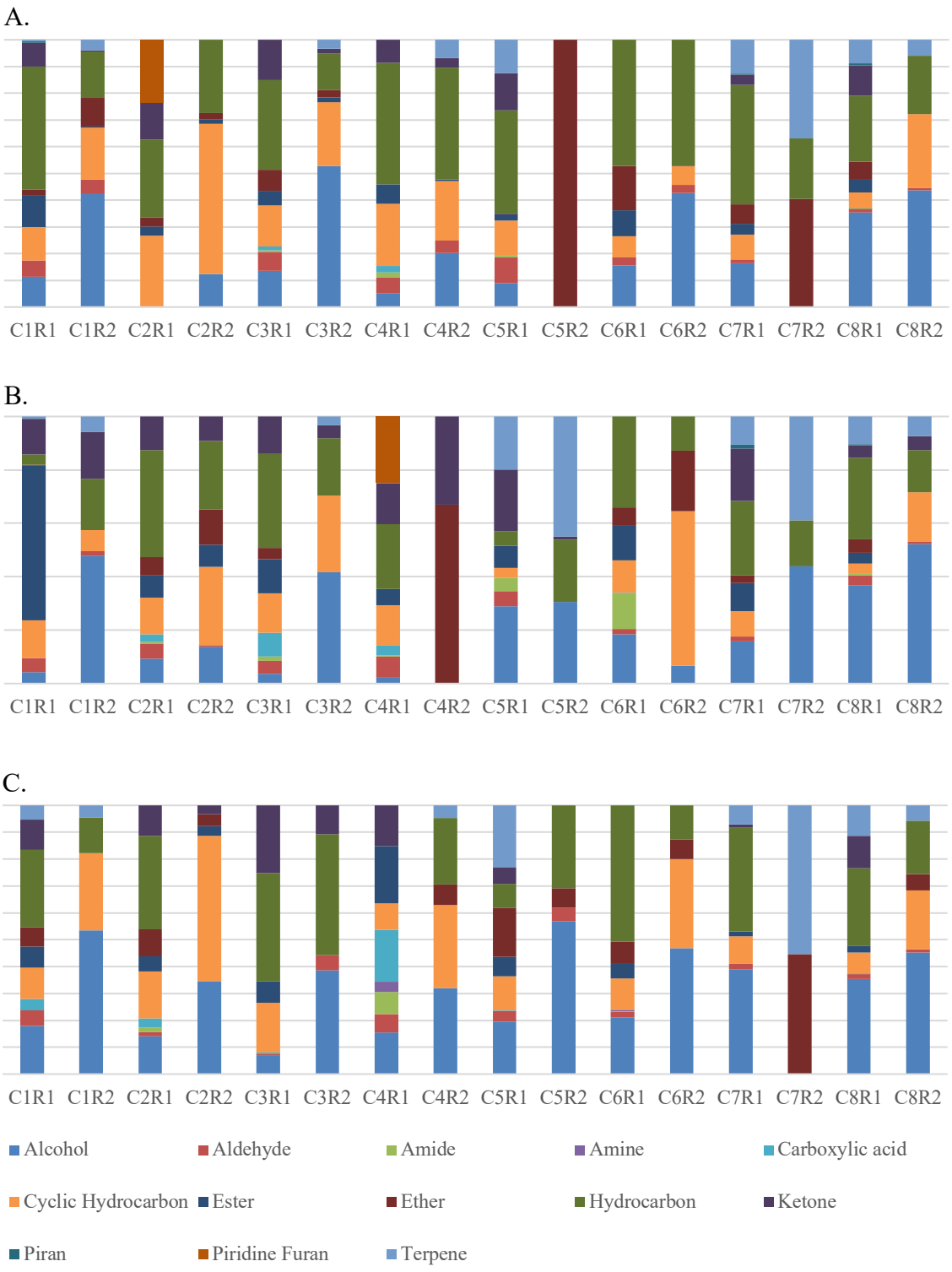


FIGURE 19. Arm (A), "Behind the ear" (B) and Nape (C) skin VOMs sampling: distribution of the VOMs by chemical group
(in %; raw data available in Appendix 10, 11 and 12)

In these assays, 82 VOMs were identified in the skin of the subjects of the control group: 67 VOMs in the arm, 68 VOMs in the ear and 69 VOMs in the nape. (Table 4) To assess the relevance of the identified VOMs, the data obtained was queried in the *Human Metabolome Database* (HMDB, version 4.0, https://www.hmdb.ca/) to obtain inputs for the biological location and possible origin of those VOMs. As shown in Table 4, many of the VOMs identified can have both an endogenous origin, as well as result from diet.

For a small number of VOMs, there is not so far metabolic information to verify their origin. Overall, it is clear that the sampling method was able to collect VOMs exhaled by the skin of the recruited subjects and not contaminants from the environment. In fact, some of the skin VOMs identified had been previously described and detected in the skin. This is the case of octanal, undecane, 6-methyl-5-hepten-2-one and D-limonene. Octanal is derived from the lipid pathway, undecane is derived from the shikimic pathway, 6-methyl-5-hepten-2-one and D-limonene are both derived from the terpene pathway, being the 6-methyl-5-hepten-2-one a carotenoid derivative [50]. Among the VOMs identified, some were present in more than half of the recruited subjects. The more frequent were 2,2-dimethyl-1-hexanol and 2,2,4-trimethyl-1-pentanol (Alcohols); benzaldehyde (Aldehyde); naphthalene, pentylcyclopropane and trans-1-methyl-2-nonyl-cyclohexane (Cyclic Hidrocarbon); 3-methylheptyl acetate, cyclobutyl tridecyl ester phtalic acid and diethyl phthalate (Esters); 1-tridecene and 2,4,4-triethyl-1-hexene (Hydrocarbons); 6-methyl-5-hepten-2-one and acetophenone (Ketones); and D-limonene (Terpene). In the case of naphthalene, although it can be present naturally in the skin, it was identified as contaminant in the SS 1mm PDMS membranes (*in vitro* assays).

Another important aspect to refer in the skin VOMs identified is that some compounds were exclusive of each of the body parts assayed. This was the case of 1-butanol and undecane exclusively identified in the arm. The metabolites found exclusively in the ear were heptanal, semustine, homosalate and n-propyl heptyl ether. Lastly, the nape was the location where more distinctive metabolites were identified: 3-ethyl-3-methyl-2-pentanol, 4-methyl-3-pentanol, 2-methylindene, 3-methyl-3-phenyl azetidine, 7-trydecylamine, 1,3-dietoxy-2-methylenepropane and 3-acetoxydodecane. It is important to refer that the amines – 2-methylindene, 3-methyl-3-phenyl azetidine, 7-trydecylamine – were the only chemical groups identified in only one body location (nape). Nevertheless, it would be very important to obtain a higher number of assays and recruited subjects to consolidate the data obtained.

TABLE 4. Characterization of the skin VOMs identified in this study

Chemical Group	CAS Number	Volatile Organic Metabolite	Arm (n=8)	Ear (n=8)	Nape (n=8)	Biological location	Possible origin
Alcohol	52340-78-0	(±)-Hydrobenzoin [L2]	6	4	6	Subcellular	Endogenous
Alcohol	100431-87-6	(S)-3-ethyl-4-methylpentanol [L2]	4	3	2	Subcellular	-
Alcohol	71-36-3	1-butanol [L2]	3	0	0	Fibroblasts, Epidermis, Biofluids	Endogenous, Food
Alcohol	36653-82-4	1-hexadecanol [L2]	2	0	2	Fibroblasts, Biofluids, Subcellular	Endogenous, Food
Alcohol	7735-42-4	1,16-hexadecanediol [L2]	2	1	0	-	-
Alcohol	7473-98-5	2-hydroxy-iso-butyrophenone [L2]	1	0	1	-	-
Alcohol	7795-80-4	2-methyl-2,3-pentanediol [L2]	1	1	1	-	-
Alcohol	2370-13-0	2,2-dimethyl-1-hexanol [L2]	6	6	7	-	-
Alcohol	123-44-4	2,2,4-trimethyl-1-pentanol [L2]	7	7	7	-	-
Alcohol	66576-22-5	3-ethyl-3-methyl-2-pentanol [L2]	0	0	2	-	-
Alcohol	5877-42-9	4-ethyl-1-octyl-3-ol [L2]	0	1	1	-	-
Alcohol	1502-05-2	Cyclodecanol [L2]	3	1	3	-	-
Alcohol	80625-44-1	E-11,13-tetradecadien-1-ol [L2]	4	6	6	-	-
Alcohol	2216-51-5	Levomenthol [L2]	2	2	1	Epidermis, Skeletal muscle, Bladder, Mouth, Nerve cells, Subcellular, Biofluids	Endogenous, Food
Aldehyde	5362-50-5	4-methyl-3-pentenal [L2]	0	0	1	-	-
Aldehyde	100-52-7	Benzaldehyde [L2]	7	8	7	Biofluids	Endogenous, Food
Aldehyde	18328-11-5	Benzenebutanal [L2]	2	2	1	-	-
Aldehyde	111-71-7	Heptanal [L2]	0	1	0	Biofluids, Subcellular	Endogenous, Food
Aldehyde	124-13-0	Octanal [L2]	2	4	3	Biofluids, Subcellular	Endogenous, Food

Amide	57-56-7	Hydrazinecarboxamide [L2]	2	3	3	-	-
Amide	7531-52-4	L-prolinamide [L2]	2	1	0	-	-
Amide	111-40-0	n-(2-aminoethyl)-1,2-ethanediamine [L2]	2	0	2	Extracellular, Subcellular	Endogenous, Food
Amide	761-65-9	n,n-dibutyl-formamide [L2]	4	4	3	-	-
Amide	2728-05-4	n,n-diethyl-4-methyl benzamide [L2]	2	0	2	-	-
Amide	13909-09-6	Semustine [L2]	0	1	0	-	-
Amine	2177-47-1	2-methylindene [L2]	0	0	1	-	-
Amine	5961-33-1	3-methyl-3-phenyl azetidine [L2]	0	0	2	Biofluids	-
Amine	22513-16-2	7-tridecylamine [L2]	0	0	1	-	-
Carboxylic acid	6303-58-8	4-phenoxy-butanoic acid [L2]	1	3	3	-	-
Carboxylic acid	334-48-5	n-decanoic acid [L2]	2	3	3	-	Endogenous, Food
Carboxylic acid	124-07-2	Octanoic acid [L2]	0	1	1	-	-
Cyclic Hydroc.	108-67-8	Mesitylene [L2]	1	1	0	Subcellular	-
Cyclic Hydroc.	91-20-3	Naphthalene [L2]	8	7	8	Biofluids, Subcellular	Endogenous, Food, Synthetic
Cyclic Hydroc.	2511-91-3	Pentylcyclopropane [L2]	7	7	5	-	-
Cyclic Hydroc.		Trans-1-methyl-2-nonyl-cyclohexane [L2]	6	5	6	-	-
Ester	118-60-5	2-ethylhexyl salicylate [L2]	3	4	3	Biofluids	-
Ester	72218-58-7	3-methylheptyl acetate [L2]	5	5	5	-	-
Ester		Cyclobutyl tridecyl ester phthalic acid [L2]	8	7	7	-	-
Ester	84-66-2	Diethyl phthalate [L2]	3	5	8	Biofluids	-

Ester	118-56-9	Homosalate [L2]	0	1	0	-	-
Ester	79-31-2	Isobutyric acid [L2]	4	5	5	Biofluids, Fibroblasts	Endogenous, Food
Ester	24851-98-7	Methyl (2-pentyl-3-oxocyclopentyl) acetate [L2]	2	4	2	Biofluids, Extracellular, Subcellular	Endogenous, Food
Ester	599-04-2	Pantolactone [L2]	2	3	4	-	-
Ether	54004-23-8	(E)-5-butoxy-2-pentene [L2]	6	4	4	-	-
Ether	629-64-1	1,1-oxybis-heptane [L2]	2	2	2	-	-
Ether	25307-84-0	1,3-dietoxy-2-methylenepropane [L2]	0	0	2	-	-
Ether	4362-22-5	2-(1-phenylethyl)-1,3-dioxolane [L2]	1	2	1	-	-
Ether	61986-67-2	2,6-dimethyl-6-trifluoro acetoxyoctane [L2]	4	6	7	-	-
Ether	60826-26-8	3-acetoxydodecane [L2]	0	0	1	-	-
Ether	116436-59-0	3-trifluoroacetoxy-6-ethyldecane [L2]	3	1	2	-	-
Ether	71112-89-5	n-propyl heptyl ether [L2]	0	2	0	-	-
Hydrocarbon	112-41-4	1-dodecene [L2]	5	4	5	Biofluids, Subcellular	-
Hydrocarbon	62184-74-1	1-nonylcyclopentane [L2]	4	3	5	-	-
Hydrocarbon	2437-56-1	1-tridecene [L2]	6	5	5	Biofluids, Subcellular	Endogenous, Food
Hydrocarbon	55534-69-5	2,2-dimethyl-(E)-4-decene [L2]	3	1	3	-	-
Hydrocarbon	7225-67-4	2,2,3,3,5,6,6-heptamethylheptane [L2]	2	1	0	-	-
Hydrocarbon	98982-97-9	2,4-dimethyl-1-heptanol [L2]	2	1	3	-	-
Hydrocarbon		2,4,4-triethyl-1-hexene [L2]	6	5	5	-	-
Hydrocarbon	107-40-4	2,4,4-trimethyl-2-pentene [L2]	6	7	4	-	-
Hydrocarbon	62108-27-4	2,4,6-trimethyldecane [L2]	3	5	2	-	-

Hydrocarbon	1974-05-6	3-bromoheptane [L2]	5	3	5	-	-
Hydrocarbon	3178-29-8	4-propylheptane [L2]	0	1	3	-	-
Hydrocarbon	56312-52-8	5-bromo-2,3-dimethyl-2-pentene [L2]	5	2	3	-	-
Hydrocarbon	13151-06-9	7-methyl-1-octene [L2]	2	2	4	-	-
Hydrocarbon	24949-42-6	7-methyl-6-tridecene [L2]	4	2	3	-	-
Hydrocarbon	16580-24-8	m-menthane [L2]	3	2	3	Extracellular, Subcellular	Endogenous, Food
Hydrocarbon	1120-21-4	Undecane [L2]	1	0	0	Biofluids, Subcellular	Endogenous, Food
Ketone	36678-43-0	(E)-4-hepten-2-one [L2]	2	7	5	Extracellular, Subcellular	Food
Ketone	51835-44-0	1-methoxyethyl benzoate [L2]	2	2	0	-	-
Ketone	19377-95-8	1,5-ditert-butyl-3,3-dimethylbicyclo [3.1.0] hexan-2-one [L2]	1	2	1	-	-
Ketone	1728-46-7	2-tert-butylcyclohexanone [L2]	1	3	2	-	-
Ketone	18641-71-9	2,4-dimethyl-3-heptanone [L2]	0	1	1	-	-
Ketone	106-35-4	3-heptanone [L2]	1	1	1	Biofluids, Extracellular, Subcellular	Endogenous, Food
Ketone	14129-48-7	4-octen-3-one [L2]	4	0	3	Extracellular, Subcellular	Endogenous, Food
Ketone	20690-70-4	5-methylene-3-heptanone [L2]	2	3	5	-	-
Ketone	110-93-0	6-methyl-5-hepten-2-one [L2]	7	8	6	Biofluids, Extracellular, Subcellular	Endogenous, Food
Ketone	98-86-2	Acetophenone [L2]	8	7	7	Biofluids, Extracellular, Subcellular	Food
Ketone	119-61-9	Benzophenone [L2]	1	1	2	Biofluids, Subcellular	Endogenous, Food, Synthetic
Piran	3056-46-0	Methyl 3,6-anhydro-β-d-glucopyranoside [L2]	3	3	1	-	-
Piridine Furan	69022-83-9	2-methyl-4-oxide-furo(3,2-b) pyridine [L2]	1	1	0	-	-
Terpene	22327-39-5	Carvone [L2]	1	1	0	Biofluids, Extracellular, Subcellular	Endogenous, Food

Terpene	5989-27-5	D-limonene [L2]		7	6	6	Biofluids, Extracellular, Epidermis, Skin, Subcellular	Endogenous, Food

[L1]Identified metabolites (GC-MS analysis of the metabolite of interest and a chemical reference standard of suspected structural equivalence, with all analyses performed under identical analytical conditions within the same laboratory) [97]
[L2]Putatively annotated compounds (spectral (MS) similarity with NIST database) [97]
[L3]Putatively characterized compound classes (physicochemical properties consistent with a particular class of organic compounds) [97]
[L4]Unknown (spectral signal that can be reproducibly detected and quantified) [97]

The results obtained revealed significative variations among subjects and assays and so the skin VOMs of the nape of two controls – one male and one female – were analysed during four consecutive days to verify if the results were consistent from day-to-day. The nape was selected for this assay as it was the body part with a bigger number of VOMs detected. Unfortunately, the results obtained in this experiment were very disappointing since the number of VOMs identified decreased by more than half. Eventually, the DVB/CAR/PDMS SPME fibre reached its usability or the GC-MS equipment was not retrieving its normal sensitivity. In any case, the contingence measures associated to the COVID-19 outbreak did not allowed to verify the cause of the lack of sensitivity observed in the referred assays.

PART 4.

CONCLUSION

Conclusions

NDDs diagnosis relies essentially on the clinical symptoms, however, neurodegeneration begins long before the patient experiences any symptoms, so there is an interest in the early diagnosis of NDDs. In this work, the characterization of the skin VOMs was explored as a promising and non-invasive tool to discover BMs for an early diagnosis of NDDs. For that purpose, a methodology suitable for the analysis of skin VOMs was developed, consisting in a PDMS membrane sampling followed by HS-SPME/GC-MS analysis. Accordingly, the 2h of US in 2-propanol was necessary to obtain a proper clean up and activation of the different membranes assayed. Among SSBV, ISP and SS membranes, the SS 1 mm was the most efficient for skin VOMs sampling. Regarding the sampling time, 1h of sampling was the most appropriate, despite some VOMs would be enriched with longer periods of sampling. However, taking in consideration the characteristics of NDDs, longer sampling times could disturb the NDDs patients. The optimized experimental layout was applied to a control group composed of 8 individuals and in three different body areas, nape, arm and ear. A total of 82 compounds – 67 VOMs in the arm, 68 VOMs in the ear and 69 VOMs in the nape, were identified, being alcohols, hydrocarbons and cyclic hydrocarbons the most abundant. Most of the VOMs identified were associated to an endogenous origin, indicating that the methodology developed is efficient in the collection of VOMs released through the skin. These results present, however, a high variation in the same subject as between subjects. A higher number of subjects and sampling procedures would be necessary to consolidate the data obtain. Unfortunately, the containment measures associated to the COVID-19 outbreak, namely the need for the social isolation of NDDs patients, does not allow the continuation of this research work during the next months. Nevertheless, the preliminary results obtained show the methodology develop is suitable for the skin VOMs sampling of NDDs patients, holding therefore a great potential to unveil putative BM for these diseases.

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APPENDICES

A1. SSBV membrane conditioning data

SSBV Contaminants	Retention time	Areas 1h	Areas 2h
TMS derivative - Oxalic acid	32.40	1.79E+06	1.76E+06
2-ethyl-1-hexanol	36.02	8.62E+06	1.02E+07
2,6-dimethyl-6-trifluoroacetoxyoctane	44.53	1.29E+06	6.07E+05
O-decyl-hydroxylamine	44.65	6.94E+05	8.78E+05
2-(2-butoxyethoxy) ethanol	49.62	2.47E+06	2.88E+06
Phenol	59.11	1.59E+06	1.22E+06
7-methyl-6-tridecene	64.32	8.50E+05	5.93E+05
2,3-dichlorophenylester-6-fluor-2-trifluormethyl benzoic acid	67.44	1.18E+06	1.51E+06

A2. ISP membrane conditioning data

ISP Contaminants	Retention time	Areas 1h	Areas 2h
n-propylheptyl ether	30.45	1.47E+06	1.60E+06
2,5-di-tert-butyl-3,4-bis(trifluoromethyl)-thiolane-3,4-dicarbonitrile	30.64	1.13E+06	0.00E+00
2,6-dimethyl-6-trifluoroacetoxyoctane	34.41	2.24E+06	0.00E+00
2-ethyl-1-hexanol	36.01	2.85E+07	7.93E+06
O-decyl-hydroxylamine	36.65	7.54E+05	0.00E+00
6-ethyl-2-methyl-decane	37.29	1.11E+06	0.00E+00
Benzaldehyde	37.62	9.27E+05	0.00E+00
2,6-dimethyl-6-trifluoroacetoxyoctane	44.52	2.26E+06	1.41E+06
Phenol	59.10	0.00E+00	1.49E+06

A3. SS 0.5mm membrane conditioning data

SS 0.5mm Contaminants	Retention time	Areas 1h	Areas 2h
1-methoxy-2-propanol	14.02	7.35E+06	0.00E+00
1,3-dioxolane-2-methanol	21.98	1.28E+06	0.00E+00
TMS derivative - Oxalic acid	32.38	2.97E+06	2.20E+06
1-(1-methylethyl)-2-nonyl-ciclopropane	34.40	2.68E+06	0.00E+00
2-ethyl-1-hexanol	36.01	2.21E+06	5.44E+06
TMS derivative - Oxalic acid	40.22	7.03E+05	0.00E+00
E-14-hexadecenal	44.52	2.07E+06	1.80E+06
2-(2-butoxyethoxy) ethanol	49.62	8.04E+05	4.40E+06
1,1-dimethyletoxybenzene	59.10	1.32E+06	1.59E+06
2,3-dichlorophenylester-6-fluor-2-trifluormethyl benzoic acid	67.44	9.09E+05	2.12E+06

A4. SS 1mm membrane conditioning data

SS 1mm Contaminants	Retention time	Areas 1h	Areas 2h	Areas 3h
2-propanol	7.23	1.31E+08	0.00E+00	0.00E+00
Ethanol	7.41	3.04E+08	0.00E+00	0.00E+00
1,1,3,3,5,5-hexamethylsiloxane	8.00	8.21E+07	0.00E+00	0.00E+00
Trimethylsilanol	9.91	5.95E+07	0.00E+00	0.00E+00
Thiobis(methylene)bis(thiomethyl)silane	12.99	7.97E+08	5.97E+07	8.64E+07
13-diisopropoxy-13-dimethyl-13-disilaciclobutane	18.13	2.93E+07	0.00E+00	0.00E+00
1-dodecene	21.44	2.86E+07	7.22E+06	1.79E+07
TMS derivative - Oxalic acid	32.30	1.23E+08	0.00E+00	3.65E+07
1,1,3,3,5,5-hexamethyltrisiloxane	34.38	1.66E+07	0.00E+00	0.00E+00

4-trifluoroacetoxytetradecane	34.60	1.26E+07	0.00E+00	0.00E+00
1-tetradecene	34.56	1.35E+07	1.42E+07	1.68E+07
2-ethyl-1-hexanol	36.17	1.91E+07	5.70E+06	8.21E+06
TMS derivative - Ethylene glycol	40.23	1.05E+07	2.61E+06	8.34E+06
Naphthalene	47.72	1.82E+07	3.32E+06	1.43E+07

A5. SS 2.4mm membrane conditioning data

SS 2.4mm Contaminants	Retention time	Areas 1h	Areas 2h	Areas 3h
2-propanol	7.24	1.31E+08	1.43E+07	0.00E+00
Ethanol	7.41	3.37E+08	0.00E+00	0.00E+00
1,1,3,3,5,5-hexamethylsiloxane	8.00	8.81E+07	5.21E+07	4.00E+07
Trimethylsilanol	9.93	5.01E+07	6.79E+06	0.00E+00
Thiobis(methylene)bis(thiomethyl)silane	12.95	8.37E+08	6.07E+08	2.61E+08
13-diisopropoxy-13-dimethyl-13-disilaciclobutane	18.17	2.99E+07	3.69E+06	0.00E+00
1-dodecene	21.41	2.05E+07	2.35E+07	2.96E+07
TMS derivative - Oxalic acid	32.08	4.79E+08	3.61E+08	0.00E+00
1,1,3,3,5,5-hexamethyltrisiloxane	34.38	1.76E+07	1.02E+07	0.00E+00
4-trifluoroacetoxytetradecane	34.60	1.92E+07	0.00E+00	0.00E+00
1-tetradecene	34.58	9.42E+06	1.21E+07	1.12E+07
2-ethyl-1-hexanol	36.16	6.97E+06	4.82E+06	1.07E+07
TMS derivative - Ethylene glycol	40.17	4.46E+07	5.00E+07	1.75E+07
Naphtalene	47.68	3.01E+07	2.46E+07	3.50E+07

A6. In vitro assays data for membrane selection

Mix compound	Retention time	SSBV	ISP	SS 0.5mm	SS 1mm	SS 2.4mm
2-Propanol	7.35	1.25E+08	5.69E+07	1.98E+07	2.73E+07	4.06E+07
n-Butyl Acetate	12.05	3.17E+08	1.94E+09	2.58E+09	3.36E+09	2.67E+09
Hexanal	12.93	1.11E+09	4.02E+08	3.56E+08	8.18E+08	7.44E+08
2-Heptanone	17.18	1.21E+08	9.49E+08	1.15E+09	8.51E+08	6.55E+08
D-Limonene	18.29	0.00E+00	0.00E+00	8.14E+07	2.37E+07	1.61E+07
Total Area	-	1.67E+09	3.35E+09	4.19E+09	5.08E+09	4.13E+09

A7. In vivo assays data for deciding skin sampling period

VOMs	Retention time	30 min	1h	2h	3h
Hydrazine carboxamide	14.43	0.00E+00	0.00E+00	3.04E+05	0.00E+00
1-butanol	14.93	1.26E+07	9.34E+06	0.00E+00	3.49E+06
3-heptanone	15.63	9.70E+05	6.98E+06	0.00E+00	9.46E+05
n-ethyl-n-methyl-1-propanaminemethyl	17.49	0.00E+00	0.00E+00	4.19E+05	0.00E+00
2-ethylhexanal	17.50	3.24E+06	1.38E+07	0.00E+00	2.08E+06
D-limonene	18.31	1.26E+06	3.68E+06	3.21E+05	8.73E+06
1H-tetrazol-5-amine	18.98	0.00E+00	0.00E+00	3.74E+05	0.00E+00
6-ethyl-2-methyl-decane	18.98	9.97E+05	1.72E+06	4.98E+05	6.70E+05
3,5-octadien-2-ol	21.17	0.00E+00	3.48E+06	0.00E+00	0.00E+00
1-dodecene	21.32	1.09E+07	3.63E+06	0.00E+00	0.00E+00

2,6-dimethyl-6-trifluoroacetoxyoctane	21.32	0.00E+00	0.00E+00	2.49E+06	0.00E+00
5-methyl-1-phenyl-1-hexanone	22.97	0.00E+00	0.00E+00	6.82E+05	6.50E+05
n-octyl phenyl ketone	22.96	1.24E+06	2.53E+06	0.00E+00	0.00E+00
Octanal	23.41	2.12E+06	1.20E+07	1.21E+06	3.61E+06
6-methyl-5-hepten-2-one	26.51	1.84E+06	6.93E+06	4.40E+06	6.67E+06
4-azidoheptane	30.00	0.00E+00	3.01E+07	1.02E+06	1.19E+06
2-butoxyethanol	30.65	4.83E+07	2.04E+06	1.94E+06	4.64E+06
3,7-dimethyl-3-octanol	32.79	0.00E+00	1.53E+07	4.23E+05	1.03E+06
1-tridecene	34.43	0.00E+00	0.00E+00	1.18E+07	1.02E+07
7-methyl-6-tridecene	35.27	0.00E+00	0.00E+00	7.64E+05	7.02E+05
Benzaldehyde	37.62	3.80E+06	1.46E+07	1.38E+06	7.78E+06
Linalool	39.04	0.00E+00	2.56E+07	2.70E+05	0.00E+00
(S)-3,4-dimethylpentanol	39.50	3.02E+05	0.00E+00	0.00E+00	0.00E+00
Acetophenone	43.73	1.05E+06	2.89E+06	0.00E+00	1.31E+06
1-methyl-1-(2-methylpropyl)-2-nonyl-cyclopropane	44.54	0.00E+00	0.00E+00	1.99E+06	2.14E+06
Phenylmethyl ester acetic acid	47.18	0.00E+00	1.91E+06	0.00E+00	0.00E+00
Naphthalene	47.59	5.21E+06	2.45E+06	1.32E+06	1.00E+06
3-isopropylbenzaldehyde	48.77	0.00E+00	4.68E+05	5.72E+05	0.00E+00
6,10-dimethyl-(E)-5,9-undecadien-2-one	52.39	0.00E+00	0.00E+00	6.16E+05	5.43E+05
Octanoic acid	61.03	0.00E+00	0.00E+00	8.28E+05	1.87E+06
1-methoxyethanol benzoate	61.57	0.00E+00	0.00E+00	4.74E+05	4.24E+05
Butylphenyl ester carbonic acid	63.38	0.00E+00	0.00E+00	1.64E+05	0.00E+00
Tetramethyl-phosphorodiamidous fluoride	63.36	0.00E+00	0.00E+00	0.00E+00	3.84E+05
n-methyl-1-octadecanamine	64.33	0.00E+00	0.00E+00	1.27E+05	5.79E+05

A8. Typical chromatogram peak identification

1: 4-methyl-2-pentanol (IS)
2: 4-oxide-furo(3,2-b)pyridine
3: D-limonene
4: 6-ethyl-2-methyl-decane
5: 1-dodecene
6: Octanal
7: 2-heptanol (IS)
8: 6-methyl-5-hepten-2-one
9: 3-octanol (IS)
10: 2,2,4-trimethyl-1-pentanol
11: 1-tridecene
12: (E)-5-butoxy-2-pentene
13: 7-methyl-6-tridecene
14: Benzaldehyde
15: 5-methylene-3-heptanone
16: Pentyl cyclopropane
17: Levomenthol
18: Acetophenone
19: 1-methyl-1-(2-methylpropyl)-2-nonyl-cyclopropane
20: Carvone
21: Naphthalene
22: (E)-4-hepten-2-one
23: Hydrobenzoin
24: 2,6-dimethyl-6-trifluoroacetoxyoctane
25: Methyl 3,6-anhydro-α-D-galactopyranoside
26: 2-methylbutyl acetate
27: 4-phenoxy-butanoic acid
28: E-11,13-tetradecadien-1-ol
29: N-decanoic acid
30: Dibutyl phthalate
31: Benzophenone

A9. Control group total areas data (CxRx – Control x, Repetition x)

Control/ Repetition	Arm	Ear	Nape
C1R1	6.57E+07	9.25E+07	1.01E+08
C1R2	3.26E+07	3.45E+07	3.18E+07
C2R1	5.42E+07	8.61E+07	1.09E+08
C2R2	1.13E+07	1.59E+07	5.40E+06
C3R1	1.19E+08	8.12E+07	7.96E+07
C3R2	2.36E+07	3.22E+07	2.58E+07
C4R1	5.75E+07	6.26E+07	1.47E+07
C4R2	2.70E+07	1.73E+06	4.31E+07
C5R1	9.44E+07	3.92E+07	6.48E+07
C5R2	5.05E+05	2.17E+07	5.87E+06
C6R1	1.17E+08	8.85E+07	1.87E+08
C6R2	3.18E+07	1.34E+07	3.73E+07
C7R1	1.89E+08	1.91E+08	1.94E+08
C7R2	2.81E+06	1.11E+07	2.13E+06
C8R1	1.27E+08	1.52E+08	2.60E+08
C8R2	3.54E+07	2.75E+07	3.66E+07

A10. Arm in vivo assays: Chemical group percentage data (CxRx – Control x, Repetition x)

Chemical group	C1R1	C1R2	C2R1	C2R2	C3R1	C3R2	C4R1	C4R2	C5R1	C5R2	C6R1	C6R2	C7R1	C7R2	C8R1	C8R2
Alcohol	11%	42%	0%	12%	14%	53%	5%	20%	9%	0%	16%	43%	16%	0%	35%	44%
Aldehyde	6%	6%	0%	0%	7%	0%	6%	5%	10%	0%	3%	3%	1%	0%	1%	1%
Amide	0%	0%	0%	0%	1%	0%	2%	0%	1%	0%	0%	0%	0%	0%	0%	0%
Amine	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Carboxylic acid	0%	0%	0%	0%	1%	0%	2%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Cyclic Hydrocarbon	13%	19%	27%	56%	15%	24%	23%	22%	13%	0%	8%	7%	9%	0%	6%	28%
Ester	12%	0%	3%	2%	5%	2%	7%	1%	2%	0%	10%	0%	4%	0%	5%	0%
Ether	2%	11%	3%	2%	8%	3%	0%	0%	0%	100%	17%	0%	7%	40%	6%	0%
Hydrocarbon	46%	18%	29%	27%	34%	13%	45%	42%	39%	0%	47%	47%	45%	23%	25%	22%
Ketone	9%	0%	14%	0%	15%	2%	9%	4%	14%	0%	0%	0%	4%	0%	11%	0%
Piran	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	1%	0%
Piridine Furan	0%	0%	23%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Terpene	1%	4%	0%	0%	0%	3%	0%	7%	13%	0%	0%	0%	13%	37%	9%	6%

A11. Behind the ear in vivo assays: Chemical group percentage data

Chemical group	C1R1	C1R2	C2R1	C2R2	C3R1	C3R2	C4R1	C4R2	C5R1	C5R2	C6R1	C6R2	C7R1	C7R2	C8R1	C8R2
Alcohol	4%	48%	9%	14%	4%	42%	3%	0%	29%	31%	18%	7%	16%	44%	37%	52%
Aldehyde	5%	2%	6%	0%	5%	0%	8%	0%	6%	0%	2%	0%	2%	0%	4%	1%
Amide	0%	0%	1%	0%	2%	0%	0%	0%	5%	0%	13%	0%	0%	0%	1%	0%
Amine	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Carboxylic acid	0%	0%	3%	0%	9%	0%	4%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Cyclic Hydrocarbon	14%	8%	14%	29%	15%	29%	15%	0%	4%	0%	12%	58%	10%	0%	4%	18%
Ester	58%	0%	8%	8%	13%	0%	6%	0%	8%	0%	13%	0%	11%	0%	4%	0%
Ether	0%	0%	7%	13%	4%	0%	0%	67%	0%	0%	7%	23%	3%	0%	5%	0%
Hydrocarbon	4%	19%	40%	26%	35%	21%	24%	0%	5%	23%	34%	13%	28%	17%	31%	16%
Ketone	13%	18%	13%	9%	14%	5%	15%	33%	23%	1%	0%	0%	20%	0%	5%	5%
Piran	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	0%	0%
Piridine Furan	0%	0%	0%	0%	0%	0%	25%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Terpene	1%	6%	0%	0%	0%	3%	0%	0%	20%	45%	0%	0%	11%	39%	10%	7%

A12. Nape in vivo assays: Chemical group percentage data

Chemical group	C1R1	C1R2	C2R1	C2R2	C3R1	C3R2	C4R1	C4R2	C5R1	C5R2	C6R1	C6R2	C7R1	C7R2	C8R1	C8R2
Alcohol	18%	53%	14%	35%	7%	39%	16%	32%	19%	57%	21%	47%	39%	0%	35%	45%
Aldehyde	6%	0%	2%	0%	0%	6%	7%	0%	4%	5%	2%	0%	2%	0%	2%	1%
Amide	0%	0%	2%	0%	0%	0%	8%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Amine	0%	0%	0%	0%	0%	0%	4%	0%	0%	0%	1%	0%	0%	0%	0%	0%
Carboxylic acid	4%	0%	3%	0%	1%	0%	19%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Cyclic Hydrocarbon	12%	29%	18%	54%	18%	0%	10%	31%	13%	0%	12%	33%	10%	0%	8%	22%
Ester	8%	0%	6%	4%	8%	0%	21%	0%	7%	0%	6%	0%	2%	0%	2%	0%
Ether	7%	0%	10%	4%	0%	0%	0%	8%	18%	7%	8%	7%	0%	45%	0%	6%
Hydrocarbon	29%	13%	35%	0%	40%	45%	0%	25%	9%	31%	51%	13%	39%	0%	29%	20%
Ketone	11%	0%	11%	3%	25%	11%	15%	0%	6%	0%	0%	0%	1%	0%	12%	0%
Piran	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Piridine Furan	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Terpene	5%	5%	0%	0%	0%	0%	0%	5%	23%	0%	0%	0%	7%	55%	11%	6%