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TD

Non-Invasive Strategy in Assessing Asthma Through Biofluids Metabolomics Exploration
Exhaled breath and urine potentialities
Michael Manuel Lima Caldeira

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DOCTORAL THESIS

Michael Manuel Lima Caldeira
DOCTORATE IN CHEMISTRY
SPECIALTY IN ANALYTICAL CHEMISTRY

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November | 2014

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SUPERVISORS
José Sousa Câmara
Sílvia Maria da Rocha Simões Carriço

UNIVERSIDADE da MADEIRA universidade de aveiro theoria poiesis praxis

Non-invasive strategy in assessing asthma through biofluids metabolomics exploration: exhaled breath and urine potentialities

Michael Manuel Lima Caldeira

Thesis submitted to Madeira University in order to obtain the degree of Doctor in Analytical Chemistry

Thesis supervised by:

Professor Doutor José Sousa Câmara

Professora Doutora Sílvia Maria da Rocha Simões Carriço

Funchal – Portugal

November, 2014

Dedication

To my wife Joana and my parents Conceição and Manuel

"The important thing is not to stop questioning.
Curiosity has its own reason for existing."

Albert Einstein

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Resumo

A asma é um problema de saúde pública com impacto significativo na população pediátrica que tem apresentado um crescimento notável ao longo dos anos. O desafio proposto para esta tese de doutoramento foi o desenvolvimento de metodologias avançadas para o estabelecimento de padrões metabolómicos em urina e ar exalado associados à asma, cuja aplicabilidade foi posteriormente explorada para avaliar o estado da doença, a adesão ou efeito da terapia e para fins de diagnóstico.

A composição volátil do ar exalado foi estudada combinando a microextração em fase sólida em modo espaço-de-cabeça (HS-SPME) com cromatografia de gás acoplada a espectrometria de massa ou com a cromatografia gasosa bidimensional abrangente acoplada à espectrometria de massa com analisador de tempo de voo de alta resolução (GC×GC-TOFMS). Estas metodologias permitiram identificar centenas de compostos de diferentes famílias químicas. A análise multivariada (MVA) permitiu concluir que o perfil metabolómico de indivíduos com asma é caracterizado por teores mais elevados de compostos associados à peroxidação lipídica, eventualmente associado a *stress* oxidativo e inflamação (alcanos e aldeídos). Com vista a futuras aplicações em contexto clínico, foi definido um conjunto de 9 metabolitos, cuja aplicabilidade foi comprovada na monitorização do estado da doença e avaliação do efeito e/ou adesão à terapia.

O metaboloma volátil global de urina também foi explorado usando um método HS-SPME/GC×GC-TOFMS, tendo sido identificados cerca de 200 compostos. Foi realizada uma análise-direccionada usando 78 metabolitos relacionados com a peroxidação lípidica e consequentemente com os níveis de *stress* oxidativo e inflamação. O padrão metabolômico não volátil urinária de asma foi estabelecido usando ressonância magnética nuclear de protão (^{1}H NMR). Esta análise permitiu identificar vias metabólicas, como o *stress* oxidativo, metabolismo dos amino ácidos e lipídico e alterações da microflora intestinal, alterações no ciclo do ácido tricarboxílico, no metabolismo da histidina, acidose láctica, e a modificação de resíduos de tirosina livre após a estimulação de eosinófilos.

Os resultados obtidos permitiram explorar e demonstrar a potencialidade da análise do perfil metabolómico do ar exalado e urina na asma. Além do desenvolvimento bem sucedido das metodologias de análise, foi possível explorar através do ar exalado e da urina vias metabólicas afetadas pela asma observando-se complementaridade entre as matrizes, bem como verificar a aplicabilidade clínica dos métodos desenvolvidos.

Palavras-chave
Asma; Ar exalado; Urina; Metabolómica; GCxGC-ToFMS; ^{1}H NMR;

Abstract

Asthma is a significant health issue in the pediatric population with a noteworthy growth over the years. The proposed challenge for this PhD thesis was the development of advanced methodologies to establish metabolomic patterns in urine and exhaled breath associated with asthma whose applicability was subsequently exploited to evaluate the disease state, the therapy adhesion and effect and for diagnostic purposes.

The volatile composition of exhaled breath was studied combining headspace solid phase microextraction (HS-SPME) with gas chromatography coupled to mass spectrometry or with comprehensive two-dimensional gas chromatography coupled to mass spectrometry with a high resolution time of flight analyzer (GC×GC–ToFMS). These methodologies allowed the identification of several hundred compounds from different chemical families. Multivariate analysis (MVA) led to the conclusion that the metabolomic profile of asthma individuals is characterized by higher levels of compounds associated with lipid peroxidation, possibly linked to oxidative stress and inflammation (alkanes and aldehydes) known to play an important role in asthma. For future applications in clinical settings a set of nine compounds was defined and the clinical applicability was proven in monitoring the disease status and in the evaluation of the effect and / or adherence to therapy.

The global volatile metabolome of urine was also explored using an HS-SPME/GC×GC–ToFMS method and *c.a.* 200 compounds were identified. A targeted analysis was performed, with 78 compounds related with lipid peroxidation and consequently to oxidative stress levels and inflammation. The urinary non-volatile metabolomic pattern of asthma was established using proton nuclear magnetic resonance (^{1}H NMR). This analysis allowed identifying central metabolic pathways such as oxidative stress, amino acid and lipid metabolism, gut microflora alterations, alterations in the tricarboxylic acid (TCA) cycle, histidine metabolism, lactic acidosis, and modification of free tyrosine residues after eosinophil stimulation.

The obtained results allowed exploring and demonstrating the potential of analyzing the metabolomic profile of exhaled air and urine in asthma. Besides the successful development of analysis methodologies, it was possible to explore through exhaled air and urine biochemical pathways affected by asthma, observing complementarity between matrices, as well as, verify the clinical applicability.

Keywords

Asthma; Exhaled breath; Urine; Metabolomics; GCxGC-ToFMS; ^{1}H NMR

Publications

Publications in international journals with referee

M. Caldeira, A.S. Barros, M.J. Bilelo, A. Parada, J.S. Câmara, S.M. Rocha, Profiling allergic asthma volatile metabolic patterns using a headspace-solid phase microextraction/gas chromatography based methodology. J. Chromatogr. A 1218 (**2011**) 3771.

M. Caldeira, R. Perestrelo, A.S. Barros, M.J. Bilelo, A. Morête, J.S. Câmara, SM. Rocha, Allergic asthma exhaled breath metabolome: a challenge for comprehensive two-dimensional gas chromatography. J. Chromatogr. A 1254 (**2012**) 87.

M. Caldeira, A.S. Barros, A. Morête, J.S. Câmara, S.M. Rocha, The unexplored potential of exhaled breath analysis in the aid of asthma clinical management, **2015**, submitted.

M. Caldeira, A.S. Barros, A. Morête, J.S. Câmara, S.M. Rocha, Asthma volatile urinary metabolome uncovered by comprehensive two-dimensional gas chromatography-time of flight mass spectrometry (under preparation).

Oral communications

M. Caldeira, J.S. Câmara, S.M. Rocha, Estabelecimento de perfis metabólicos em crianças com asma alérgica e crianças controlo, 4as Jornadas de Bioquímica da Universidade de Aveiro, Aveiro, Portugal, **April 2010**.

M. Caldeira, J.S. Câmara, S.M. Rocha, In-deep study of compounds from oxidative stress in breath: a challenge to allergic asthma diagnosis, 12th International Symposium on Extraction Technologies, Poznan, Poland, **September 2010**.

M. Caldeira, A.S. Barros, J.S. Câmara, S.M. Rocha, Asthma molecular diagnosis in the clinical practice: reality or utopia?, 6as Jornadas de Bioquímica da Universidade de Aveiro-6th Biochemistry day – Pharmaceutical Biochemistry, Aveiro, Portugal, **April 2012**.

Poster communications

M. Caldeira, A.S. Barros, J.S. Câmara, S.M. Rocha, Development of complete factorial design to assess of allergic respiratory diseases metabolomic patterns by SPME/GC-qMS based methodology, International Conference on Breath and Breath Odor Research, Dortmund, Germany, **April 2009**.

M. Caldeira, A.S. Barros, A. Parada, J.S. Câmara, S.M. Rocha, Development of a complete factorial design to assess allergic respiratory diseases metabolomic patterns by a solid-phase microextraction gas chromatography-mass spectrometry based methodology**,** 6º Encontro Nacional de Cromatografia, Funchal, Portugal, **December 2009**.

M. Caldeira, J.S. Câmara, S.M. Rocha, Allergic asthma exhaled breath: from one dimensional to comprehensive two-dimensional chromatographic analysis, 34nd International Symposium on Capillary Chromatography and 7th GCxGC Symposium, Riva del Garda, Itália, **May 2010**.

M. Caldeira, J.S. Câmara, S.M. Rocha, Profiling oxidative stress markers in allergic asthma by analysis of exhaled breath using comprehensive two-dimensional chromatography, Exhaled Breath Analysis Conference: From Sensors to Devices and Applications, Barga, Italy, **October 2010**.

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List of Abbreviations

Abbreviation/ Acronyms	Significance
1D	One dimensional
^{1}H NMR	Proton nuclear magnetic resonance
AIT	Allergen specific immunotherapy
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
Car/PDMS	Carboxen / polydimethylsiloxane
CLTs	Cysteinyl leukotrienes
CW/DBV	Carbowax/divinylbenzene
DC	Direct current
DVB/CAR/PDMS	Divinylbenzene/carboxen/polydimethylsiloxane
EBC	Exhaled breath condensate
FeNo	Fractional exhaled nitric oxide
FEV1	Forced expiratory volume 1
FVC	Forced vital capacity
GC×GC–ToFMS	Comprehensive two-dimensional gas chromatography coupled to mass spectrometry with a high resolution time of flight analyzer
GC–MS	Gas chromatography coupled to mass spectrometry
GINA	Global Initiative for Asthma
GMC-SF	Granulocyte macrophage colony stimulating factor
HS-SPME	Headspace solid phase microextraction
ICS	Inhaled corticosteroids
IgE	Immunoglobulin E
IL	Interleukin
ISAAC	International Study of Asthma and Allergies in Childhood
LABAs	Long acting beta$_2$ agonists
LC-MS	Liquid chromatography coupled to mass spectrometry
LLE	Liquid-liquid extraction
LOO-CV	Leave one out cross validation
LPO	Lipid peroxidation
LTC4	Leukotriene C4
LTMs	Leukotriene modifiers
LTRA	Leukotriene receptor antagonists
LTRI	Leukotriene receptor inhibitors
MCCV	Monte Carlo cross validation

Abbreviation/ Acronyms	Significance
MDGC	Multidimensional gas chromatography
MVA	Multivariate analysis
PA	Polyacrylate
PCA	Principal component analysis
PDMS	Polydimethylsiloxane
PDMS/DVB	Polydimethylsiloxane/ divinylbenzene
PLS-DA	Partial least squares discriminating analysis
PQN	Probabilistic normalization quotient
RF	Radio frequency
RI	Retention indices
RNS	Reactive nitrogen species
ROC	Receiver operating characteristics
ROS	Reactive oxygen species
SABAs	Short acting $beta_2$ agonists
SPE	Solid-phase extraction
TCA	Tricarboxylic acid
Th	T-helper
TNF-α	Tumor necrosis factor alpha
UPLC-MS	Ultra performance liquid chromatography coupled to mass spectrometry
VOCs	Volatile organic compounds

CHAPTER 1
INTRODUCTION

1.1 Asthma

1.1.1 Asthma: definition, symptoms and clinical diagnosis

Asthma is a complex chronic disorder of the airways characterized by variable and recurring symptoms, airflow obstruction, bronchial hyperresponsiveness, and an underlying inflammation[1]. These characteristics interact and determine the clinical manifestations, asthma severity and treatment response. The symptomatology associated with asthma is coughing, wheezing, breathlessness and chest tightness that varies from person to person in severity and frequency (Figure 1.1).

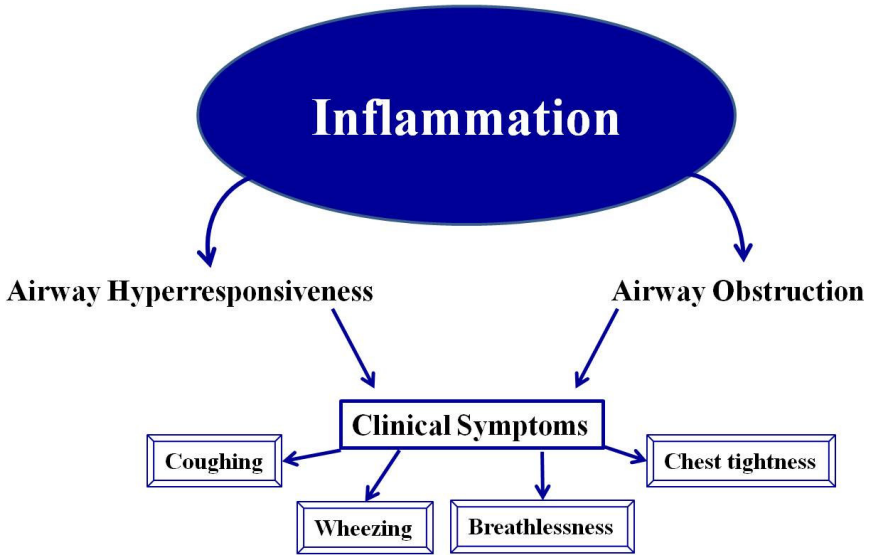


Figure 1.1 – The interaction between airway inflammation and clinical symptoms[1].

Asthma severity is divided into four groups: intermittent, persistent-mild, persistent-moderate and persistent-severe. Asthma control can also be classified as well, not well and poorly controlled[2]. Both asthma severity and control are assessed by criteria, such as symptoms presence, nighttime awakenings, normal activity impairment and peak flow among others. Asthma severity classification is highlighted when initiating therapy whereas asthma control is emphasized to monitor and change therapy as needed. Actually, asthma control is weighted equally with asthma severity to

establish the adequate therapy. Nevertheless, asthma severity can change over time and therefore continuing care is highly recommended[3].

Asthma complexity leads to an evolving concept. Previously, asthma emphasized the duality of allergic asthma and non-allergic asthma. More recently, research in this field led to separate into subgroups based upon clinical, physiologic, and pathologic characteristics. Thus, an array of phenotypes and endotypes arose. Asthma phenotypes include allergic asthma, severe steroid-resistant asthma and asthma induced by exposure to air pollution, cigarette smoke, diesel exhaust particles, obesity, aspirin and exercise[4]. Eighty percent of childhood asthma has been reported to be allergic[5]. The triggers that cause allergic asthma are known as allergens. These comprise a large array of substances that two important characteristics: (1) they are almost always organic and (2) are always harmless to non-allergic individuals. Common sources include animal proteins (cat and dog allergens), dust mites, cockroaches, fungi and pollens.

Asthma symptoms can manifest at any time in life, although many people develop asthma as children. In this PhD thesis, the focus was on pediatric asthma. Diagnosing asthma in children can be a difficult task to perform differing from pre-school children, school-aged children, adolescents and adults in several ways. In early life, asthma is quite variable and not fully grasped. Early childhood wheezing and symptoms related to asthma are heterogeneous disorders with several phenotypes and variable expressions during infancy. Another issue is related to the use of objective lung function tests in children, as these procedures poses a challenge for children to perform. Asthma control in children is also a complex issue due to the anatomic differences, as for example the smaller size of airways and lower inspiratory flow rate, affecting medication deposition in the affected airways. In addition to these aspects, there are a number of comorbidities that have similar symptoms to the ones caused by asthma, as well as, asthma is also associated with other pathologies such as rhinitis, hay fever, atopic eczema and rhinosinusitis. These comorbidities have a role in asthma clinical expressions and are documented to influence asthma management and control[6].

In children younger than 6 years old, lung function tests lack accuracy and the physician relies on the information that the parents and even the child give. The diagnosis may be in a halt until months have passed of symptom observation. Children younger than age 3 that have asthma symptoms the physician doctor may use a wait-and-see approach as the effects of medication are still not clear. In children 6 years old

and older, pulmonary function tests are performed: auscultation, spirometry and bronchodilator response test[1].

Spirometry is a relatively simple and non-invasive method to measure the full lung inflation air volume and flow using forced manoeuvres[7]. It plays an important role in diagnosis and managing respiratory diseases; nevertheless there is no standard reference tests to accurate determine the sensitivity or specificity of spirometry to establish airflow limitation[8]. Gender, age, height, weight and ethnic group are factors that influence the obtained values[9]. The volume of air exhaled forcefully in 1 second is denominated forced expiratory volume 1 (FEV1) and the forced vital capacity (FVC) is the volume that can be maximally forcefully exhaled, so FVC contains FEV1. To detect an obstruction the ratio FEV1/FVC has to be lower than 0.7 and FEV1 less than 80% of the predicted value. FVC is highly dependent on the effort and collaboration of the subject and to complete a maneuver subjects must expel air forcefully and completely at least three times. This is extremely difficult for elderly subjects, children and those with severe airflow limitation. Another disadvantage in spirometry is that it is not recommended for asymptomatic individuals, being used for those with respiratory symptoms and there is no benefit in using spirometry to monitor disease status. This limitation leads to underdiagnosis in younger subjects and overdiagnosis in elderly subjects, which leads to false positives and false-negatives depending on age[10].

The determination of nitric oxide (NO) levels in exhaled breath is also an available tool that may complement asthma diagnosis. NO induced by inflammatory mediators in the airways are reportedly increased in asthmatic patients[11]. Several reports have been published to demonstrate its clinical value. NO is produced when L-arginine is catalyzed by nitric oxide synthases (NOS) into L-citrulline[12]. NO can be measured online allowing the evaluation with a real-time display. In the offline mode exhaled breath is collected into containers for posterior analysis. NO values are affected by several factors such as age, race, gender, atopy, diet, nasal NO contamination, measurement techniques, exhalation flow rate, among others. Although a connection between NO levels and eosinophils in blood, bronchoalveolar lavage fluid (BALF), sputum, and bronchial biopsies may correlate to eosinophilic inflammation, there are confounders that might result in higher eosinophilic content. Other pathologies that cause inflammation other than asthma, as atopic eczema reported by Zinelli and co-authors[13], increase the levels of NO found in exhaled breath. Recently, Hastie *et al.*[14] reported that NO levels, among other biomarkers, are poor surrogates in the accurate

prediction of sputum eosinophil and neutrophil percentages. The link between NO and treatments remain unclear, nevertheless lower values of NO are reported with the administration of short acting β-agonists[15]. Using NO in the clinical practice is still debated due to the inconsistencies and due to the large number of factors that can affect the outcome.

Whenever the doctor suspects that asthma is related to allergens evaluation can be performed by skin prick tests. This test is performed by pricking the skin with a pin that contains a small dosage of the allergen, and if an immune-response arises in the form rash or urticaria the conclusion is that the patient has a hypersensitivity to that allergen (Figure 1.2).

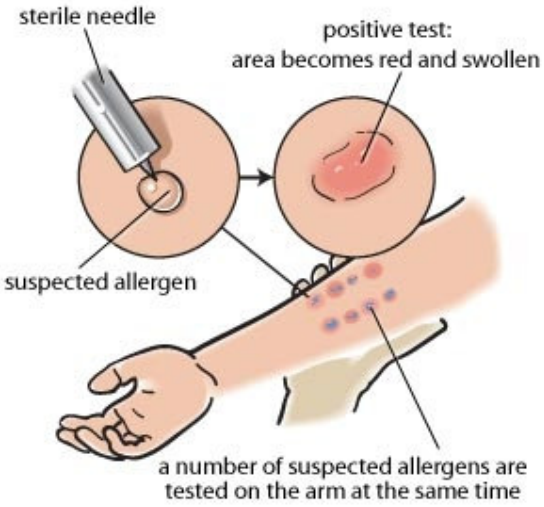


Figure 1.2 – Skin prick test[16]

The advantage of this method is the quick interpretation (15-20 minutes) after pricking the skin, accompanied with visual indication of the results. Nevertheless, this test is not practical with patients with extensive eczema, dermographism, urticaria, and moreover cannot be applied in subjects that are consuming anti-histamines or other medications that may interfere with the results and lacks procedure worldwide standardization and allergen panels[17]. Although there was a considerable progress in asthma diagnosis, management and treatment, in the past years the development of new methods that allow standardization, more reliable results, and the follow-up of therapy, to yield better asthma controlled status, led to considerable research that take into consideration

important parameters such as airway inflammation, instead of symptoms and lung function.

The invasive method of obtaining samples such as BALF, bronchoscopy or bronchial biopsy are normally used to measure airway inflammation and to give more detailed information of asthmatic patients. Sputum induction is performed by inhaling isotonic or hypertonic solutions by nebulisation to induce airway secretion that can be expectorated[18]. BALF is an important tool in the diagnosis of several lung diseases in which a bronchoscope is passed through the mouth or nose into the lungs and a liquid is squirted in and collected for examination. The use of lung tissue in asthma is also a possibility using samples obtained during the bronchoscopy procedure yielding important information on tissue resident cells[19]. Although these procedures indicate airway inflammation more accurately, the invasiveness impairs their regular use in the clinical practice creating the necessity to assess the inflammation status in a less invasive manner. Blood is a less invasive method than sputum induction and bronchoscopy. It can be assumed that it can be used to indirectly measure the state of asthma as the inflamed tissue releases chemoatratractants and cytokines, which recruit activated immune cells from peripheral blood. Another possibility for atopy evaluation is the determination of allergen specific Immunoglobulin E (IgE) from blood samples. IgE antibodies are detected and measured by allergen-specific IgE antibody assays. The amount of bound allergen-specific IgE is interpolated from a standard calibration curve linked to WHO IgE standard[20].

1.1.2 Costs, incidence and prevalence

According to the Global Initiative for Asthma (GINA) it is reported that 300 million individuals suffer from asthma, and is the most chronic disease among children[21]. Asthma represents a substantial burden on society and is considered an important public health issue. Asthma is associated with high direct and indirect costs, being between 1-3% of the total medical expenditures in most countries. Around 30 million people in Europe suffer from asthma[22]. In Europe, it is projected that these cost represent about €18 billion[23,24] (Figure 1.3).

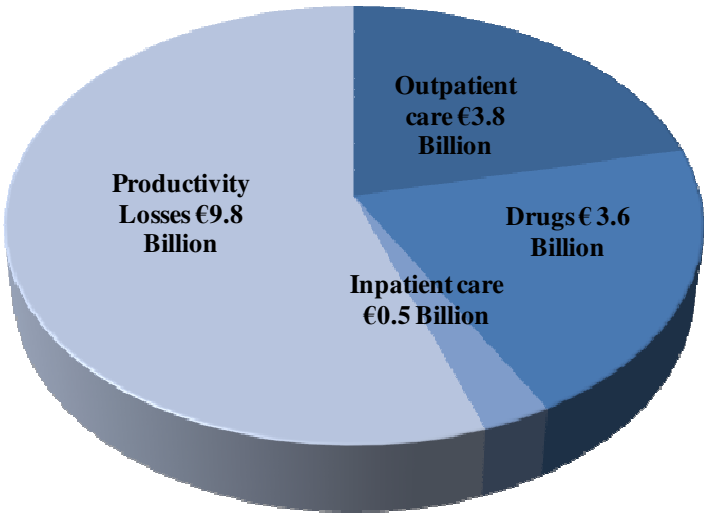


Figure 1.3 – Cost of care for asthma in Europe (data from 2003)[24].

These elevated costs are related to disease severity, poor illness control and underdiagnosis (direct costs) in addition to the costs related to time off work or school (indirect costs). It can be observed from Figure 1.3 that more than 50% of the costs are due the indirect costs of asthma not only of those affected by the pathology but also the caregivers. In England, up to 69% of parents had to miss working days due to their child asthma and 13% have been reported to quit their jobs[25]. The direct costs have also increased in the primary and secondary care, in addition to hospital admissions and treatment costs. The expenses associated with asthma drugs represent about 20% of the total costs in Europe[25]. Asthma, therefore, has a significant socioeconomic impact not only on patients, but on the whole family and consequently reducing life quality[26,27].

Incidence and prevalence are two important concepts. Incidence is the risk evaluation to develop a new condition in a specific period of time whereas prevalence is the population portion that has the said condition[28]. Over the last years, it has been observed a striking increase in asthma incidence mainly in the urban areas of developed countries. It is thought that urbanization and industrialization are the main contributors to this tendency. Developing countries have also shown signals in following this pathway. Nevertheless, globally in the past 5-10 years data suggests stabilization of asthma incidence[29]. Asthma prevalence, despite of hundreds of reports, is difficult to determine as there is a lack of a precise and well-defined definition of asthma that makes the comparison of reported prevalence rather difficult[30] (Figure 1.4). Globally the prevalence of asthma for different countries varied between 1 and 18%[31] accordingly to the data listed in 2012 GINA report.

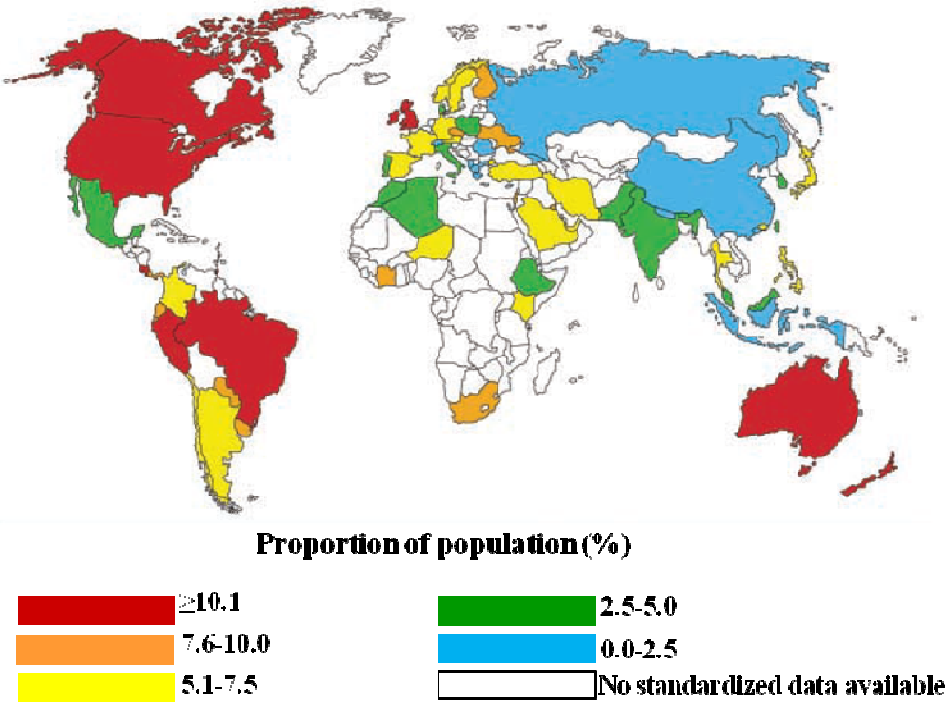


Figure 1.4 – Worldwide prevalence of asthma[32]

There is a sharp increase in global prevalence over the last 40 years, increasing by 50% each decade. The data from hospitalization due to asthma reflects an increase in asthma severity and poor disease management, especially in young children[33].

In Portugal, the latest asthma prevalence study (2012) has shown that 6.8% of the Portuguese population has asthma currently whilst 10.5% had lifetime asthma[34]. Before this, and according to the International Study of Asthma and Allergies in Childhood (ISAAC) performed in 4 different Portuguese cities (Coimbra, Funchal, Portimão, Porto), children between the ages of 6-7 asthma prevalence was 13%, whilst asthma in teenagers (13-14 years old) varied between 9% (in 1995) and 12% (2002)[35].

1.1.3 Disease management

After diagnosing asthma and assessing its severity, an action plan is required. The control is focused in decreasing the risk of asthma attacks, the impairment, the symptoms frequency and intensity and whatever functional restrictions that the patient may possess - such as lung function decline[1].

The control of asthma symptoms can be achieved by either a non-pharmacological and/or a pharmacological management (Figure 1.5). The non-pharmacological management can be performed before or after the disease onset being designated primary and secondary prophylaxis, respectively. House dust mite, animal allergens (dog or cat fur, for example), tobacco smoke, pollen, specific foods (for example, eggs or milk) are some examples of triggers if avoided can help in controlling asthma symptoms.

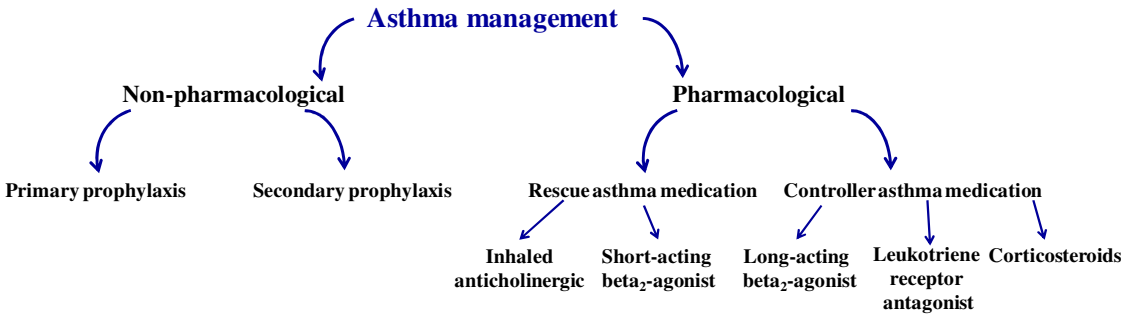


Figure 1.5 – Non-pharmacological and pharmacological asthma management[36]

Asthma treatment is an intricate pathway involving several steps that aims to achieve asthma control (Figure 1.5). The objective is to promote the decrease on medication intake to the lowest possible whilst the asthma condition remains stable. Nevertheless, treatment is approached in a step wise fashion, i.e., if the asthma severity increases the frequency and medications increases or if it diminishes the opposite is observed[36]. Asthma is treated with two types of medicines: controller asthma (long-term control) and rescue asthma (quick-relief) medications. Long-term control medicines help reduce airway inflammation and prevent asthma symptoms. Quick-relief, or "rescue," medicines alleviate asthma symptoms that may flare up. The initial treatment will depend on the asthma severity. In case of allergic asthma, many triggers can set off or worsen your asthma symptoms.

The pharmacological management aims to control the disease, which entails several parameters to be achieved such as no daytime or nighttime symptoms, no need for rescue medications, no exacerbations, normal lung function, no limitation in activity and all with minimal medication side effects. Asthma medication can be divided into two main classes: medication for long-term control that includes inhaled corticosteroids (ICS), leukotriene modifiers (LTMs) and long acting beta$_2$-agonists (LABAs). For quick relief of airflow obstruction and symptoms there are available short-acting beta$_2$-

agonists (SABAs), anticholinergics and systemic corticosteroids. ICS are the most effective long term control medication for asthma that reduces inflammation in the airways and clinically there is a reduction of symptom severity, improvement of asthma control, diminished airway hyperresponsiveness, hospitalization reduction and prevention of exacerbation, among others. Moreover, ICS suppresses cytokine generation and the release of inflammatory mediators[37]. LTMs are divided into two classes: leukotriene-receptor antagonists (LTRA) and leukotriene-receptor inhibitors (LTRI). Leukotrienes are potent biochemical mediators that contract airway smooth muscle, increase vascular permeability, increase mucus secretions and activate inflammatory cells in airways of patients with asthma. LTRA prevents leukotriene binding whilst LTRI focus on synthesis inhibition[38]. As for inhaled LABAs, their actions is to relax airway smooth muscle by stimulating $beta_2$-receptors and are used as an adjunct to inhaled CCS to provide long-term control of asthma symptoms[39]. SABAs provide quick relief of symptoms relaxing the muscles lining the airway within 5 minutes, increasing the airflow thus relieving asthma symptoms. Nevertheless, SABAs do no control the inflammation. Asthma management is a continuous appreciation of the patient's asthma status as shown in Figure 1.6.

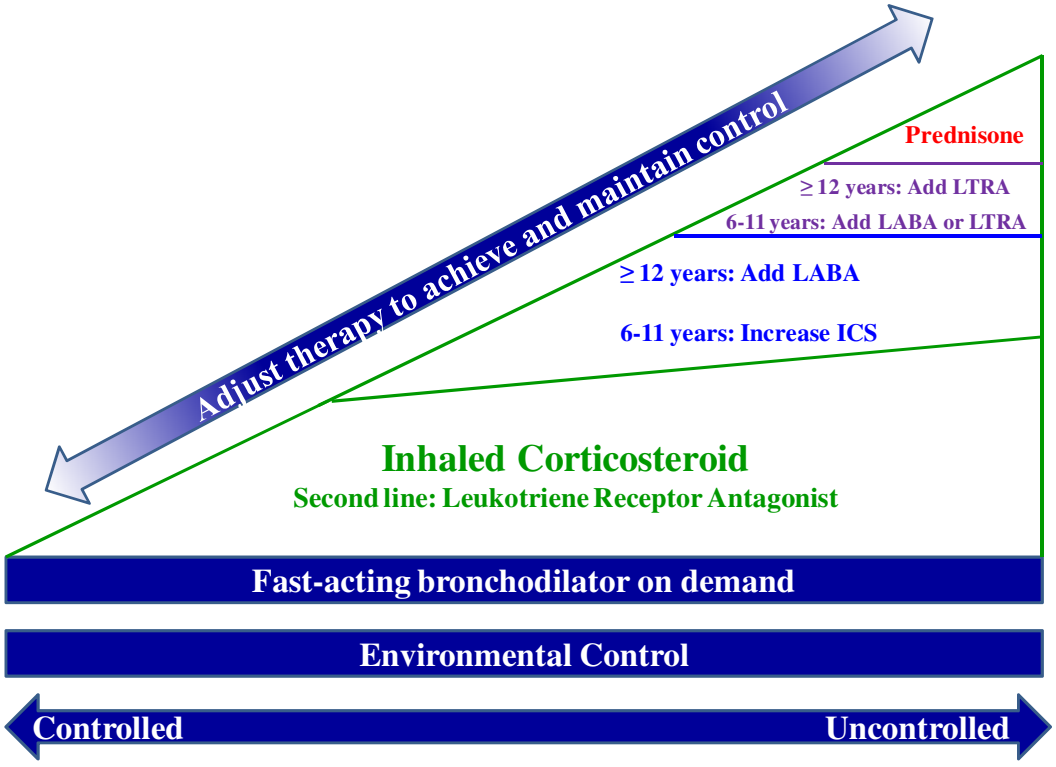


Figure 1.6 – Asthma management continuum[40] (ICS- Inhaled Corticosteroid; LABA- long acting $beta_2$-agonists; LTRA- Leukotriene Receptor Antagonist).

Allergen-specific immunotherapy (AIT) is a treatment used whenever asthma symptoms cannot be controlled by medication and environmental control, who cannot tolerate the medication, or do not comply with the medication regimen. AIT entails the administration of allergen extracts to provoke clinical and immunologic tolerance to the allergen. AIT can be administered subcutaneously or sublingually. This therapy stimulates desensitization and allergen specific tolerance in addition to the inhibition of allergic inflammation in affected tissues[41]. Several disadvantages limits a broader applicability, as for example, unwanted secondary effects, and poor efficacy/specificity that are mainly related to a poor quality of natural allergen extracts and the treatment takes several years[42].

For those suffering from the severe type of asthma and that have allergies Omalizumab® can be considered. This drug is a humanized, monoclonal anti-IgE antibody that stops the allergy cascade by binding to the circulating IgE. This medication is subcutaneously injected every 2 to 4 weeks usually related to the total IgE levels. This therapy is used whenever in patients 6 years of age or older with severe, persistent allergic asthma and elevated serum IgE levels whose symptoms cannot be controlled with ICS. This drug lowers the IgE levels regardless allergen specificity preventing free IgE from interacting with mast cells, basophils, macrophages, dendritic cells, among other cells[43].

1.1.4 Asthma Pathophysiology

Sensitization to the allergen has to occur in order for an individual to develop allergic asthma. This process can happen years before any clinical symptoms begin. Allergic sensitization is the result of a intricate interaction between the allergen and the host. After the allergen has been inhaled antigen-presenting cells as dendritic cells, which are present in ephithelium lining of the airways and nose, internalise, process and expresses the allergen on their surface. Afterward, the allergens are presented to immune response cells as T-lymphocytes that differentiate into T-helper (Th) 2-type cells. The activation of these cells produce inflammatory cytokines and consequently allergen-specific B-cells propagate and IgE-antibodies that are able of binding to a particular allergen are produced. Allergen-specific antibodies are produced whenever stimulation occurs by different allergens. IgE then connects to high affinity receptors on

mast cells so that the allergen specific receptor site is potentially accessible for the allergen. The process is completed with the production of allergen specific IgE-antibodies (Figure 1.7).

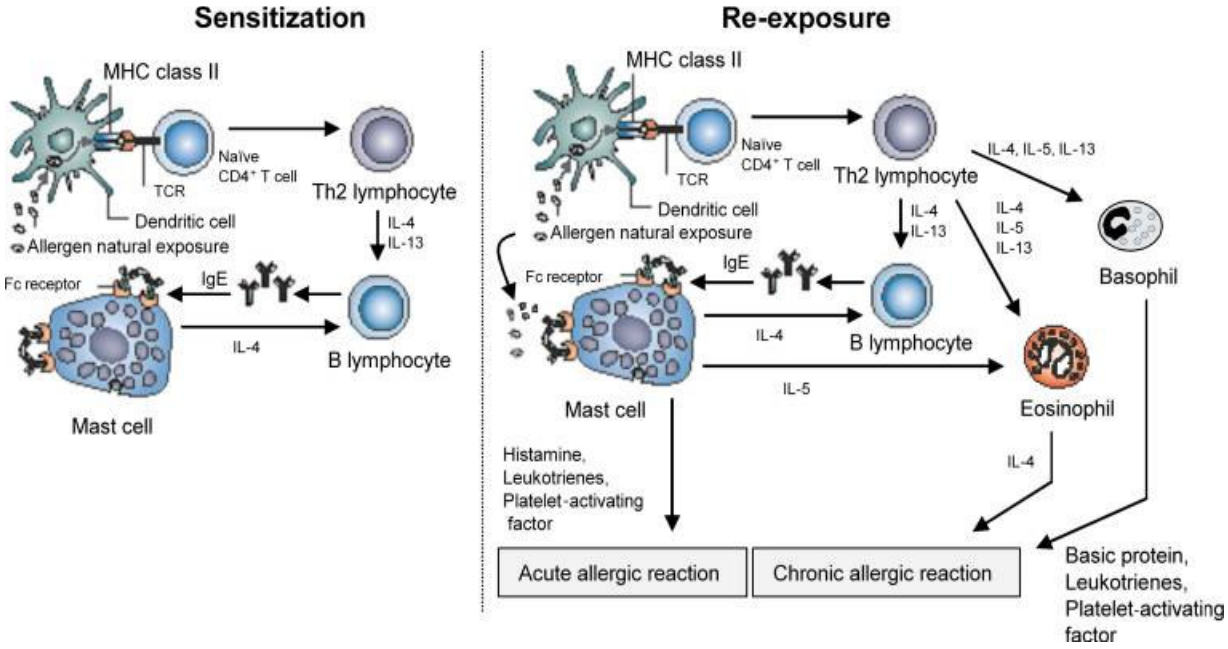


Figure 1.7 – Allergic asthma pathophysiology representation of sensitization and re-exposure[44](CD- Cluster of Differentiation; IL- Interleukin; TCR- T-cell receptor).

Allergen re-exposure makes the immune system react more agressively and in a rapid memory response way. The allergen connects to the allergen-specific IgE on mast cells that leads to the release of the previously formed mediators. Whenever a sufficient number of mast cell/basophil- bound IgE antibodies are bound to the allergen cell degranulation occurs, leading to the release of inflammation mediators such as histamine chemokines, lymphokines, eicosanoids, proinflammatory cytokines and growth factors is initiated in an intra-cellular signalling process. The T_H2 lymphocyte is very important in asthma pathogenesis as it produces cytokines involved in the initiation and maintenance of the inflammation cascade. Cytokines, such as interleukin (IL) 3, granulocyte macrophage colony-stimulating factor (GMC-SF) and IL-5, play a critical role in the differentiation, proliferation, survival and activation of eosinophils[45]. Eosinophils secret cytotoxic granule proteins and produce lipid-derived pro-inflammatory mediators such as leukotriene C4 (LTC4), mediate the damage to the airway epithelium[46]. IL-3 and to a lesser extent IL-4, are critical in the development of mast cells[47], which play a prominent role in the acute-phase response. IL-4 and IL-13

are required for isotype switch to IgE. IL-1 and tumor necrosis factor alpha (TNF-α) are potent pro-inflammatory cytokines that are involved in the upregulation of adhesion molecules on vascular endothelium, an essential step in the migration of leukocytes to sites of inflammation[48].

The response of the immune system is usually split in two phases: early and late phase reaction. Early phase reaction is the immediate hypersensitivity where the chemical mediators, as histamine, prostaglandins, leukotrienes and thromboxane, are released by mast cells producing characteristic allergic reaction responses. Some examples are edema, mucus secretion and sneezing in the respiratory tract, vasodilation in the nose and bronchoconstriction in the lung that leads to wheezing. In the late phase reaction, that usually occurs 4-6 hours after the early phase reaction, the continued allergic response leads to cellular infiltration, fibrin deposition and tissue destruction. This reaction increases bronchial reactivity, edema and extra inflammatory cell recruitment. Eosinophils, T lymphocytes and macrophages, as well as, the epithelial, endothelial and smooth muscle cells support the airway inflammation chronic symptoms. These cells are also thought to play an important role in the formaiton of inflammatory mediators like chemokines, cytokines, and leukotrienes[44]. IgE plays an important tole in the response that the immune systems yields to allergens as it triggers mas cell mediator release that directs to early and late phase reactions[49].

The role of oxidative stress in asthma pathogenesis is still in debate. Oxidative sress is observed on asthma patients and it may be derived from the inflammation or it may a contributory factor to asthma pathogenesis as several reports have been made on the role of oxidative stress in the immulogic diseases. ROS act as transfer stimulating signals as a critical intracellular second menssenger modulating immune responses. Oxidative stress increases airway inflammation as it induces pro-inflammatory mediators, which in its turn enhaces bronchial hyperresponsiveness, stimulates bronchspasm and increases mucus secretion. In fact, intracellular ROS, whenever NADPH oxidase activity occurs, increases temporarily in many types of cellular stimuli that include antigens, infections, chemical mediators and growth factors[50,51].

Asthma metabolic disorders

Asthma produces alterations on a person's physiology and hypothetically the metabolic profile of an asthmatic is different from a healthy individual. Therefore, the

metabolic signature would be different for differentiated conditions[52]. The asthma complexity is reflected in the myriad of biochemical pathways involved, as shown in Figure 1.8.

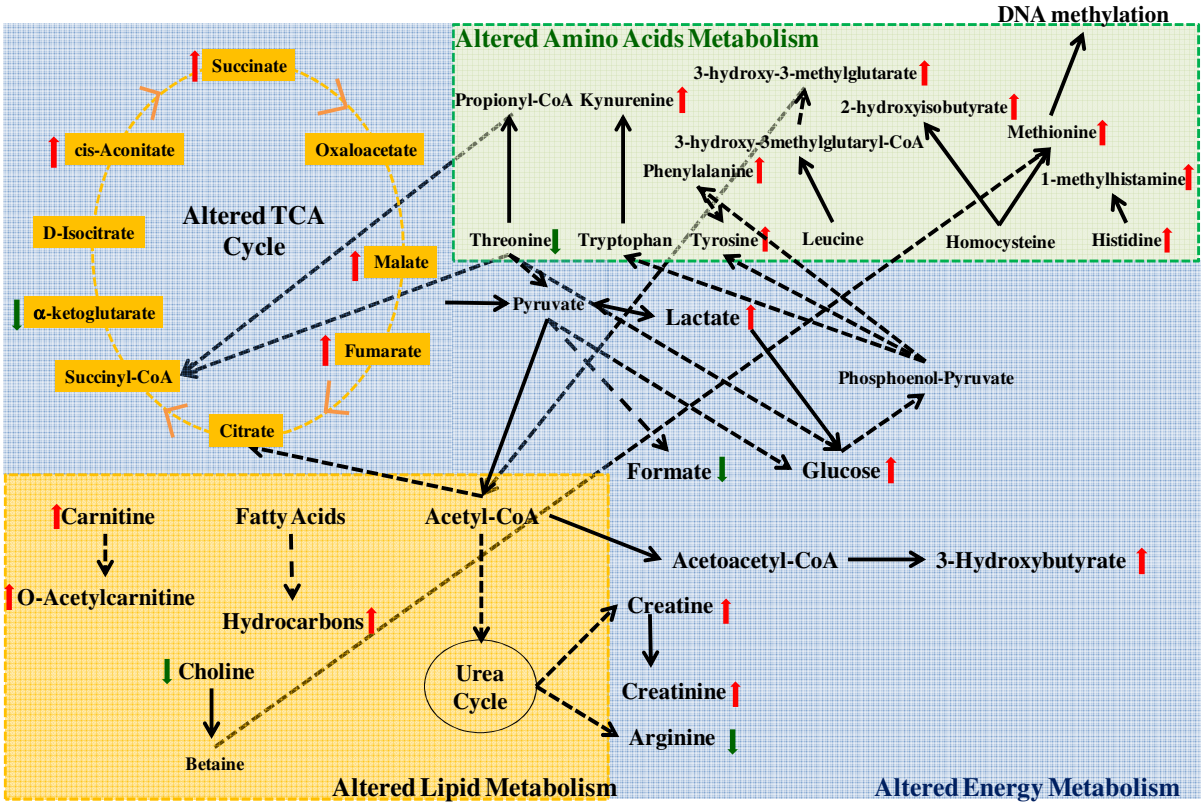


Figure 1.8 – Main altered biochemical pathways in asthma[53].

Compounds and the inherent metabolic pathways have been identified, such as oxidative stress (that damages lipids, proteins and DNA), lactic acidosis, tricarboxylic acid (TCA) cycle, the amino acids and energy metabolisms.

Oxidative stress and lipid peroxidation (LPO) is a lipid metabolism dysfucntion [54] and asthma is characterized by sistemic and localized oxidative stress and subsequent LPO. Inflammatory cells (activated eosinophils, neutrophils, macrophages and monocytes) and epithelial/smooth muscle cells can generate reactive oxygen species[50]. Free radicals reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in the body by various endogenous systems, exposure to different physiochemical conditions or pathological states. A free radical can behave as an oxidant or reductant as they can accept or donate an electron from other molecules. Table 1.1 shows the three phases of the LPO mechanism

Table 1.1 - LPO mechanism phases

Phase	Description
1	Initiation: formation of free radicals
2	Propagation: free radical chain reactions
3	Termination: formation of non radical products

The initiation can begin in a single event that can convert hundreds of fatty acids into lipid hydroperoxides. The length of the propagation depends on many factors as the lipid-protein ratio, the fatty acid composition, oxygen concentration and the presence of antioxidants within the membrane that might interrupt the chain reaction[55]. Propagation takes place by the reaction of lipid radical with a methylene group of a non-oxidized unsaturated fatty acid molecule to yield another lipid hydroperoxide and another fatty acid radical, which can react with oxygen to start a chain reaction[56]. These reactions occur until termination that occurs when there is a combination of two radicals forming a non radical product or by the presence of hydrogen or electron donor[57]. Some examples of important oxygen-containing free radicals in several disease states are for example superoxide anion, hydroxyl, hydrogen peroxide and peroxynitrite. Free radical formation occurs continuously in the cells as a consequence of both enzymatic and non-enzymatic reactions. Figure 1.9 shows the link of allergens and ROS in asthma.

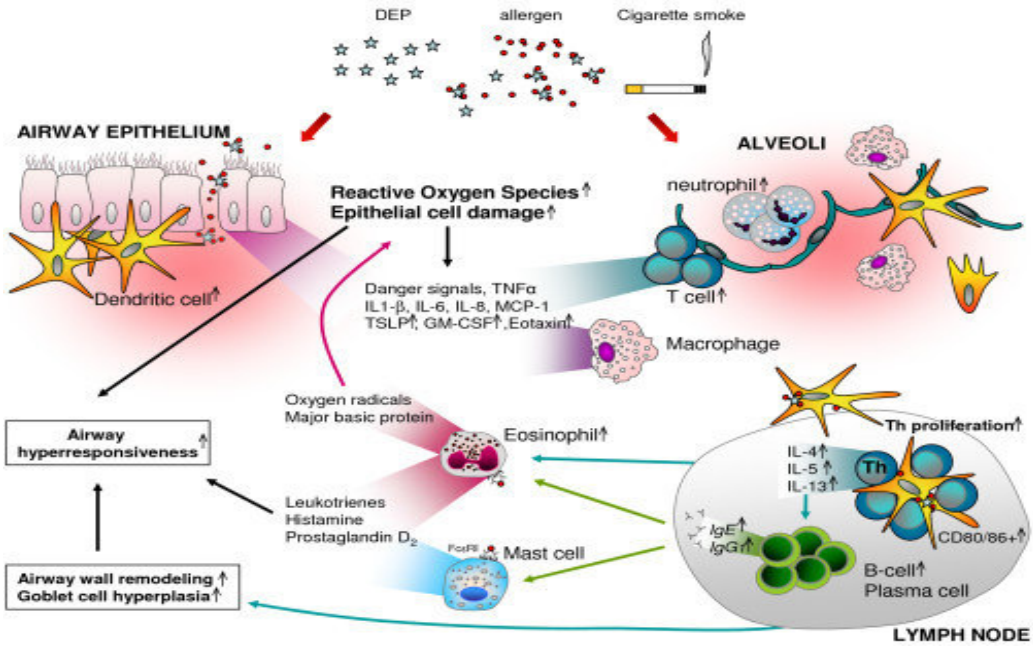


Figure 1.9 – Allergens and reactive oxygen species linked in asthma[58] (DEP- Diesel Exhaust Particles; GM-CSF- granulocyte macrophage colony-stimulating factor; MCP- Monocyte chemotactic protein; TNFα- tumor necrosis factor alpha).

If an imbalance between the free radicals and the antioxidant defenses is observed, a condition known as oxidative stress arises. Free radicals alter lipids, proteins and DNA and are known to generate a number of human diseases. Oxidative stress is thought to have a role in the development of human diseases such as cancer, cardiovascular disorders and immunologic diseases[59].

Lipid metabolism is also affected by asthma pathology as shown in the reports by Ho[53], Saude *et al.*[77] and Jung *et al.*[81] (Table 1.2). Lipid metabolism has shown to contribute to asthma pathophysiology in childhood[60].

Table 1.2 - Lipid Metabolism related compounds by Nuclear Magnetic Ressonance

Author	Compound	↑	Biofluid
Jung *et al.*[81]	methionine	Asthma	Serum
	choline	Healthy	
Saude *et al.*[77]	carninitine	Asthma	Urine
	O-acetylcarnitine	Asthma	

The latter author has demonstrated that there is a strong and direct influence of metabolic pathways on the immune mechanisms. Saude *et al.*[77] has described that the urinary levels of carnitine are high in asthma patients with unstable status. This compound has a role in oxidative metabolism in mitochondrial and hypoxemic stress. The identified compounds in lipid metabolism pathways are identified in Table 1.1.

Oxidative stress has been proven to affect smooth muscle contraction, induce airway hyperresponsiveness and increase mucus secretion and epithelial shedding within respiratory cells. Excessive ROS production in asthma can trigger key enzymatic and non-enzymatic reactions, leading to an antioxidant-oxidant imbalance in respiratory airways which promotes state of oxidative stress in asthma[61].

There is evidence that free radicals are influential components of the molecular mechanisms involved in the pathogenesis of asthma, namely airway inflammation, and determinant of asthma severity[61]. Inflammatory cells and resident cells (epithelial and smooth muscle cells) can generate ROS that can attack proteins to form carbonyls and react with nitrogen species and tyrosine to form nitrotyrosine. It has been shown that nitrotyrosine formation is increased after allergen exposure[50]. ROS also reacts with lipids to release isoprostane. 8-isoprostane has been reported to be elevated in the exhaled breath condensate of asthma patients[62]. ROS excess production also correlates with airway hyperresponsiveness, as for example, in the immediate formation of O_2^-

when an asthma attack occurs being high at the sites of antigen challenge that persists through the late asthmatic phase[63]. NO also plays an important role in asthma. There is evidence that the autoxidation of NO results in nitrite, which is a substrate for eosinophil peroxidase and myeloperoxidase[64]. NO reacts with superoxide that yields $ONOO^-$ and consequently can nitrate tyrosine residues and damage enzymes, structural and functional proteins[65]. ROS production by neutrophils also correlates with asthma severity as demonstrated by the levels of 3-bromotyrosine, a product of eosinophil peroxidase and eosinophil, which was 3 times higher in the bronchoalveolar lavage fluid in individuals with asthma when compared with control subjects[66].

Beside the previously identified compounds, volatile organic compounds (VOCs) have also been associated with asthma (Figure 1.10).

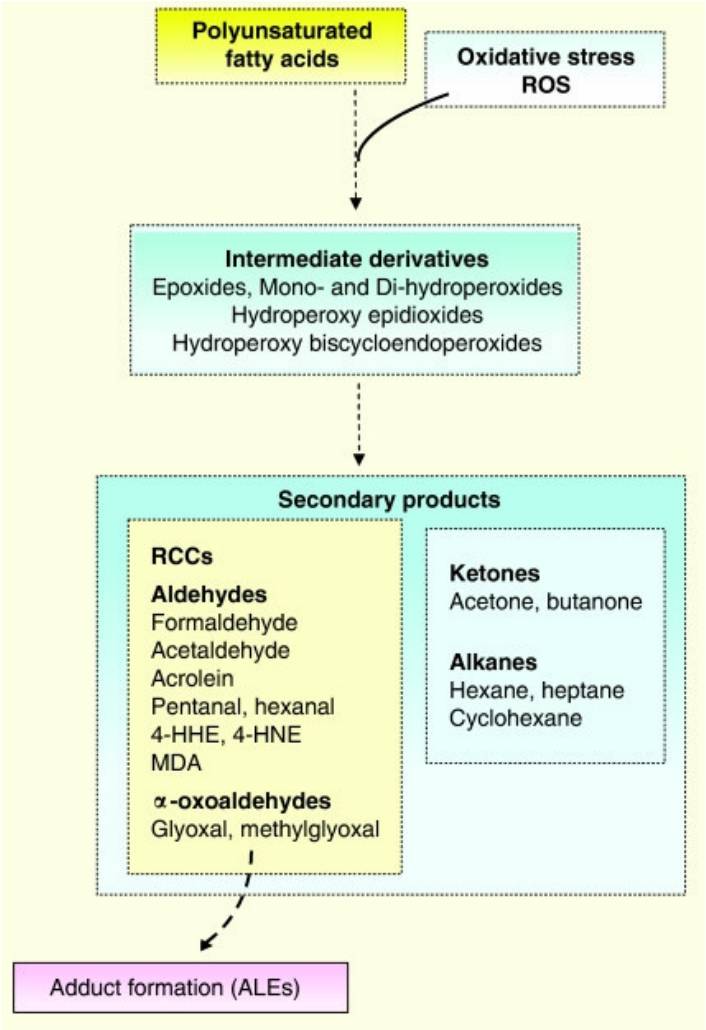


Figure 1.10 – Secondary products formation in LPO (ALEs - advanced lipoxidation end products)[67].

VOCs are generated during pulmonary physiologic and pathologic processes, such as LPO and inflammation[68]. The production of secondary compounds constitutes a minor degradation pathway and they comprise an array of chemical families, from aldehydes, ketones and alkanes with the latter being more stable not reacting with proteins. Dallinga *et al.*[69] and Ibrahim *et al.*[70] have reported C_{13} alkanes in the characterization of asthmatic population through exhaled breath (Table 1.3). Although the mechanism by which these alkanes are formed (namely, ramified alkanes) is still not well understood there are been reports on the volatile composition of lung cancer cell lines where these metabolites have an important role[71].

Table 1.3 - Oxidative stress and LPO secondary compounds in asthma studies analysing exhaled breath by GC-MS

Author	Compound
Dallinga *et al.*[69]	(branched) hydrocarbon ($C_{13}H_{28}$) (branched) hydrocarbon ($C_{13}H_{28}$) unsaturated hydrocarbon ($C_{15}H_{26}$) (branched) hydrocarbon ($C_{11}H_{24}$)
Ibrahim *et al.*[70]	2,6,11-trimethyl-dodecane (branched) hydrocarbon ($C_{13}H_{28}$)

Increased ROS is known to damage DNA, lipids, proteins and carbohydrates affecting cellular functions and increase inflammation (Figure 1.11).

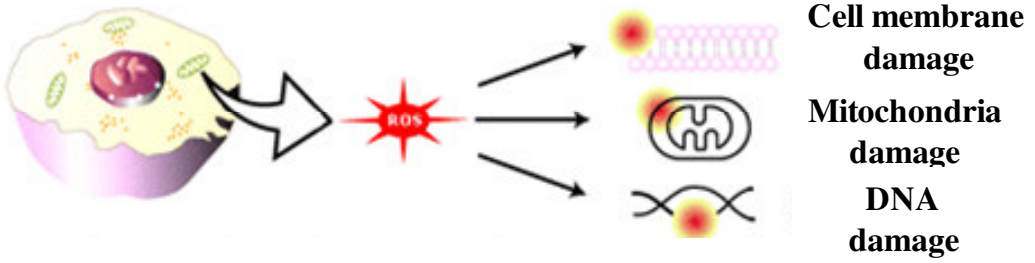


Figure 1.11 – Possibilities of the damage caused by ROS [72].

The oxidative damage to DNA is known to affect nuclear or mitochondrial DNA leading to mutation, gene expression changes[73]. For example, it has been shown that hydrogen peroxide induces DNA strand breaks in airway epithelial cells that leads to modifications in the epithelial cell-specific gene expression[74]. DNA strand breaks also showed to influence type 2 alveolar epithelial cells and shows a relationship to the development of lung disease[73] (Figure 1.12).

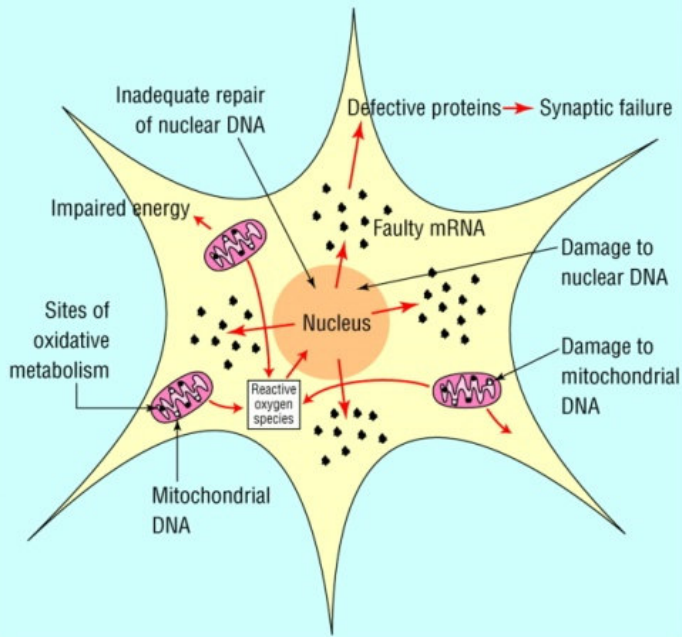


Figure 1.12 – Reactive oxygen species damage nuclear and mitochondrial DNA[75].

ROS's protein modification affects their biological function that in several occasions are important to initiate and maintain inflammation. An example is the loss of antioxidant capacity of the superoxide dismutases known to catalyze superoxide to hydrogen peroxide and subsequently to water. Moreover, inflammatory cells can secrete enzymes that damages oxidatively the protein by producing reactive brominating, chlorinating and nitrogen species[76].

Other metabolic pathways are affected by asthma like the TCA and urea cycles. The TCA cycle is the central metabolic hub of the cell, which is the final common pathway for the oxidation of amino acids, fatty acids and carbohydrates. It is a fundamental metabolic pathway for energy production through aerobic respiration that takes place in the mitochondria. Saude *et al.*[77] analyzed human urine from asthmatic individuals using proton nuclear magnetic resonance (^{1}H-NMR) and found compounds related to the TCA that were critical for the discrimination attained between healthy individuals, outpatient asthmatics and even asthmatics from emergency department. Ho *et al.*[53] studied in sensitized mice BALF that also identified compounds that are related to the TCA cycle.

Succinate, fumarate, lactate and cis-acotinate concentrations were higher in asthmatics when compared to the healthy children whilst threonine and α-oxoglutarate levels are higher for the healthy children. Ho *et al.*[53] found increases in malate, lactate

and creatinine mice BALF that are energy metabolism related compounds. The behavior of these energy-related compounds may be explained by an increased burden and subsequent demand of a higher energy intake by the inflammatory cells. Higher levels of lactate are a consequence of poor oxygenation (hypoxia), in which the mitochondria are unable to produce adenosine triphosphate in a sufficient rate to supply the cell. Subsequently, glycolysis is increased and the pyruvate excess is converted in lactate that is released to the bloodstream. For example, lactate was also found to be elevated in patients with lung cancer[78]. Malate is a TCA cycle intermediate and there are no reports that describe this compound to be altered in asthma or any lung related pathology with the exception of Ho *et al.*[53]. Nevertheless, other intermediates of the TCA cycle succinate, fumarate and cis-aconitate, have been reported by Saude *et al.*[77] to be altered in asthmatics which supports the evidence that TCA cycle is affected by this pathology. Creatine takes part in the energy supply to muscles, as for example airway smooth muscles[79] and as shown by Saude *et al.*[77] the levels of this compound is increased in the asthmatic patients. This behavior is also attributed to the increased respiratory burden[77]. Creatinine is a downstream compound of creatine, and according to Ho *et al.*[53], it has higher values in sensitized mice which corroborate the hypothesis of the promotion of energy metabolism, but by the urea cycle. The information is compiled in Table 1.4.

Pyruvate is an end product of glycolysis and is converted to acetyl CoA involved in the TCA cycle[80]. Pyruvate has been identified in asthmatic sera[81], nevertheless there is no reference of asthma influencing this particular compound but compounds that arise from different pathways have been reported. Lactate levels were increased in the sera of asthma patients[81], as well as, in urine[77].

Table 1.4 - Energy metabolism affected by asthma

Author	Metabolites	↑	Metabolic pathways	Analytical technique	Biofluid
Saude *et al.*[77]	α-oxoglutarate	**Healthy**	TCA Cycle	NMR	Human Urine
	succinate	**Asthma**			
	fumarate	**Asthma**			
	threonine	**Healthy**			
	lactate	**Asthma**			
	cis-conitate	**Asthma**			
Ho *et al.*[53]	malate	**Asthma**	TCA Cycle	GC-MS LC-MS	Mice BALF
	lactate	**Asthma**			
	creatinine	**Asthma**	Urea cycle		
	creatine	**Asthma**			

Lactate concentration rises whenever lactic acidosis occurs[82], that typically occurs when cells receives too little oxygen (hypoxia)[83] and it is considered as a distinct form of metabolic acidosis. Lactic acidosis is more frequent in adult patients with severe asthma[84], nevertheless it does occur in the pediatric population to a lower extent[85]. Lactate levels are also increased in lung cancer patients, as reported by Rocha *et al.*[78]. Glucose results from non-carbohydrate carbon substrate, such as pyruvate and lactate[86], and the concentration of this compound has been reported to be higher in urine of asthmatic patients when compared to the healthy control levels[77]. Nonetheless, in sera glucose has decreased levels[81], but in this study lactate levels, as previously mentioned, are increased which could explain this result. Ho *et al.*[53] has also demonstrated lower levels of carbohydrate related compounds (mannose, galactose and arabinose) and suggested that these compounds could be used to yield lactate and malate to provide extra energy. Formate can also be formed from pyruvate and in serum the concentration is lower for asthmatics than in children[81], nevertheless in exhaled breath condensate the levels of formate are 40% higher in asthmatic samples when compared to children considering this result as an indication of the airway S-nitrosothiol depletion[87].

The protein and amino acid metabolisms are reportedly changed by the asthma pathology. Ho *et al.*[53] and Saude *et al.*[77] also noticed the alteration in the protein and amino acid metabolism and the compounds that supported this hypothesis are shown in Table 1.5.

Table 1.5 - Amino acid metabolism affected by asthma

Author	Metabolites	↑	Analytical technique	Biofluid
Saude *et al.*[77]	2-hydroxyisobutyrate	Asthma	NMR	Human Urine
	3-OH-3-methylglutarate	Asthma		
	1-methylhistamine	Asthma		
	tryptophan	Asthma		
	threonine	Healthy		
	kynurenine	Asthma		
Jung *et al.*[81]	methionine	Asthma	NMR	Human sera
	histidine	Asthma		

The protein metabolism is a set of biochemical processes (Figure 1.13) that comprise the protein and amino acid synthesis, as well as, protein breakdown by catabolism.

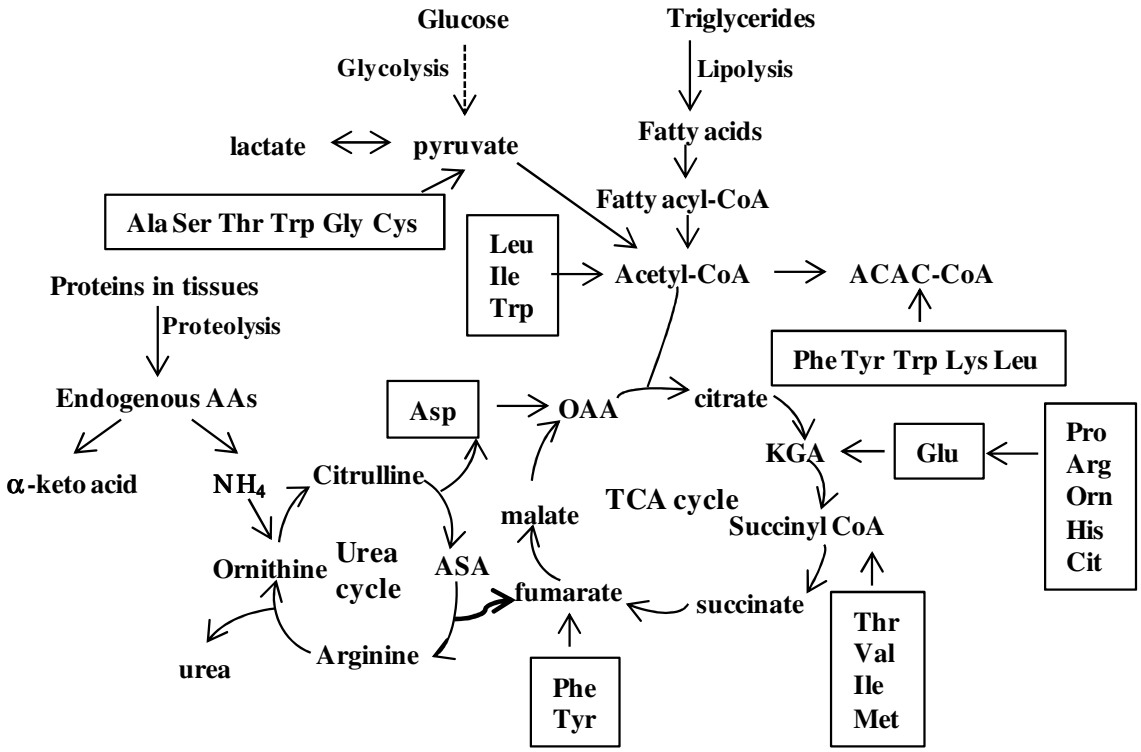


Figure 1.13 – Example of amino acid involvement in other biochemical pathways[88] (AAs – Amino Acids; Ala- Alanine; Arg- Arginine; Asp- Aspartic acid; ASA- Argininosuccinate; Cit- Citrulline; Cys- Cysteine; Ser-Serine; Gly- Glycine; His- Histidine; KGA- α-Ketoglutaric Acid; Leu- Leucine; Ile- Isoleucine; Lys- Lysine; Met- Methionine; OAA- Oxaloacetic Acid; Phe – Phenylalanine; Orn- Ornithine; Pro- Proline; Succinyl CoA- Succinyl Coenzyme A; Thr-Threonine; Trp-Tryptophan; Tyr- Tyrosine; Val- Valine)

Another reported alteration is related to the histidine metabolism. Histamine is formed by decarboxylation reaction catalyzed by the enzyme histidine decarboxylase. Jung *et al.*[81] have shown that histidine is elevated in the sera of asthmatic patients showing that this biochemical pathway may be influenced by asthma. A downstream compound of histamine (Figure 1.14), 1-methylhistamine, was reportedly increased in urine of asthmatic patients[81].

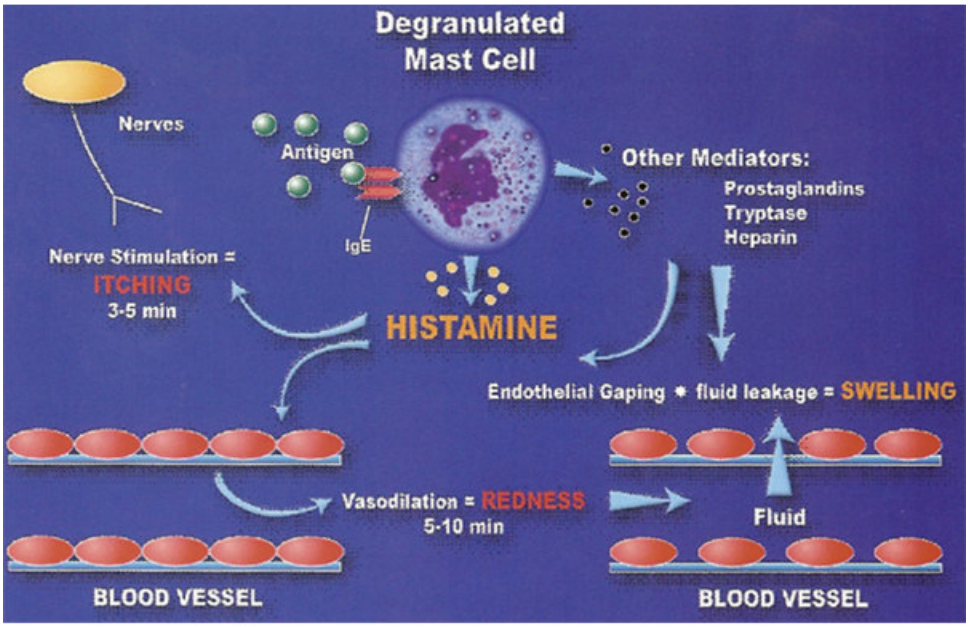


Figure 1.14 - Role of histamine in allergic reaction[93].

Histamine is a known airway constrictor and influences the cells types that play a role in immune and inflammatory reactions. In asthmatics BALF, the histamine concentration has been shown to be higher[89]. The release of histamine, during allergic reactions, is activated by immunologic and non-immunologic triggers from cytoplasmic granules of mast cells and basophils[90]. Upon antigen challenge, histamine levels increase as well as IgE stimulated mast cells. Depending on the location, histamine release causes symptoms as acute rhinitis, bronchospasm, and flare responses, among others. The majority of acute symptoms in allergic asthma are the result of fast activity of histamine on vascular endothelium and bronchial and vascular smooth muscle cells[91].

Histamine elicits such responses as vasodilation, smooth muscle contraction, mucus hypersecretion, and edema as part of the immediate-phase allergic reaction[92,93]. It has also a significant immune/pro-inflammatory properties that are mediated by numerous cell types, including macrophages, T lymphocytes, epithelial and endothelial cells and their products, and other key regulatory molecules[94].

Kynurenine pathway is a major route for tryptophan catabolism. Tryptophan is a precursor of several biochemical pathways, such as kynurenine, serotonin, melatonin and protein synthesis[95]. The kynurenine pathway exists in varying extents in most cell types, among them infiltrating macrophages and endothelial cells. The first stable intermediate in the tryptophan catabolism is kynurenine. Subsequently, several free-radical generators can be formed[96]. The evidence that the kynurenine pathway is

important in the immune system regulation has been increasing in the past years[97]. The up-regulation of the kynurenine pathway has been associated to several pathologies, which include lung and breast cancer[98]. Kynurenine was found in higher concentrations in asthmatics than in healthy children[77]. Its concentration was also reported for interstitial lung disease to be higher when compared to normal subjects[99].

Phenylalanine is an essential amino acid and it reportedly has adverse effects on asthma[100] as phenylketonuria is associated with increased plasma IgE and atopic dermatitis[101]. It has also been associated with the production of tyrosine and catecholamines, as for example epinephrine[102]. Tyrosine residues react with ROS, as peroxynitrite to form a stable product nitrotyrosine that reportedly induces hyperresponsiveness in airways of guinea pigs, inhibits pulmonary surfactant and damages pulmonary epthilelial cells[103]. Tyrosine can also be catabolized to fumarate and acetoacetate[80], which are intermediaries of the TCA cycle which may be influenced by asthma[77].

Methionine is involved in several processes such as the biosynthesis of cysteine, taurine, lecithin, phosphatidylcholine and other phospolipids (Figure 1.15). Methonine is reported to be elevated in asthmatic sera[81]. It has been apparently involved in the DNA methylation phenomena[104].

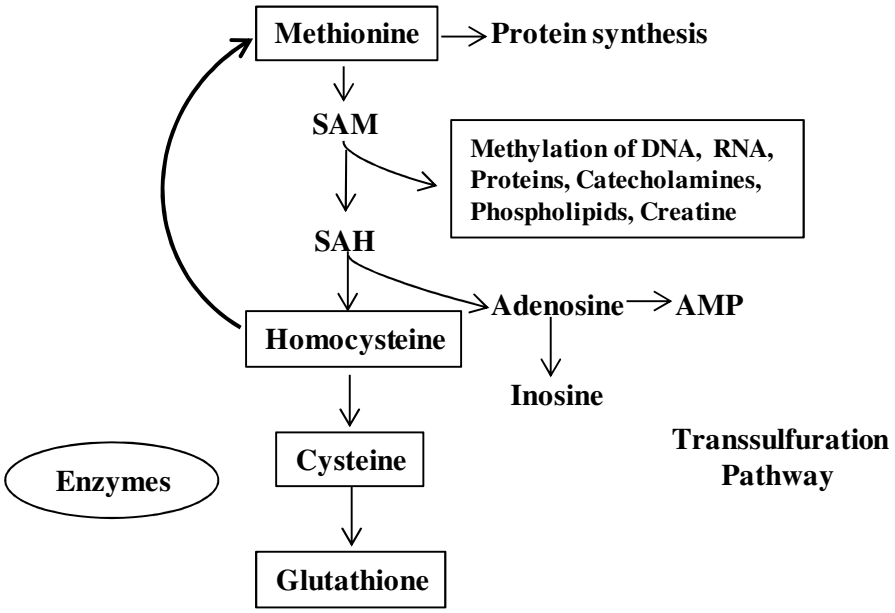


Figure 1.15 – Methionine pathway to cysteine and glutathione[105] (AMP- Adenosine monophosphate SAH- S-adenosyl-homocysteine; SAM- S-adenosyl methionine).

Asthma also affects the leucine metabolism[77]. 3-Hydroxy-3-methylglutarate, which arises from leucine catabolism, is reported to be in higher concentration in unstable asthmatics when compared to the levels obtained with healthy children. 3-Hydroxy-3-methylglutarate converts to 3-hydroxy-3-methylglutaryl-CoA and acetoacetate that can be converted to acetyl-CoA which plays a role, for example, in the TCA cycle and the urea cycle. This means that 3-hydroxy-3-methylglutarate may have an indirect role in these cycles influencing the normal outcome in these metabolic pathways. It has also been shown that 3-hydroxy-3-methylglutarate induces lipid and protein oxidative damage[106].

1.2 Metabolomics

1.2.1 Concept

Metabolomics is an extensive measurement of the overall compounds in a biological system. This allows uncovering a wide range of endogenous and exogenous compounds and has a significant role in investigating physiological status, diagnosing diseases, pattern discovery for disease characterization and identification of affected biochemical pathways due to diseases or treatment. These small compounds play a significant part in the biological systems and are candidates to understand disease phenotypes[107].

These compounds are downstream products of numerous biochemical processes and can be a sensitive measure of an organism's environment-gene interactions, in the identification of disease biomarkers and drug discovery[108]. The metabolome is complementary to transcriptomics and proteomics, nevertheless it has advantages since it reflects more closely the activities of the cells at a functional level, and the changes it reflects are expected to be amplified relative to the changes in the transcriptome and proteome[109] (Figure 1.16).

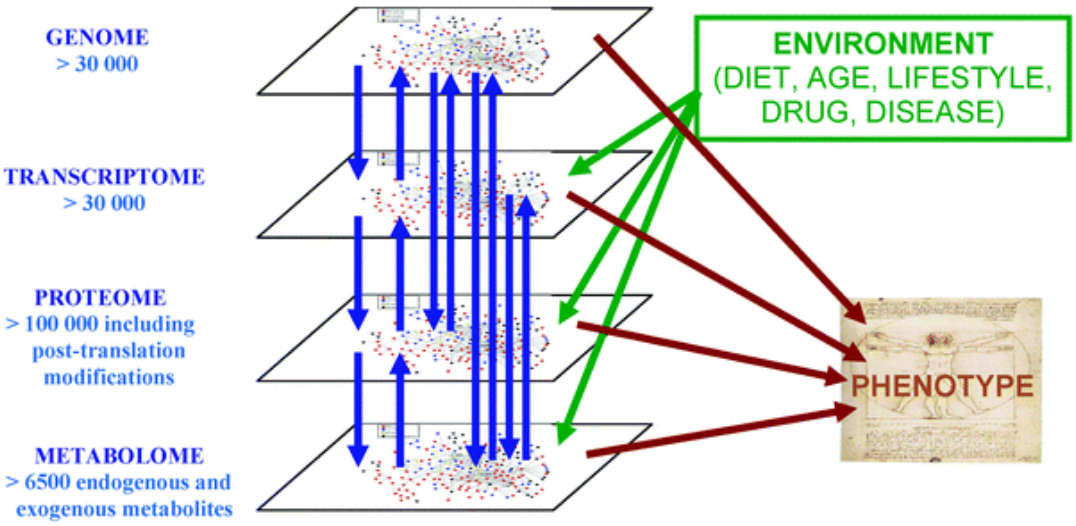


Figure 1.16 – The interactions between the different omics fields[110].

The environmental, developmental stimuli and/or genetic mutation can lead to alterations in cells, biofluids or even cell media. With these changes, the amount of the intermediate pathway metabolites and/or even the terminal metabolites may vary. The metabolites typically range from pmol to mmol and all compounds, if possible, should

be analyzed in a single analysis. Nevertheless this is unlikely to occur due to technological issues and a holistic approach is employed[111]. This approach, instead of identifying and quantifying each compound of the biological fluid, treats the obtained spectra as a unique classifiable metabolic fingerprint. With the use of an array of statistical tools, the spectral profiles of blood, tissue, urine or exhaled breath are classified in specific categories, conditions or disease states. All compounds are looked at once and a pattern of compounds may be formed for a determined status of the biological system understudy[112].

Figure 1.17 shows the workflow of the main steps involved in the metabolomic analysis for this PhD thesis. The experimental metabolomic workflow included the selection of the biological material to be studied. Direct analysis would be ideally performed; nevertheless it is an infrequent option. It was followed by sample preparation and subsequent measurement using the selected analytical chemistry methods and instrumentation. The raw data obtained was processed and normalized to yield a data matrix for statistical analysis. The compounds that discriminated the groups were identified and this information was biochemically interpreted and biological insights were achieved[113].

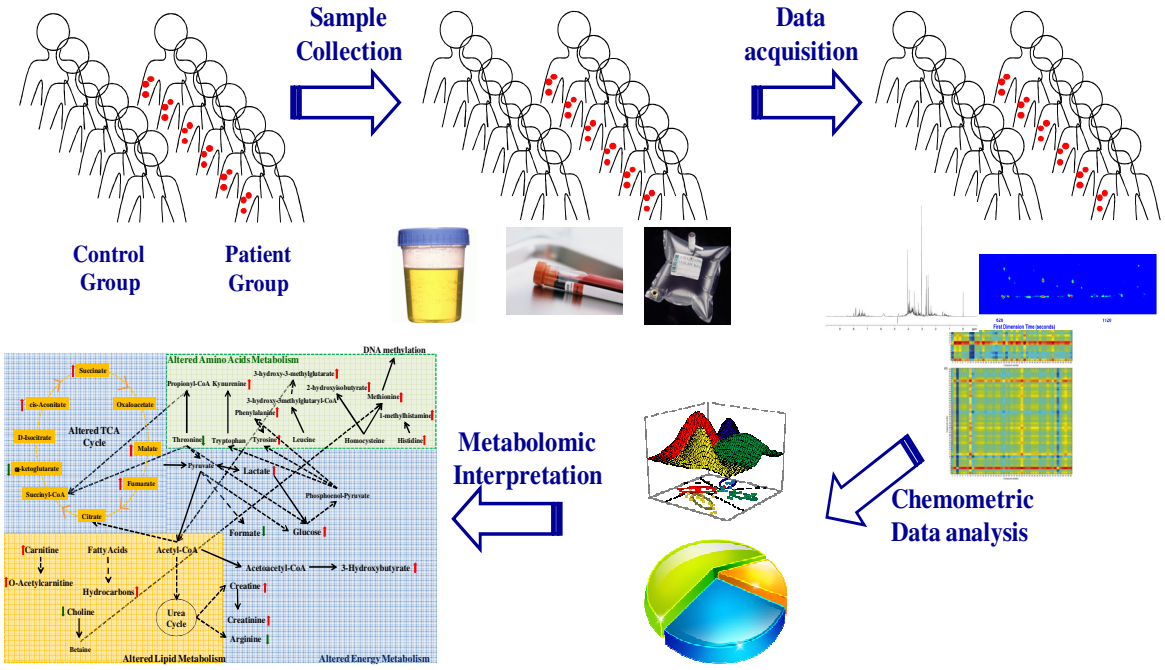


Figure 1.17 – Metabolomic analysis workflow (adapted from Zhang *et al.*[114]).

1.2.2 Analytical techniques in metabolomics: MS or NMR based methodologies

Several analytical techniques are applied to profile the diverse compounds of a given biofluid or tissue, which makes metabolomic an interdisciplinary field of science that may combine analytical chemistry, NMR[115], Fourier transform-infrared spectroscopy (FT-IR)[116] and MS coupled to separation techniques, including GC-MS[117], GC×GC-ToFMS[118], liquid chromatography-mass spectrometry (LC-MS)[119], FT-MS[120] and ultra high pressure liquid chromatography-mass spectrometry (UPLC-MS)[121] with sophisticated multivariate data analysis[107]. For a comprehensive metabolome study the parallel application of several techniques followed by data integration should be sought to have a broader perspective and the most complete information.

MS techniques, often coupled with other techniques, is extensively used in metabolomics offering high selectivity and sensitivity in the identification of several metabolites that are known end-products of cellular processes. The combination with chromatographic separation due to compound separation on a time dimension reduces spectra complexity and offers information on the analytes physicochemical properties. GC-MS has been widely used for metabolomics and can provide efficient and reproducible results[122]. The coupling of powerful separation and detection systems enables unbiased analysis of thousands of known and unknown compounds that can provide novel insights into the biological system under study. It is possible to analyze both volatile and nonvolatile compounds after derivatization. The MS electron ionization energy of 70 eV yields reproducible mass spectra and highly transferable electron ionization MS spectral libraries (as the National Institute of Standards and Technologies library) allowing the identification of compounds match to the spectral library. Retention indices are another tool useful to confirm compound identification on compounds that have similar mass spectra but are separated with chromatography.

Although GC-MS provides adequate information for a variety of analytical tasks, it does not resolve the numerous components present in complex biological samples. Technological advances led to development of comprehensive two-dimensional gas chromatography (GC×GC). One dimensional GC in most cases provides adequate resolution, however does not resolve the numerous components present in biological samples[118]. In GC×GC two capillary columns with different stationary phases are

connected through a modulator and this setup increases separation efficiencies and peak capacities[123]. GC×GC has superior resolving power, which is suited for the analysis of low-molecular weight analytes in complex samples; sensitivity is enhanced due to solute band re-concentration and provides a two-dimensional chromatogram with chemically similar compound patterns[124]. The use of GC×GC coupled with a time of flight mass spectrometer (ToFMS) has been used in the analysis of the volatile profile for diabetes biomarker discovery[125] or in the analysis of specific compounds, as reported by Snyder *et al.*[126] for l-β-methylamino-alanine. GC×GC-ToFMS has also been applied in the metabolic footprinting of tumorigenic and nontumorigenic uroepithelial cells revealing the advantages that this technique presents and the potentiality that it demonstrated in the analysis of bladder cancer[127]. Hartonen *et al.*[128] characterized cerebrospinal fluid and serum samples using GC×GC which led to distinct metabolomic profiles mainly identifying sugar and amino acid derivatives.

NMR has been extensively used in providing unbiased information about compound profiles as it is a straight forward technique, largely automated and non-destructive. Extensive research has been performed using NMR because this technique allows analyzing the pattern of compounds in a target matrix instead of all individual compounds making it a more realist and suitable approach. In addition, it permits the identification of primary and secondary compounds. In the NMR spectrum the signals are proportional to their molar concentration which makes the direct comparison possible, without the need to construct calibration curves for each compound. Nevertheless, the NMR application has a major drawback, the low sensitivity. Another limitation is the considerable signal overlap in the NMR spectra as it has hundreds of overlapping signals that may amper signal identification and accurate peak integration[129]. Nevertheless, several studies have been performed using NMR in a wide range of pathologies and have been extensively reviewed such as in HIV-infected biofluids[130], cancer[131], neurological diseases[132], cardiac[133], paediatric respiratory diseases and bronchiolitis[134], among others.

1.2.3 Metabolomics in asthma

Asthma is complex with several biochemical pathways affected. Additionally, this complexity translates to the clinical practice. Therapy responsiveness is not identical for each patient or even consistent in individual patients over time. There is considerable variability when it comes to asthma control that can be related to known asthma triggers (for example, allergen exposure) or unknown triggers. Nevertheless, a non-controlled status asthmaticus is associated to increased levels of airway inflammation and the objective measurement of this phenomenon would be of significant value in the clinical practice. This knowledge would result in selecting the right therapy, adjust medication dose, possibly separate asthma phenotypes and offer an objective classification of asthma. Figure 1.18 summarizes what is available in the clinical practice and under development.

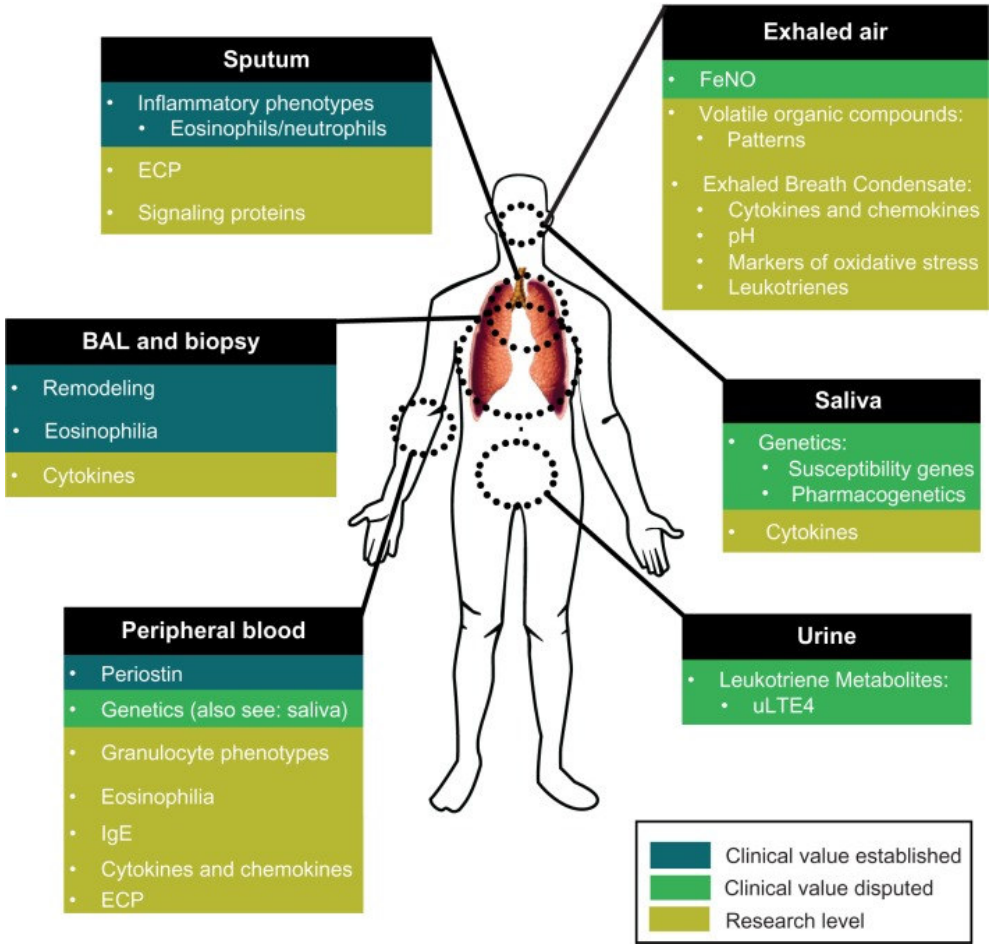


Figure 1.18 – Asthma biomarkers overview: present and future?[135] (ECP- Eosinophil Cationic Protein; FeNO- Fractional exhaled nitric oxide; uLTE- Urinary Leukotriene;)

As previously verified, asthma diagnosis and management is based on asthma symptoms combined with lung function tests and normally these do not reflect accurately airway inflammation[136]. Bronchoscopy and bronchoalveolar lavage (BAL) are the used tests to assess airway inflammation, but are generally too invasive. With this in mind, it is necessary to find asthma biomarkers or pattern of biomarkers that deal with the issues of identifying clinical asthma phenotypes, optimize diagnosis, guide treatment and eventually enlighten the biochemical pathways that are affected by asthma.

Exhaled breath

Exhaled breath is a rich source of information from a molecular point-of-view due the thousands compounds that are comprised in this biofluid. Over the past years exhaled breath has emerged as a new endpoint yielding useful biomarkers. The composition of exhaled breath has been correlated to several disease statuses. Exhaled breath has been extensively studied for the cancer (lung, breast, colorectal), asthma, cystic fibrosis, chronic pulmonary disease, interstitial lung diseases, among others. The identified compounds in exhaled breath have been classified as inorganic compounds (carbon dioxide, oxygen and nitric oxide), non-volatile compounds (isoprostanes, leukotrienes) and VOCs (hydrocarbons, aldehydes, ketones, sulphur compounds, alcohols)[137].

Thomas *et al.*[138] reviewed the potential of exhaled breath condensate (EBC) in the diagnosis and evaluation of the inflammation of asthmatic children. Several markers are reported such as pH, nitric oxide products, glutathione and aldehydes, hydrogen peroxide, eicosanoids, leukotrienes and cytokines. EBC is obtained by cooling using a collection device, which allows collecting different respiratory particles, droplets and water vapor.

Murata *et al.*[139], using a cohort of 29 asthmatics and 33 healthy individuals, showed that pH had lower levels in the asthma group and that the hydrogen peroxide values were elevated. Nevertheless, it was also found out that these values did not correlate with the asthma control test scores, the fractional exhaled nitric oxide (FeNo) or even with the pulmonary function. Similarly to NO, a single marker is unlikely to reflect various pathologies present in a heterogeneous disease such as asthma and additional markers of airway inflammation would be necessary.

EBC is also composed by other compounds that may be helpful asthma markers such as leukotrienes and isoprostanes that are considered inflammation biomarkers. Balanzá[140] has shown that LTB4, as well as, 8-isoprostane concentrations were increased in asthmatic children when compared with healthy individuals and were able to differentiate two degrees of asthma severity. EBC methodologies remain controversial due to standardization issues and a high intra-individual variability[141].

The measurement of VOCs in exhaled breath is a metabolomic approach to study molecular signatures of respiratory disease as it contains a complex mixture of potentially thousands of VOCs. These compounds derive from the metabolic processes in the airways and the presence and/or concentration of the different compounds may be influenced by airway inflammation. It is the least invasive method to obtain information about airway inflammation and oxidative stress (Figure 1.19).

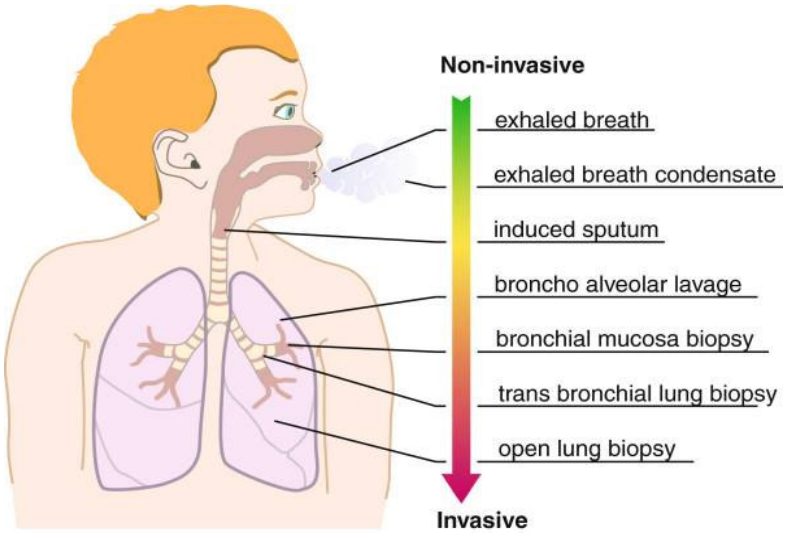


Figure 1.19 – Representation of non-invasive to invasive sampling techniques[142].

To determine the VOCs, two different methods can be applied: the use of a polymer-based gas sensor array (electronic nose) or GC-MS. The electronic nose represents a total expired VOC profile in which individual compounds cannot be identified and mimics the human nose. Dragonieri *et al.*[143] has shown that an electronic nose has the ability to differentiate asthma patients from controls; however distinguishing different asthma severity degrees was not possible.

The use of GC-MS or any related techniques is considered the gold standard in volatile analysis and the use of these techniques have been applied in analysis of exhaled breath in asthma populations. Recently, the reports of VOCs in asthma have increased and an

example is the study made by Gahleitner *et al.*[144] attaining discrimination between asthmatic and healthy children using a panel of 8 compounds, namely 1-(methylsulfanyl)propane, ethylbenzene, 1,4-dichlorobenzene, 4-isopropenyl-1-methylcyclohexene, 2-octenal, octadecyne, 1-isopropyl-3-methylbenzene and 1,7-dimethylnaphtalene. Robroeks *et al.*[145] using a pattern of 6 volatile compounds (*p*-xylene, 3-methylpentane, 2-ethyl-4-methyl-1-pentanol, an unknown compound, 1-phenyl-1-butene and 4,6,9-nonadecatriene) were able to predict exacerbations of childhood asthma. Ibrahim *et al.*[146] built a model using 15 compounds (2,6,10-trimethyldodecane, 2,6,11-trimethyldodecane, benzyl alcohol, 3,4-dihydrobenzonitrile, 2-methyldecane, 1-methyl-4-(1-methylethylidene)cyclohexene, 2,2,-dimethyl-3-oxo butanoic acid ethyl ester, 4-nitroso ethylester benzoic acid, 2-butylcyclohexanol, 5,5-dibutylnonane, 4-ethenyl-1,2-dimethylbenzene, 2,6-bis(1,1-dimethylethyl)-2,5-cyclohexadiene, 2-butanone, pentadecanal and allyl methyl sulphide) with an accuracy of 86% and were able to classify clinically relevant disease phenotypes. Dallinga *et al.*[147] developed a diagnostic tool for asthmatic children with a 92% classification rate and relatively high specificity (95%) and sensitivity (89%) with a total of ten compounds (two branched hydrocarbons ($C_{13}H_{28}$), carbon disulphide, 1-penten-2-one, butanoic acid, 3-(1-methylethyl)-benzene, an unsaturated hydrocarbon, ($C_{15}H_{26}$), benzoic acid, p-xylene, and a branched hydrocarbon ($C_{11}H_{24}$)).

Urine

Urine is non-invasive, accessible, "informative", stable body fluid. Bouatra *et al.*[108] have compiled the information on the studies that have been performed to identify asthma biomarkers using urine as biofluid/matrix. The premise relies in the fact that asthma affects the body as a whole and alterations in the metabolism may be reflected in urine.

Cysteinyl leukotrienes (CLTs) are major eicosanoids products released from various cells including eosinophils, neutrophils and mast cells. CLT_4 and D_4 are quickly catabolized to LTE_4 and this compound appears in urine providing an indication of LTs production[135]. Misso *et al.*[148] measured LTE_4 in urine showing that there was no statistical difference between the asthmatics and the controls and it varied widely both in the control group, as well as, in the asthmatics. More recently, Balgoma *et al.* [149] developed a method using LC-MS/MS to quantify eicosanoid compounds in urine of

atopic asthmatics and discovered increases in compounds of prostaglandin D2, CLTs and isoprostanes after allergen provocation.

A study performed by Saude *et al.*[77] using urine of stable and unstable asthmatic patients/healthy children and 70 compounds were measured. Multivariate tools were used in the discrimination of the tree groups with high classification rates. The obtained results allowed extrapolating some biochemical pathways affected by asthma. Mattarucchi *et al.*[150] characterized urine of asthmatic and healthy groups by LC-MS attaining discrimination between both groups and identified compounds that are supposedly correlated with inflammatory diseases.

1.3 Biofluid Analysis: breath and urine

1.3.1 Methodological aspects of sampling procedures

Several reports have been made with highly promising results using exhaled breath and urine with an assortment of compounds characterizing asthma. Nevertheless, the regular use of these discoveries have not been introduced in the clinical practice due to technical obstacles such as a sampling, preconcentration and analysis, as well as, methodological issues such as normalization and data expression[151]. A brief discussion will be made addressing the studied matrices, exhaled breath and urine.

Exhaled breath

Exhaled breath is largely composed by nitrogen, oxygen, carbon dioxide, water vapor and inert gases. Several hundred volatile compounds of exogenous and endogenous sources comprise a small portion of exhaled breath. Exhaled breath potential to determine normal or abnormal physiology has been used for centuries beginning with Hippocrates. Subjective impressions of exhaled breath odor has been used to distinguish uncontrolled diabetes, liver, renal or dental diseases[152]. In the 1970's, Linus Pauling showed that exhaled breath was a complex gas containing more than 200 VOCs[153]. Since then, the identification and quantification of compounds in exhaled breath has been gained interest.

The collection of exhaled breath is one of the most important steps in breath analysis (Figure 1.20) and can be made in one of two ways:

- Mixed expiratory sampling
- Alveolar sampling

Mixed expiratory sampling is an easy and commonly used sampling method as it only requires spontaneously breathing by the subjects to a collection system and requires no additional sampling equipment. In this sampling procedure total breath, which included dead space air, is collected and a disadvantage can arise and be attributed to inaccuracy as the sample is diluted by dead space air. In order to circumvent this disadvantage, a breath capture system can be used, and adding a capnograph to the system only alveolar breath can be sampled.

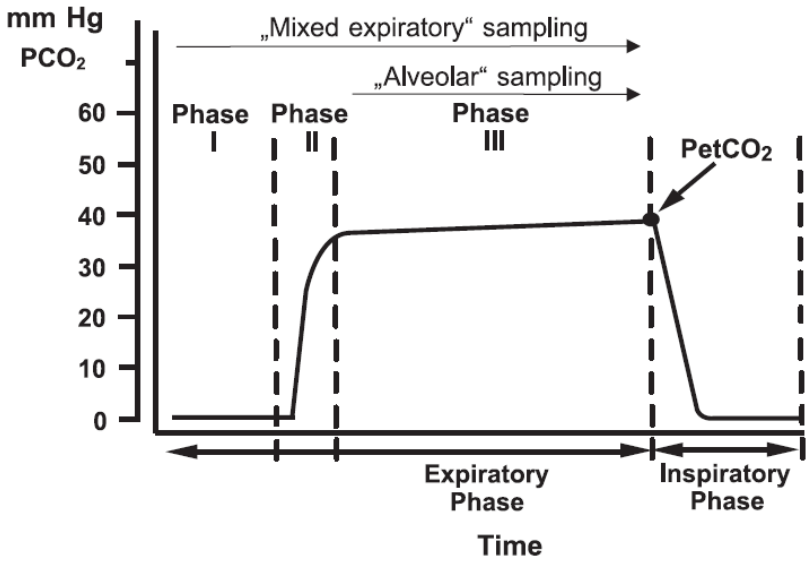


Figure 1.20 – Capnograph waveforms with mixed expiratory and alveolar sampling (Phase I- Respiratory baseline, Phase II- Expiratory Stroke, Phase III- Expiratory Plateau; $PetCO_2$- partial pressure of end-tidal carbon dioxide

The main advantage of sampling alveolar exhaled breath relates to accuracy as the sample is not diluted in dead space air, nevertheless the complexity and expenses of the collection apparatus are two important disadvantages[154]. In dead space air no exchange is made, comes from the mouth, trachea and bronchioles, and averages about a third of the tidal volume. The remaining fraction is denominated alveolar air. Analyzing exhaled breath directly would be ideal; however in many situations this is not feasible and breath has to be stored in appropriate containers.

The breath container where exhaled breath is captured into also plays an important role in exhaled breath analysis. Breath can be collected into several containers such as transparent or black Tedlar® bags (PTFE-polytetrafluoroethylene), BIOVOCS®, flexfoil bags PET/NY/AL/CPE-polyethylene terephthalate/nylon/aluminium foil/chlorinatedpolyethylene), nalophan bags, thermal desorption tubes, micropacked sorbent traps and metal canisters. The most employed breath container is an inert bag, for example Tedlar® or Teflon. The volunteer is asked to breath into the bag that is taken to the laboratory for analysis. The ease in which the volunteer performs this task and the ability of reusing the container after appropriated cleansing are two big advantages. Nevertheless, there are some disadvantages in these bags. There is a risk of decomposition/loss of the compounds due to the interaction of sample constituents (reactive chemistry of the sample) modifying the sample composition and consequently

altering the outcome. Storage is also an important parameter in breath analysis as most chemical compounds found in breath are highly reactive and perishable. This disadvantage can be overcome by experimentally determine the most adequate bag [158,155] or determine how long exhaled breath can be maintained in the container without any significant loss[156]. The use of pre-concentration methods can be applied, as for example sorbent traps or solid-phase microextraction (SPME) which can diminish the decomposition issues. Stability assays have been performed to determine the compounds stability in the polymer bags[157,158]. Condensation also presents as a problem, namely in water-soluble compounds. It has been shown that there are differences between dry and wet samples by 10% and this number has risen up to 40% in heavier compounds. The volume of breath in the Tedlar® bag is also a parameter of interest as it has been shown that smaller volumes may affect the stability of the sample due to permeation problems. The actual recommendation by Mochalski and co-authors[158] is to store breath for a maximum of 6 hours filling the maximum volume of the Tedlar® bag[159].

Another issue with polymer bags is related with the possible chemical contamination from the bag itself. Appropriate cleaning procedures must be adopted to circumvent these issues. Tedlar® bags often release *N,N*-dimethylacetamide and phenol, septa may release compounds such as carbon disulfide or 3-methylpentane and plasticizers from tubings and/or valves may contaminate breath samples. Another approach is to collect breath directly to a trapping system avoiding the use of containers or even into glass vials or metal canisters, nevertheless this adds complexity to the design and also increases the expenses.

Another issue that has to be addressed in exhaled breath analysis is the background contamination and the decision whether the identified compound are endogenous or exogenous. To overcome this drawback, room air volatile composition can be determined and taken into account as high concentrations of substances that can be inspired can impact the concentration of endogenous compounds. Another parameter that influences the possible outcomes of exhaled breath is the intra-individual variability that represents a possible issue in breath analysis. The intra-individual variability represents the variability associated with replicate exhaled breath samples from the same subject, and it is verified that natural biological intra-individual variability can be expected, as well as, related to diet or lifestyle changes [160,161].

Urine

The primary route of removing water-soluble waste products is urination. Urine is a transparent, amber-colored fluid that contains high amounts of urea, inorganic salts, creatinine, ammonia, organic acids, various water-soluble toxins and pigmented products of hemoglobin breakdown. The kidneys also remove excess water, sugars and numerous other compounds. Currently, urine is analyzed to evaluate urinary glucose, bilirubin, ketone bodies, nitrates, leukocyte esterase, specific gravity, hemoglobin, urobilinogen and protein. Other more detailed urine analysis can be used to verify renal diseases such as bladder, ovarian and kidney diseases.

The use of urine for medical purposes dates back to ancient Egypt and throughout time the examination of color, cloudiness, smell and even taste was used to help in the identification of a wide range of diseases. As an important biological fluid, urine has been subjected to a detailed chemical analysis over the last 100 years and normal reference values have been documented for more than 100 urine ions and compounds. In addition to this information, several research groups have studied urine using a myriad of technologies and more than 3000 metabolites have been identified.[108] Urine is biofluid collected in a non-invasive way with great information potential to add to standardized studies that may be performed. So, there are been several studies that analyzed this matrix using an assortment of analytical techniques for several pathologies. In asthma, one can argue that urine is not directly linked to the lung tissue, yet urine is an integrated biofluid and it can reflect localized and systemic changes; nevertheless, this also is a disadvantage as this can difficult the determination of the origin of the metabolic changes. Another concern is the possible alteration of the compounds excretion by kidney or any liver diseases.

After weighing the advantages and disadvantages of the matrix itself, strategies have to be employed to minimize variability and remove sample bias. A number of factors can influence considerably the obtained metabolome and this encompasses sampling time, diet, age, environment, exercise, among others. Beside these factors, other parameters may also influence the possible outcome such as collection method, collected volume, collection time, processing and storage. Harmonizing urine collection, handling and storage is determinant to yield meaningful discoveries and outcomes.

Urine samples can be obtained in a random fashion, in timed sampling and/or 24-h sampling. The random sampling can take place at any time of the day but does not take into account the excretion variation and usually a correction is necessary, as for example the ratio to a compound such as creatinine[162]. The 24-h sampling is burdensome but yields a more complete depiction of the excretion. The recovery of the first morning urine is also frequently used[163].

Another methodological issue with urine collection is the type of container used. The use of a container designed to facilitate the collection, ensure an optimal transport, free of interfering agents and made of non-absorbing materials is also a factor that has to be taken into consideration[163].The use of high throughput and sensible MS and NMR based technology requires just a few microliters to milliliters of urine to proceed to a full metabolomic analysis.

Urine stability during storage is an important consideration so that it provides valid data and reliable information. The effects of freezing urine have been performed: at -80ºC immediately or stored at 4ºC for 24-h before being frozen. In general there were no statistical differences between the metabolic profiles of the samples stored in the two conditions[164]. Klein and co-authors studied the stability of selenium compounds in urine and reached to the conclusion that urine samples can be stored at -80ºC up to 4 weeks without measurable changes[165]. Urine contains components that precipitate when cooled in refrigerators and centrifugation is necessary as non-centrifuged samples have shown different metabolomic profiles[163].

Despite the influence that the previous factors that may affect the urine metabolome outcome, an approach that is common to several groups is to consider that these possible confounding factors may not be controlled and that the compounds that allow the discrimination between a group with a determined pathology and the control groups is sufficient and intra-individual variability is supplanted.

1.3.2 Breath and urine characterization based on solid phase microextraction combined with gas chromatography analysis

Sample preparation is often a necessary step and the main purpose is to extract the analytes from complex biological matrices, which removes matrix components that may interfere with the analysis. In the case of low-level compounds a pre-concentration

step is necessary to achieve the detection limits of the chosen analytical technique. Conventional sample preparation techniques, such as liquid-liquid extraction (LLE) and solid-phase extraction (SPE) (Figure 1.21) are often not feasible as analytes might be incompletely extracted and losses occur if the extract is concentrated. Solvent can interfere with analytes during gas chromatography process, for example compounds can be cut off with solvent delay, and solvent-free preparation techniques are often chosen. Traditional extraction techniques, such as LLE and SPE, have been extensively used in the preparation of biological samples. These techniques have some disadvantages, which include complicated and time-consuming operations, being difficult to automate, require solvents, usually involving large quantities of sample and usually the sample amount is limited. These multi-step procedures are prone to analyte losses. Therefore, the use of sample preparation techniques that can be easily automated, miniaturized, with a high throughput performance, using little or no solvent and with the possibility to couple on-line with analytical equipment has been subject of interest in metabolomic analysis[166].

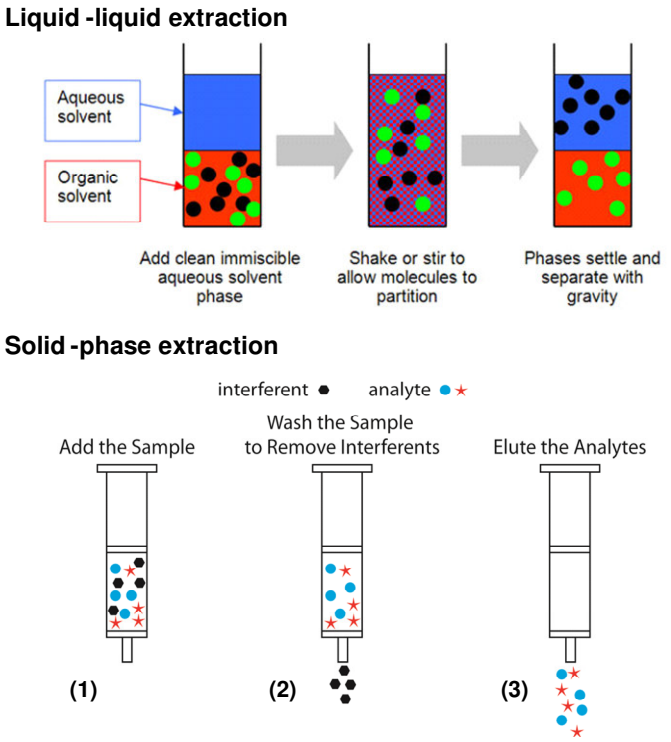


Figure 1.21 – Comparison between liquid-liquid and solid phase extraction[167].

SPME is an example used in the analysis of volatile and semi-volatile compounds in metabolomics[168]. SPME, an extraction technique, introduced by Pawliszyn in 1990s[169], convenes these characteristics and has been extensively used in the metabolomics extraction procedures. The first report using SPME as the extraction technique occurred in 1997 with the analysis of ethanol, isoprene and acetone in healthy volunteers[170] whilst the analysis of urine by SPME was first reported in 1995 in the analysis of thinner components[171]. Since then, SPME has been used in urine analysis for potential cancer biomarkers[172,173,174], in the analysis of cell cultures of cancer[175], in the comparison of volatile compounds from melanoma and benign skin[176], lung cancer chemotherapy control by breath profile[177], determination of acetone in human breath[178], among many other studies in the investigation of several pathologies. In spite of the technique simplicity, there are parameters that have to be optimized before analyzing exhaled breath and/or urine that will be discussed subsequently.

SPME was developed to facilitate sample preparation, reduce solvent use, easy to operate and to be field compatible. There are several phase geometries: fiber, disk, thin film, stirrer, suspendend particles and vessel. The most used is the fiber, a modified syringe that contains a stainless steel microtubing within its syringe needle with a fused-silica fiber tip with about 1cm coated with an organic polymer. Sampling can be made by immersion/direct extraction (DI-SPME) or by headspace extraction (HS-SPME). In DI-SPME the fiber is put directly in the sample whereas in HS-SPME the compounds are extracted from the gas phase that is in equilibrium with the sample. On HS-SPME, the fiber has no direct contact with the sample, therefore the matrix effect and background effect are reduced, increasing the lifetime of the SPME fibers and increasing the selectivity by extracting volatile and semi-volatile compounds[179]. In this PhD thesis, HS-SPME mode was chosen. After the coated fiber is exposed using the pre-determined SPME conditions it is inserted in the injection port of the chromatograph to release the analytes, and in GC this is performed resorting to thermal desorption.

The SPME extraction is non-exhaustive, which means that a small fraction of compounds are extracted representing the compounds overall composition. In this PhD thesis, it was decided to select the conditions that promoted greater extraction efficiency, i.e., a compromise between the number of extracted compounds, an acceptable chromatographic intensity and time.

Upon exposure the fiber enters in a mass-transfer process driven by the second law of thermodynamics and the amount of analyte sorbed are expressed by:

$$n = \frac{C_0 \times V_f \times V_s \times K}{K \times V_f + V_s}$$

Where C_0 is the initial concentration of analyte, V_f is the fiber volume, V_s is sample volume (or headspace) and K is equal to $K_{fh} \times K_{hs}$ (K_{fh} is the partition coefficient between the fiber coating and headspace and K_{hs} the partition coefficient between the headspace and the sample).

The aforementioned SPME parameters namely fiber coating, temperature and extraction time, stirring effect, salt addition, sample pH among others, are usually experimentally optimized.

There are several commercially available SPME coating fibers with different polarities, film thicknesses and with different type of interaction with the compounds. There are liquid and solid coatings and the performance is quite different. Initially, extraction begins by analyte adsorption at the extraction phase-matrix interface followed by analyte diffusion into the bulk of the extraction-phase. If the diffusion coeficient of the analytes is high, absorption occurs with the analytes fully partitioning between the two phases. However if the diffusion coeficient is low, the analyte remains in the interface and adsorption occurs.The fibre coatings consist of one, two or three polymers. The fibers with two or three polymers have a synergetic effect of adsorption and absorption. When compared to the fibers with one polymer, a higher retention capacity and a higher sensitivity are achieved. Some examples are shown in Table 1.6.

Table 1.6 – Commercially available SPME coatings

Coating type	Film thickness (µm)	Polarity
Polydimethylsiloxane (PDMS)	7 30 100	Nonpolar
Polyacrylate (PA)	85	Polar
Polydimethylsiloxane/ divinylbenzene (PDMS/DVB)	65	Polar
Carbowax/divinylbenzene (CW/DBV)	65	Polar
Carboxen / polydimethylsiloxane(Car/PDMS)	85	Bipolar
Divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS)	55/30	Bipolar

Temperature has a significant effect on the kinetics of the SPME process as it determines the vapour pressure of the analytes above the condensed phase, as well as, the compounds solubility on liquid samples. The increase of temperature results in an

increase of the analyte's Henry's constant, an increase in the the diffusion coefficient, and a decrease in the extracted amount at equilibrium (as the distribution constant decreases with temperature increase) [180]. Consequently, increasing temperature will lead to the improvement of the extraction time. Nevertheless, a compromisse has to be achieved as elevated sampling temperatures decrease the fibre/sample partition coefficients leading to the decrease of the amount extracted in equilibrium.

Another highly important parameter is the extraction time. Theoretically, if the period of time is long enough a concentration equilibirum can be reached between the extraction phase and the matrix and exposing the fiber longer will not result in the accumulation of more analytes. However, in a practical point-of-view the equilibrium time is defined as the time required to extract 95% of the analyte[181]. The optimum extraction time is independent of sample concentration and is a compromisse between the desired length, sensitivity and repeatability and reaching equilibrium provides the highest sensitivity. However, equilibrium extraction times tend to be long[182]. This is not convenient in laboratory assays and a compromisse should be reached.

In liquid samples, such as urine, an optimizable parameter is ionic strength. Adding salt to a sample solution increases the constant Kfs improving sensitivity in most applications. The analytes mass transport from the aqueous phase to the headspace is improved and consequently the extraction efficiency is increased. Sodium chloride with high purity is usually added and this addition changes the properties of the boundary phase. Also the compound solubility decreases in the aqueous phase, a process known as salting-out where the water molecules form hydration shperes around the salt molecules. The water content that dissolve the analytes molecules is reduced and the compounds pass to the headspace. At the saturation point the compounds may interact with the salt ions in solution and this reduces the extraction efficiency[183]. Agitation can also improve extraction efficiency and increase extraction efficiency. This is provided by the use of a magnetic stirring bar and the compounds are transported from the solution to the proximity of the fiber. The effect caused by the depletion zone that is produced close to the fiber is reduced and the analytes diffusion coefficients is decreased. pH adjustment can also improve method sensitivity converting analytes that might be present in the sample into the neutral forms, so it is another parameter that can be optimized in sample such as urine[182].

After the extraction procedure is completed the exhaled breath and urine volatile and semi-volatile compounds are generally analyzed using gas chromatographic

techniques. GC-MS is the powerful combination of two analytical tools: gas chromatography for the gas-phase separation of the components in complex mixtures, and mass spectrometry that allows the identification of these components. GC-MS was developed in the 1950's and it has been considered a highly valuable tool in any analytical chemistry laboratory[184]. Since its development the technique had numerous advances that allowed an in-depth sample characterization. The main advantages of GC-MS are sensitivity, compound identification, and widely available instrumentation at relatively low cost (compared to other equipments)[185]. This analytical technique has been extensively used in exhaled breath[186, 187, 188] and urine analysis[189, 190, 191].

Briefly, in GC-MS the components of a mixture are physically separated and selectively distributed between the mobile phase (which is an inert carrier gas) and a stationary phase present in inner column wall or as column packing particles. Nowadays, the majority of used columns are capillary with the stationary phase coated in the inner wall. The separation occurs due to different distribution coefficients of the single components of the mixture and the chromatographic process is a result of repeated sorption and desorption processes as the analytes move through the stationary phase carried by the inert gas. The GC instrumentation consists of a sample introduction device, a column housed in a temperature programmable oven, an interface, a mass spectrometer and a data collecting system. There are several detection systems available; nevertheless as the quadrupole mass analyzer was used in this study a more detailed description will be made. The quadrupole is a powerful analyzer for GC-MS systems as it presents several advantages such as the simplicity, size, reasonable cost and rapid scanning. This analyzer uses the ions trajectories stability in oscillating electric fields separating ions according to their m/z ratios. Quadrupole analyzers are constituted by four rods with circular, or ideally, hyperbolic section having a fixed direct current (DC) and alternating radio frequency (RF) voltages applied to the ions. These ions, produced in the source, are focused and passed in the middle of the rods and the motion depends on the electric fields, so the ions of a particular m/z will be in resonance and pass through to the detector. The ion motion is directly proportional to the mass, voltage on the quadrupole and RF. The RF voltages are varied, thus bringing different m/z ions to the detector forming a mass spectrum. Before entering the analyzer, the ions go through a potential to give the ions a determined speed to pass along the center of the quadrupole[192].

As for compound identification, standard injection and mass spectrum inspection are normally used. In addition, another strategy to improve identification confidence may employed, as for example retention indices (RI) values. After the injection of an n-alkane series, usually ranging from C_6 to C_{20}, in the same GC column and chromatographic method the RI values can be calculated using the van den Dool and Kratz equation[193]. The obtained values can then be compared with literature values obtained in equal or similar chromatographic columns used experimentally. The RI values are relatively independent from individual chromatographic system conditions such as column length/diameter, film thickness, carrier gas velocity, and pressure. These values are obtained by normalizing the retention times to an adjacent n-alkane which makes RI values based on carbon number of the molecule.

Although GC-MS yields significant analytical results, the matrices complex nature usually requires longer GC runs. It has also been shown that some peaks are formed by two or more compounds, a process denominated co-elution. For these reasons, in the last decades, research has been performed to overcome these issues and increase resolving power. The overall resolving power of 1D GC can be described by the peak capacity that is the maximum number of peaks that can be place, side by side, into the available one-dimensional separation space at a given resolution. In 1D GC, values of peak capacity of 1000 are very difficult to obtain[194]. A trustworthy option that has emerged is multidimensional gas chromatography (MDGC), namely GC×GC.

MDGC is a robust and reliable technique that exceeds the separation capacity of single dimensional chromatography. MDGC incorporates multiple sequential separations of different mechanisms (for example, different column selectivity) with a transfer process between dimensions that serve to effectively split individual retentions[195].

GC×GC is reported by Liu and Phillips[196] for the first time in 1991. The authors incorporated a thermal modulator between two columns that traps, focus and injects small amounts from the first column to the second column. When compared to one dimensional (1D-GC) the GC×GC presents several advantages:

1. higher peak capacity, the highest number of chromatographic peaks is significantly enhanced due to the product of the peak capacity of both dimensions;

2. signal enhancement due to analyte refocusing followed by fast chromatography avoiding band broadening and the signal to noise ratio is improved due to improved analyte separation and;
3. the ability to produce structured chromatograms. The structuration obtained of the 2D chromatographic space can be highlighted. Due to the complementary separation mechanisms of the two columns, a spatial distribution of the analytes occurs in the 2D space according to their chemical properties allowing the separation in compound classes. This can be used to reduce analysis time and the identification is more trustworthy as characteristic patterns are formed in an ordered manner[197]. For example, the use of non-polar capillary column in ^{1}D leads to separation by volatility whilst the use of a polar column in ^{2}D separates the analytes by polarity.

These advantages makes GC×GC a powerful tool that has been used in different applications that are not easily solved in 1D GC [198]. For this purpose, multidimensional chromatography is a technology that uses two orthogonal separation mechanisms to increase resolution power and peak capacity[199]. The theoretical peak capacity of these systems is the sum of the peak capacities of the first and second dimensions multiplied by the number of heart-cuts[200].

In GC×GC, two separations based on different separating mechanisms are applied to originate orthogonality. Orthogonality in GC×GC is created using columns that provide independent separation mechanisms in the first and second dimension[201]. The separation on the GC system is based on two parameters: the analytes volatility and the interaction with the stationary phase. The use of a non-polar column, where volatility is the only parameter of interest, and a polar column that will separate the analytes by specific interactions; but also by volatility is a common column set-up. Usually, using this set-up a structure chromatogram is obtained and it is possible to perform group-type identification[202]. Ryan *et al.*[203] studied separation orthogonality using different sets of ^{1}D and ^{2}D columns and concluded that if the columns used are very different the separation on the second dimension is maximized. The in-series connection is performed through an interface known as the modulator. An example of the interface is the cryomodulator. This interface cuts small fractions of the eluate from ^{1}D by cryofocusing and re-injecting into the second column. Nevertheless, to maintain

the [1]D separation the interface cuts the fractions so that they are no larger than one quarter of peak width. Then each individual fraction is refocused and injected in the second column which is a very fast process when compared to the first column separation[204]. Large series of high-speed second-dimension chromatograms are transformed to form a two-dimensional chromatogram that is usually performed by laboratory-written program. The visualization can be made in a contour plot (colored or not) or in a 3D plot[202] (Figure 1.22).

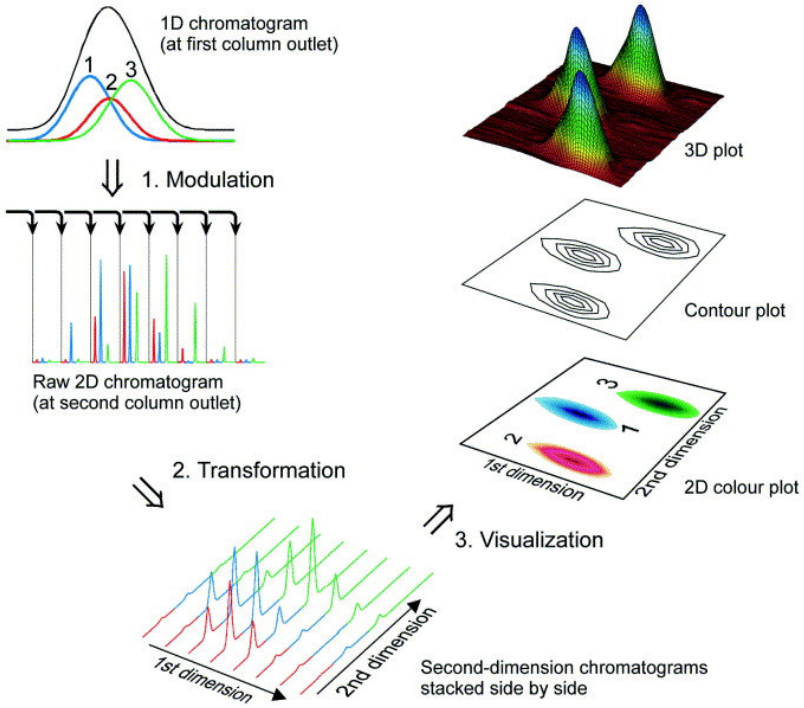


Figure 1.22 - GC×GC generation and visualization[202].

There are several detectors that can be used in combination with GC×GC, as for example, the flame ionization detector (FID), electron capture detector or ToFMS. The detectors used in GC×GC have to be very fast due to the fast separation in the second dimension that lead to peaks with typical widths between 100-600ms[205]. In the developed study, the GC×GC was combined with a ToFMS. ToFMS separation relies in the mass separation principle with the determination, by time measurement, of ion mass-to-charge ratio. The ions velocity, attained by homogeneous electrostatic field acceleration, depends on the mass-to-charge ratio. So ions with the same charge will possess the same kinetic energy and heavier ions will have lower velocities and lighter ions will have higher velocities[206]. ToFMS is ideal in the characterization for fast gas

chromatographic separation due to two main attributes: high spectral acquisition rates (a few hundred full-mass-range spectra per second) and spectral continuity across the chromatographic peak profile for a single-component peak. Spectral continuity means that for all the points on a chromatographic peak the ion abundance ratios for the different masses are the same[207]. Using the ToF spectrometer, even though concentration changes during peak elution, spectral continuity is not affected. The use of quadrupole MS or other scanning instruments the analyte concentration changes which results in skewed spectra that is resolved by averaging spectra across the apex of the chromatographic peak[208].

The use of comprehensive two-dimensional gas chromatography coupled to mass spectrometry with a high resolution time of flight analyzer (GC×GC–ToFMS) has brought a significant advantage in research and in analytes identification in several fields. Over the past years the use of GC×GC–ToFMS in the analysis of exhaled breath and urine compositions has gained interest in the scientific community. In exhaled breath it has been used in the detection of breath metabolome demonstrating the added value of this technique. Much more analytes were identified than in the 1D GC-MS reports due to the improved separation and increased sensitivity[209,210].The GC×GC–ToFMS was also used in urine analysis in infant urine finding several organic acids that would not be possible using 1D GC[211]. Other applications using GC×GC–ToFMS concerned the bladder cancer metabotyping[212] and in the analysis of anabolic agents in doping control[213].

1.3.3 Urine characterization based on proton based nuclear magnetic ressonance

NMR to analyze biofluids goes back to early 1980s. Actually, NMR is applied in several areas as disease diagnosis, drug discovery, microbiology, nutrition, toxicology, plant and environmental sciences. NMR is widely used in metabolomics applications as this technique is resolute, reproducible, reliable, and the data obtained is easily quantified and robust. Additionally, NMR requires minimal or even no sample preparation/separation and is of non-destructive nature. A major advantage in using NMR to obtain a metabolomic profile in a biological sample is that the acquisition can take from 1-15 min with acceptable sensitivity to be able to differentiate subtle

biological differences[214]. The major drawback is related to the sensitivity level when compared with MS-base methods[215].

NMR is able to detect a wide range of structurally different compounds in a single run in the micromole per liter range. The number of compounds in human fluids are estimated between 2000-3000 and a maximum of about 20 000 compounds. The smaller compounds, including sugars, amino acids and bioactive products that are paramount in several metabolic pathways, are of great interest to researchers. Usually these compounds act as signaling functions at very low concentrations. The complexity of the urine ^{1} H NMR spectrum can be observed with several resolved peaks (Figure 1.23).

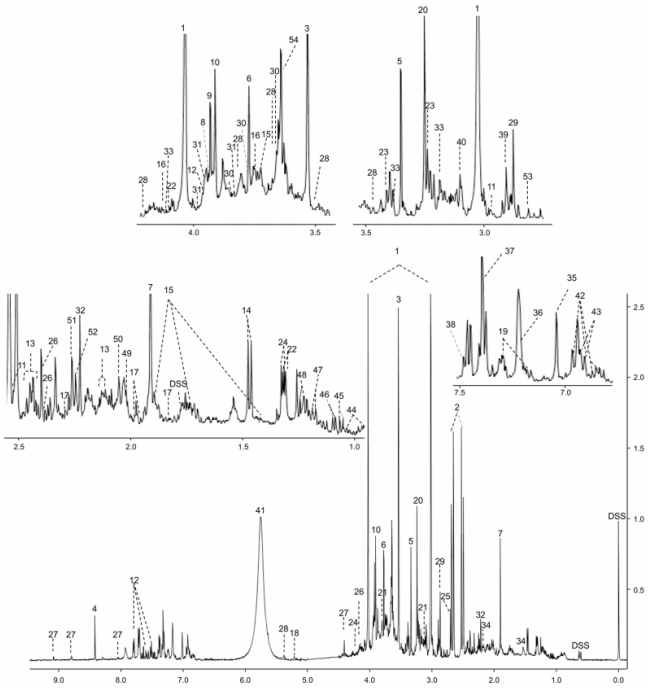


Figure 1.23– Example of a urine ^{1}H NMR spectrum[108]

Several ^{1}H NMR urine studies have been performed and have been extensively reviewed. Bouatra and co-authors[108] have built a database of detectable compounds in human urine. These authors found out that by NMR several hundred compounds were reported in a study performed with 22 healthy children up to 209 unique compounds have been identified and each compound was indisputably identified and quantified. These high-dimensional datasets are attained by bucketing. A bucket is a small slice of the spectrum and the integral is calculated to obtain a variable for each bucket. This procedure reduces the total number of variables and compensates the possible

misalignments. Beside inorganic ions and gases, the most abundant constituents in a healthy individual are urea, creatinine, hippuric acid and citric acid. Other compounds include branched-chain amino acids, organic acids, N-acetylated amino acids, hydroxycarboxylic acids, aromatic signals (for example, indoxylsulfate, histidine, phenylacetylglutamine), sugars, phenylacetate derivatives, among others[216].

NMR and metabolomics have been mainly applied in the human disease diagnosis field. The metabolomic studies using urine have provided important information on several pathologies such as cancer[217,218], inborn errors of metabolism[219], prenatal disorders[220], cardiovascular pathologies[221], respiratory diseases[222], neurological pathologies[223] and kidney pathologies[224], among others.

1.4 Data pre-processing and multivariate analysis

Multivariate analysis (MVA) plays an exceptionally significant role in metabolomic analysis. The several compounds found in the studied biofluid, the lack of reference NMR and MS spectra for all the compounds and the inherent variability in each sample due to the individuality of the each organism highlights the role of MVA. The identification of the chemical features that characterize the object of study against the large background of compounds that define the system, the variations attained by disturbing the studied system by experimental and environmental factors and even the fluctuation on data spectra due to instrument instability or sampling variability requires a robust MVA methodology to verify the trends in the data set. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) are tools used in metabolomics do determine relative differences between two data sets[225].

A metabolomic data analysis consists in several steps: pre-processing, pre-treatment, processing, post-processing, validation and interpretation. Data pre-processing consists in the obtention of clean data from raw instrumental data. Deconvolution, peak-picking, target analysis, alignment, phasing, baseline correction and bucketing are pre-processing tools used in NMR spectra and MS chromatograms (the last three tools are NMR specific). Pre-treatment involves normalization procedures, centering or mean-centering, scaling (autoscaling, range or pareto scaling), transforming the data (using logaritm, square root or box-cox) and examining for outliers[226].

In this PhD thesis, the unsupervised method of PCA and supervised methods of PLS-DA were employed due to the multivariate nature of the obtained data The basic principles will be described. Previously the normalization is discussed as it is an intrinsic part of the adopted MVA for the performed studies.

1.4.1 Data normalization in metabolomics

Metabolomic data needs to be transformed before proceding to MVA as the raw data varies due to experimental or biological reasons. This situation has to be previously dealt with so that the intended goals can be accomplished. The experimental verified

variation can be due to human error (sample preparation and/or extraction), instrument variation (temperature alteration in the instrument, sample degradation or even loss in equipment sensibility in the runs), different sets, laboratories and even different analytical platforms. Another possibility are biological discrepancies, as for example different biofluid concentrations, which might be mistaken as possible features of interest. This unwanted variation can be detected as in weight or volume of biological samples or even cells number, or remains undetected as for instance the aforementioned biofluid concentration or other factors that might influence as form/size of cells.

With this in mind, normalization procedures can identifiy and eliminate the observed and unnoticed factors mentioned taking a central role in metabolomic data analysis, as MVA results depends on the adopted procedure. These features are identified and eliminated by the normalization procedure and the selection of the appropriate method has to take into account a number of factors. Usually normalization is data and experiment-dependent but should also depend on the goal of the statistical analysis. This procedure can be performed by using an internal standard or by applying scaling factors for the samples under study for the complete dataset such as normalization by average, median, maximum, standard deviation, among others.

In the present work the most classical case of normalization, mean normalization, was chosen in exhaled breath and urine volatile data. This procedure consists in dividing each row of a data matrix by its average, thus neutralizing the influence of any hidden factor. In urine data obtained by ^{1}H NMR three normalization techniques were tested, namely total area normalization, creatinine[227] and probabilistic normalization quotient (PQN)[228]. After the appropriate normalization method is selected, MVA tools are used to develop models and identify biologically revelant features for future analysis.

1.4.2 Principal component analysis and partial least squares - discriminant analysis

The most popular tools for MVA in metabolomics are PCA and PLS[229]. Essentially these techniques are used to distinguish the classes in highly complex datasets, although there are other factors that may cause class variability. Generally a data matrix X, which contains N observation row vectors of K variables each, is the

most common manner to depict the acquired data. The data matrix X inputed is the spectral data obtained by MS and/or NMR.

PCA is widely applied in statistical analysis in chemometrics in general. PCA is a orthogonal linear transformation of the data preserving the variance of the original data. In metabolomics it is the starting point of MVA being used for exploratory purposes and to reduce the obtained data[230]. It may establish intrinsic class-related patterns or clusters although PCA is an unsupervised method and may enable the detection of outliers in the data set. PCA is desgined to extract and display the systematic variation in a data matrix X and the projection of each sample into a plane (defined by the first two components) makes it possible to visualize all samples[231].

Though the unsupervised PCA yields an unbiased dimensionality reduction, only when the within-group variation is sufficiently less than the between-group variation a group structure is revealed. As a result supervised MVA tools, such as PLS-DA, are applied in metabolomic experiments where class membership of each observation is inputted. Class memberships are usually coded in the matrix to Y component and this way forced to be orthogonal[229]. PLS-DA is a powerful tool in the metabolomic data classification[232]. The information may be quantitative or qualitative and the information is used to focus the model plane to capture the Y-related variation in X . PLS focus on maximizing the variance of the dependent variables explained by the idependent ones instead of reproducing the empirical covariance matrix[233]. When the information provided by Y is qualitative, the method is denominated by PLS-DA to make the distinction from the quantitative.

1.4.3 Statistical models validation

Validation is a crucial step to guarantee the reliability of any developed statistical models. For example, the most common method PLS-DA[234] overfits models to data where completely random variables have excellent class separation.

The categorical variable Y indicates class membership, meaning that values of -1 and 1 represent the healthy and the asthmatics under study, respectively. Nevertheless, due to the properties of regression models, the prediction is not inevitably the exact value. The challenges of PLS-DA are classification procedure and precise estimation of the quality of the obtained models and thereby differences between two classes. To

verify the quality of the obtained discrimination models several tools have been developed, among them cross validation[235]. Regression model validation determines the if the relationships between the variables, obtained from regression analysis, truly describes the data. In the present work , an out-of-sample evaluation were considered.

Cross validation is widely applied in chemometrics as the use of this tool is indispensable to validate data from supervisioned tests such as PLS-DA, and if not mentioned, it is simply referred as the leave-one-out cross validation (LOO-CV). LOO-CV is a practical and reliable fashion to test the significance of the developed models. Briefly, LOO-CV uses one observation for validation purposes whilst the remaining observations are considered the training data and the process is repeated until each observation is used once for validation[236]. LOO-CV has the disadvantage of being computationally expensive and often causes overfitting, and on average, gave an under-estimation of the true predictive error[237]. MCCV first reported by Cook[238] and tested for chemometric purposes by Xu and Liang[239], can avoid an unnecessary large model and therefore decreases the risk of over-fitting for the calibration model. MCCV splits the data into a learning set or a test set and the model developed on the learning set and the error evaluated in the test set. The test set estimates are averaged over the learning-testing random splits and each case only appears in the learning set or the test set, but not in both[240]. MCCV substancially reduces the variance of the split-sample error estimate[241]. MCCV yields the statistical model classification rate, sensitivity and specificity. The classification rate indicates the samples correctly identified, the sensitivity yields the percentage of positives that are correctly identified and specificity measures the percentage of negatives to be true negatives. Receiver operating characteristics (ROC) curves plot sensitivity versus (1-specificity). This graphical illustration gives the proportion of true positives against the false positives. Also, the values of R^2 could be used to assess the the degree of the model to the data in spite not being a cross validation evaluation. However, if R^2 is much higher that Q^2 obtained in MCCV it possibly indicates model overfitting[229].

1.5 Aims and outlines of this PhD thesis

Following several steps, the work developed in this PhD thesis ultimately intended the development simple methodologies in the analysis of non-invasively obtained samples for asthma regarding for their clinical applications. The matrices under study were exhaled breath and urine that are promising biofluids that over the years have attracted scientific and clinical interest. These matrices are rich sources of information, obtained in a non-invasive manner and effortlessly. Among several available analytical techniques, GC-MS, GC×GC-ToFMS and ^{1}H NMR were chosen to obtain the most complete volatile and non-volatile information. The chosen extraction methodology for the study of exhaled breath and urine volatile composition was HS-SPME. This technique has been widely used in research in the clinical context as it possesses several advantages that make this technique meritorious in the analysis of the aforementioned matrices. In metabolomics, the use of multivariate tools is essential. These tools are necessary as the datasets are of multivariate nature due to the instrumentation availability and the complexity of systems and processes. These tools allow the extraction of relevant data with several variables using all the variables simultaneously forming a metabolomic pattern that may characterize the pathology.

In Chapter 1, the state-of-the-art is described, namely asthma definition, the metabolomic alterations produced, definition of metabolomics and used analytical techniques and biofluid analysis. The results of this thesis are then presented as follows. Chapter 2 begins with the optimization of the SPME parameters, as well as, of exhaled breath sampling parameters with the use of GC-MS. The optimized conditions were then applied to a set of asthmatic and healthy children and multivariate tools were applied. A GC×GC–ToFMS method was then developed to confirm and enrich the previous information using a new cohort of children. To add robustness to the developed statistical models, an external validation method (MCCV) was applied. Subsequently, using prediction tools new exhaled breath samples were collected to verify the possibility to use the developed method in a clinical practice environment. Case studies were included to appraise the method response versus the clinical status of the subject under study. In Chapter 3, the analysis of the urine volatile composition was made using an in-lab developed method. The same strategy was used as in exhaled breath and multivariate/validation tools were used. To complement this study, the non-volatile composition was accessed by ^{1}H NMR. This allowed obtaining more

information on asthma using this matrix, in addition to the development of statistical models that could be certainly used for future endeavors. Chapter 4 has the general conclusions of this PhD thesis, as well, recommendations for future research.

1.6 References

(1) National Asthma Education and Prevention Program, Third Expert Panel on the Diagnosis and Management of Asthma. Expert Panel Report 3: Guidelines for the Diagnosis and Management of Asthma. Bethesda (MD): National Heart, Lung, and Blood Institute (US); 2007 Aug. Section 2, Definition, Pathophysiology and Pathogenesis of Asthma, and Natural History of Asthma. Available from: http://www.ncbi.nlm.nih.gov/books/NBK7223/

(2) Colice G.L., Categorizing Asthma Severity: An Overview of National Guidelines. Clin. Med. Res. (2004) 2: 155-163.

(3) Pollart S.M., Elward K.S., Overview of Changes to Asthma Guidelines: Diagnosis and Screening. Am. Fam. Physician. (2009) 79: 761-767

(4) Kim H.Y., DeKruyff R.H., Umetsu D.T., The many paths to asthma: phenotype shaped by innate and adaptive immunity. Nat. Immunol. (2010) 11: 577-584

(5) Johansson S.G., Lundahl J., Asthma, atopy, and IgE: what is the link? Curr. Allergy Asthma Rep. (2001) 1: 89-90

(6) Boulet L.P., Boulay M.È., Asthma-related comorbidities. Expert. Rev. Respir. Med. (2011) 5: 377-393.

(7) Ferguson G.T., Enright P.L., Buist A.S. Higgins M.W., Office spirometry for lung health assessment in adults: a consensus statement from the National Lung Health Education Program. Chest (2000) 117: 1146–61

(8) Liang B-M., Lam D.C.L., Feng Y-L., Clinical applications of lung function tests: A revisit. Respirology (2012) 17: 611-619

(9) Barreto L.M., Duarte M.A., Moura S.C.D.O, Alexandre B.L., Augusto L.S., Fontes M.J.F., Comparison of measured and predicted values for maximum respiratory pressures in healthy students. Fisioter. Pesqui. (2013) 20: 235-243

(10) Aggarwal A.N., Gupta D., Argawal R., Jindal S.K., Comparison of the Lower Confidence Limit to the Fixed-Percentage Method for Assessing Airway Obstruction in Routine Clinical Practice. Resp. Care (2011) 56: 1778-1784

(11) Kharitonov S.A., Yates D., Robbins R.A., Logan-Sinclair R., Shinebourne E.A., Barnes P.J., Increased nitric oxide in exhaled air of asthmatic patients. Lancet (1994) 343: 133–135

(12) Huynh N.N., Chin-Dusting J., Amino acids, arginase and nitric oxide in vascular health. Clin. Exp. Pharmacol. Physiol. (2006) 33: 1-8

(13) Zinelli C., Caffarelli C., Strid J., Jaffe A., Atherton D.J., Measurement of nitric oxide and 8-isoprostane in exhaled breath of children with atopic eczema. Clin. Exp. Dermatol. (2009) 34: 607–612.

(14) Hastie A.T., Moore W.C., Li H., Rector B.M., Ortega V.E., Pascual R.M., Peters S.P., Meyers D.A., Bleecker E.R.; National Heart, Lung, and Blood Institute's Severe Asthma Research Program. Biomarker surrogates do not accurately predict sputum eosinophil and neutrophil percentages in asthmatic subjects. J. Allergy Clin. Immunol. (2013) 132: 72-80.

(15) Peirsman E.J., Carvelli T.J., Hage P.Y., Hanssens L.S., Pattyn L., Raes M.M., Sauer K.A., Vermeulen F., Desager K.N., Exhaled nitric oxide in childhood allergic asthma management a randomised controlled trial. Pediatr. Pulmonol. (2014) 49: 624-631.

(16) http://healthierchoices.ca/TestsAndProcedures_details.aspx?TP_id=2&Type=2 (reviewed in 09/11/2014)

(17) Heinzerling L., Mari A., Bergmann K.C., Bresciani M., Burbach G., Darsow U., Durham S., Fokkens W., Gjomarkaj M., Haahtela T., Bom A.T., Wöhrl S., Maibach H., Lockey R., The skin prick test - European standards. Clin. Transl. Allergy. (2013) 1: 3-13

(18) Paggiaro P.L., Chanez P., Holz O., Ind P.W., Djukanović R., Maestrelli P., Sterk P.J., Sputum induction. Eur Respir J Suppl. (2002) 37: S3-S8

(19) Snell N., Newbold P., The clinical utility of biomarkers in asthma and COPD. Curr. Opin. Pharmacol. (2008) 8: 222-235.

(20) Cox L., Williams B., Sicherer S., Oppenheimer J., Sher L., Hamilton R., Golden D.; American College of Allergy, Asthma and Immunology Test Task Force; American Academy of Allergy, Asthma and Immunology Specific IgE Test Task Force. Pearls and pitfalls of allergy diagnostic testing: report from the American College of Allergy, Asthma and Immunology/American Academy of Allergy, Asthma and Immunology Specific IgE Test Task Force. Ann. Allergy Asthma Immunol. (2008) 101: 580-92.

(21) Global Strategy for Asthma Management and Prevention. Global Initiative for Asthma (GINA), 2006. Available from www.ginasthma.org Date last updated, 2012

(22) Samolinski B., Fronczak A., Kuna P., Akdis C.A., Anto J.M., Bialoszewski A.Z., Burney P.G., Bush A., Czupryniak A., Dahl R., Flood B., Galea G., Jutel M., Kowalski M.L., Palknone S., Papadopoulos N., Raciborski F., Sienkiewicz D., Tomaszewska A., Von Mutius E., Willman D., Wtodarczyk A., Yusuf O., Zuberbier T., Bousquet J., Prevention and control of childhood asthma and allergy in the EU from the public health point of view: Polish Presidency of the European Union, Eur. J. All.Clin. Immun. (2012) 67: 726-731

(23) European Respiratory Society and the European Lung Foundation. European lung white book. Brussels, Belgium: European Respiratory Society and the European Lung Foundation, 2003.

(24) http://www.chemicalshealthmonitor.org/spip.php?rubrique8 (reviewed in 11/12/2013)

(25) European Lung white book, Chapter 11 Childhood Asthma, European Respiratory Society (2013)

(26) Barnes P.J., Jonsson B., Klim J.B., The costs of asthma, Eur. Respir. J. (1996) 9: 636-642.

(27) http://www.chemicalshealthmonitor.org/spip.php?rubrique8 (reviewed in 11/12/2014)

(28) Rothman K.J., Epidemiology: An introduction (2012), Second Edition, Oxford University Press, New York, New York, USA

(29) Chang C., Asthma in children and adolescents: a comprehensive approach to diagnosis and management. Clin. Rev. Allergy Immunol. (2012) 43: 98-137

(30) Bousquet J., Bousquet P.J., Godard P., Daures J.P., The public health implications of asthma Bulletin of the World Health Organization, (2004) 83: 548-554

(31) From the Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) 2011. Available from: http://www.ginasthma.org/.

(32) Masoli M., Fabian D., Holt S., Beasley R.; Global Initiative for Asthma (GINA) Program. The global burden of asthma: executive summary of the GINA Dissemination Committee report. Allergy (2004) 59: 469-478.

(33) Braman S.S., The global burden of asthma, Chest 2006, 130: S4-S12.

(34) Sa-Sousa A., Morais-Almeida M., Azevedo L.F., Carvalho R., Jacinto T., Todo-Bom A., Loureiro C., Bugalho-Almeida A., Bousquet J., Fonseca J.A., Prevalence of asthma in Portugal – The Portuguese National Asthma Survey. Clin. Transl. Allergy. 2 (2012) 2:15

(35) International Study of Asthma and Allergies in Childhood (ISAAC): Worldwide variations in the prevalence of asthma symptoms: the International Study of Asthma and Allergies in Childhood (ISAAC). Eur. Respir. J. 1998, 12: 315-35

(36) British Thoracic Society Scottish Intercollegiate Guidelines Network, British Guideline on the Management of Asthma, Thorax (2008) 63: Suppl 4: iv1-121

(37) Barnes P.J., Corticosteroid effects on cell signalling, Eur. Respir. J., (2006) 27: 413-416

(38) Berger W.E., New approaches to managing asthma: a US perspective, Ther. Clin. Risk. Manag. (2008) 4: 363-379

(39) Barnes P.J., Scientific rationale for inhaled combination therapy with long-acting β2-agonists and corticosteroids, Eur Respir. J. (2002) 19: 182-191.

(40) Lougheed M.D., Lemière C., Dell S.D., Ducharme F.M., FitzGerald J.M, Leigh R., Licskai C., Rowe R.H., Bowie D., Becker A., Boulet L.H., Canadian Thoracic Society Asthma Management Continuum – 2010 Consensus Summary for children six years of age and over, and adults. Can. Respir. J. (2010) 17: 15-24.

(41) Lin S.Y., Erekosima N., Suarez-Cuervo C., Ramanathan M., Kim J.M., Ward D., Chelladurai Y., Segal J. B., Allergen-Specific Immunotherapy for the Treatment of Allergic Rhinoconjunctivitis and/or Asthma: Comparative Effectiveness Review. Agency for Healthcare Research and Quality (US) (2013) Available from: http://www.ncbi.nlm.nih.gov/books/NBK133240/

(42) Focke M., Swoboda I., Marth K., Valenta R., Developments in allergen-specific immunotherapy: from allergen extracts to allergy vaccines bypassing allergen-specific immunoglobulin E and T cell reactivity. Clin. Exp. Allergy (2010) 40: 385-97.

(43) Kopp M.V., Omalizumab: Anti-IgE therapy in allergy. Curr. Allergy Asthma Rep. (2011) 11: 101-106

(44) Taher Y.A., Henricks P.A., van Oosterhout A.J. Allergen-specific subcutaneous immunotherapy in allergic asthma: immunologic mechanisms and improvement. Libyan J. Med. (2010) 5: 5303

(45) Hercus T.R., Thomas D., Guthridge M.A., Ekert P.G., King-Scott J., Parker M.W., Lopez A.F., The granulocyte-macrophage colony-stimulating factor receptor: linking its structure to cell signaling and its role in disease. Blood (2009) 114: 1289-1298.

(46) Jönsson F., Daëron M., Mast cells and company. Front. Immunol. (2012) 20: 3-16.

(47) Hu Z.Q., Zhao W.H., Shimamura T., Regulation of mast cell development by inflammatory factors. Curr. Med. Chem. (2007) 14:3044-3050.

(48) Advances in Combination Therapy for Asthma and COPD, (2012) Jan Lötvall, Wiley-Blackwell, John Wiley & Sons, Ltd , Hoboken , USA
(49) van Ree R., Hummelshøj L., Plantinga M., Poulsen L.K., Swindle E. Allergic sensitization: host-immune factors. Clin. Transl. Allergy. (2014) 4: 12-21.
(50) Sahiner U.M., Birben E., Erzurum S., Sackesen C., Kalayci O., Oxidative Stress in Asthma. World Allergy Organ. J. (2011) 4:151-158.
(51) Cho Y.S., Moon H-B., The Role of Oxidative Stress in the Pathogenesis of Asthma. Allergy Asthma Immunol. Res. (2010) 2: 183-187
(52) Admako D.J., Skyes B.D., Howe B.H., The metabolomics of asthma: novel diagnostic potential. Chest (2012) 141: 1295-1302
(53) Ho W.E., Xu Y.J., Xu F., Cheng C., Peh H.Y., Tannenbaum S.R., Wong W.S., Ong C.N., Metabolomics reveals altered metabolic pathways in experimental asthma. Am. J. Respir. Cell. Mol. Biol. (2013) 48:204-211.
(54) Borza C., Muntean D., Dehelean C., Săvoiu G., Şerban C., Simu G., Andoni M., Butur M., Drăgan S. (2013)., Prof. Rodrigo Valenzuela Baez (Ed.), ISBN: 978-953-51-0944-0, InTech, DOI: 10.5772/51627. Available from: http://www.intechopen.com/books/lipid-metabolism/oxidative-stress-and-lipid-peroxidation-a-lipid-metabolism-dysfunction.
(55) Halliwell B., Chirico S., Lipid peroxidation: its mechanism, measurement, and significance. Am. J. Clin. Nutr. (1993) 57: S715-S724.
(56) Storey K.B., Functional Metabolism: Regulation and Adaptation (2004), John Wiley and Sons Inc., New Jersey, USA.
(57) Shils M.E., Shike M., Modern Nutrition in Health and Disease (2006), Lippincott Williams & Wilkins, Philadelphia, USA,.
(58) Maes T., Provoost S., Lanckacker E.A., Cataldo D.D., Vanoirbeek J.A., Nemery B., Tournoy K.G., Joos G.F., Mouse models to unravel the role of inhaled pollutants on allergic sensitization and airway inflammation. Respir. Res. (2010) 11: 7-38
(59) Lobo V., Patil A., Phatak A., Chandra N., Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn. Rev. (2010) 4: 118-126
(60) Cottrell L., Neal W.A., Ice C., Perez M.K., Piedimonte G., Metabolic abnormalities in children with asthma. Am. J. Respir. Crit. Care Med., (2011) 183: 441-448.
(61) Zuo L., Otenbaker N.P., Rose B.A., Salisbury K.S., Molecular mechanisms of reactive oxygen species-related pulmonary inflammation and asthma. Molec. Immun. (2013) 56: 57-63
(62) Montuschi P., Corradi M., Ciabattoni G., Nightingale J., Kharitonov S.A., Barnes P.J., Increased 8-isoprostane, a marker of oxidative stress, in exhaled condensate of asthma patients. Am. J. Respir. Crit. Care Med. (1999) 160: 216-220.
(63) Wedes S.H., Khatri S.B., Zhang R., Wu W., Comhair S.A., Wenzel S., Teague W.G., Israel E., Erzurum S.C., Hazen S.L. Noninvasive markers of airway inflammation in asthma. Clin. Transl. Sci. (2009) 2: 112–117.
(64) van Dalen C.J., Winterbourn C.C., Kettle A.J., Mechanism of nitrite oxidation by eosinophil peroxidase: implications for oxidant production and nitration by eosinophils. Biochem. J. (2006) 394: 707-713.
(65) Pacher P., Beckman J.S., Liaduet L., Nitric Oxide and Peroxynitrite in Health and Disease. Physiol. Rev. (2007) 87: 315-424.
(66) Wu W., Samoszuk M.K., Comhair S.A., Thomassen M.J., Farver C.F., Dweik R.A., Kavuru M.S., Erzurum S.C., Hazen S.L. Eosinophils generate brominating oxidants in allergen induced asthma. J. Clin. Invest. (2000) 105: 1455–1463.
(67) Negre-Salvayre A., Coatrieux C., Ingueneau C., Salvayre R., Advanced lipid peroxidation end products in oxidative damage to proteins. Potential role in diseases and therapeutic prospects for the inhibitors. Br. J. Pharmacol. (2008) 153: 6-20
(68) Boots A.W., van Berkel J.J., Dallinga J.W., Smolinska A., Wouters E.F., van Schooten F.J., The versatile use of exhaled volatile organic compounds in human health and disease. J. Breath Res. (2012) 6: 027108.
(69) Dallinga J.W., Robroeks C.M., van Berkel J.J., Moonen E.J., Godschalk R.W., Jöbsis Q., Dompeling E., Wouters E.F., van Schooten F.J., Volatile organic compounds in exhaled breath as a diagnostic tool for asthma in children. Clin. Exp. Allergy (2010) 40: 68-76
(70) Ibrahim B., Basanta M., Cadden P., Singh D., Douce D., Woodcock A., Fowler S.J., Non-invasive phenotyping using exhaled volatile organic compounds in asthma, Thorax (2011) 66: 804-809
(71) Ligor M., Ligor T., Bajtarevic A., Ager C., Pienz M., Klieber M., Denz H., Fiegl M., Hilbe W., Weiss W., Lukas P., Jamnig H., Hackl M., Buszewski B., Miekisch W., Schubert J., Amann A.,

Determination of volatile organic compounds appearing in exhaled breath of lung cancer patients by solid phase microextraction and gas chromatography mass spectrometry. Clin. Chem. Lab. Med. (2009) 47: 550–560.
(72) http://www.whfoods.com/genpage.php?dbid=19&tname=faq (reviewed on 18/09/2014)
(73) Barker G.F., Manzo N.D., Cotich K.L., Shone R.K., Waxman A.B., DNA Damage 2008) 153: 6-20
(73) Boots A.W., van Berkel J.J., Dallinga J.W., Smolinska A., Wouters E.F., van Schooten F.J., The versatile use of exhaled volatile organic compounds in human health and disease. J. Breath Res. (2012) 6: 027108.
(73) Dallinga J.W., Robroeks C.M., van Berkel J.J., Moonen E.J., Godschalk R.W., Jöbsis Q., Dompeling E., Wouters E.F., van Schooten F.J., Volatile organic compounds in exhaled breath as a diagnostic tool for asthma in children. Clin. Exp. Allergy (2010) 40: 68-76
(73) Ibrahim B., Basanta M., Cadden P., Singh D., Douce D., Woodcock A., Fowler S.J., Non-invasive phenotyping using exhaled volatile organic compounds in asthma, Thorax (2011) 66: 804-809
(73) Ligor M., Ligor T., Bajtarevic A., Ager C., Pienz M., Klieber M., Denz H., Fiegl M., Hilbe W., Weiss W., Lukas P., Jamnig H., Hackl M., Buszewski B., Miekisch W., Schubert J., Amann A., Determination of volatile organic compounds appearing in exhaled breath of lung cancer patients by solid phase microextraction and gas chromatography mass spectrometry. Clin. Chem. Lab. Med. (2009) 47: 550–560.
(73) http://www.whfoods.com/genpage.php?dbid=19&tname=faq (reviewed on 18/09/2014)
(73) Barker G.F., Manzo N.D., Cotich Induced by Hyperoxia: Quantitation and Correlation with Lung Injury. Am. J. Respir. Cell Mol. Biol. (2006) 35: 277–288
(74) Henricks P.A., Nijkamp F.P., Reactive oxygen species as mediators in asthma. Pulm. Pharmacol. Ther. (2001) 14: 409-20
(75) Mann D.M., Molecular biology's impact on our understanding of aging. BMJ (1997) 315: 1078-1081
(76) Comhair S.A., Erzurum S.C., Redox control of asthma: molecular mechanisms and therapeutic opportunities. Antioxid. Redox Signal. (2010) 12: 93-124
(77) Saude E.J., Skappak C.D., Regush S., Cook K., Ben-Zvi A., Becker A., Moqbel R., Sykes B.D., Rowe B.H., Adamko D.J., Metabolomic profiling of asthma: diagnostic utility of urine nuclear magnetic resonance spectroscopy. J. Allergy Clin. Immunol. (2011) 127: 757-764
(78) Rocha C.M., Carrola J., Barros A.S., Gil A.M., Goodfellow B.J., Carreira I.M., Bernardo J., Gomes A., Sousa V., Carvalho L., Duarte I.F., Metabolic signatures of lung cancer in biofluids: NMR-based metabonomics of blood plasma. J. Proteome Res. (2011) 10: 4314-4324.
(79) Bolton C.F., Sepsis and the systemic inflammatory response syndrome: neuromuscular manifestations. Crit Care Med (1996) 24: 1408–1416.
(80) Berg J.M., Tymoczko J.L., Stryer L. Biochemitry (2002), 5th Edition, New York: W H Freeman
(81) Jung J., Kim S.H., Lee H.S., Choi G.S., Jung Y.S., Ryu D.H., Park H.S., Hwang G.S., Serum metabolomics reveals pathways and biomarkers associated with asthma pathogenesis. Clin. Exp. Allergy (2013) 43: 425-433.
(82) Phypers B., Pierce J.M., Lactate physiology in health and disease. Contin. Educ. Anaesth. Crit. Care Pain (2006) 6: 128-132.
(83) Luft F.C., Lactic Acidosis Update for Critical Care Clinicians. JASN (2001) 12: S15-S19.
(84) Rashid A.O., Azam H.M., DeBari V.A., Blamoun A.I., Moammar M.Q., Khan M.A. Non-anion gap acidosis in asthma: clinical and laboratory features and outcomes for hospitalized patients. Ann. Clin. Lab. Sci. (2008) 38: 228-234.
(85) Meert K.L., McCaulley L., Sarnaik A.P., Mechanism of lactic acidosis in children with acute severe asthma. Pediatr. Crit. Care Med. (2012) 13: 28-31.
(86) Lehninger A., Nelson D.L., Cox M.M., Nelson D.L., Lehninger Principles of Biochemistry (2004), W H Freeman & Co, New York.
(87) Greenwald R., Fitzpatrick A.M., Gaston B., Marozkina N.V., Erzurum S., Teague W.G., Breath formate is a marker of airway S-nitrosothiol depletion in severe asthma. PLoS One (2010) 5: e11919.
(88) Zhao Y., Fu L., Li R., Wang L.N., Yang Y., Liu N.N., Zhang C.M., Wang Y., Liu P., Tu B.B., Zhang X., Qiao J., Metabolic profiles characterizing different phenotypes of polycystic ovary syndrome: plasma metabolomics analysis. BMC Med. (2012) 10: 153 -168
(89) Jarjour N.N., Calhoun W.J., Schwartz L.B., Buse W.W., Elevated bronchoalveolar lavage fluid histamine levels in allergic asthmatics are associated with increased airway obstruction. Am. Rev. Respir. Dis. (1991) 144: 83-87
(90) Falcone F.H., Hass H., Gibbs B.F., The human basophil: a new appreciation of its role in immune responses. Blood (2000) 96: 4028-4038.

(91) Gelfand E.W., Role of Histamine in the Pathophysiology of Asthma: Immunomodulatory and Antiinflammatory Activities of H1-Receptor Antagonists. Am. J. Med. (2002), 113: 2-7
(92) Naclerio R., Clinical manifestations of the release of histamine and other inflammatory mediators. J. Aller. Clin. Immun. (1999) 3: S382-S385.
(93) http://biotechhelpline16.blogspot.pt/2012/09/hypersensitivity-i.html (reviewed in 11/12/2014)
(94) Zitt M.J., The role of nonsedating antihistamines in asthma therapy. Allergy Asthma. Proc. (2003) 24: 239-252.
(95) Boccuto L., Chen C.F., Pittman A.R., Skinner C.D., McCartney H.J., Jones K., Bochner B.R., Stevenson R.E., Schwartz C.E., Decreased tryptophan metabolism in patients with autism spectrum disorders. Mol. Autism. (2013) 4: 16
(96) Chen Y., Guillemin G.J., Kynurenine Pathway Compounds in Humans:Disease and Healthy States. Int. J. Tryptophan Res. (2009) 2: 1-19.
(97) Mándi Y., Vécsei L., The kynurenine system and immunoregulation. J. Neural Transm. (2012) 119: 197-209.
(98) Platten M., Wick W., Van den Eynde B.J., Tryptophan catabolism in cancer: beyond IDO and tryptophan depletion. Cancer Res. (2012) 72: 5435-5440.
(99) Meyer K.C., Arend R.A., Kalayoglu M.V., Rosenthal N.S., Byrne G.I., Brown R.R., Tryptophan metabolism in chronic inflammatory lung disease. J. Lab. Clin. Med. (1995) 126: 530-540.
(100) Fogarty A., Broadfield E., Lewis S., Lawson N., Britton J., Amino acids and asthma: a case-control study. Eur. Respir. J. (2004) 23: 565-568.
(101) Gupta K.B., Verma M., Nutrition and asthma. Lung India (2007) 24: 105-114.
(102) Fernstrom J.D., Fernstrom M.H., Tyrosine, phenylalanine, and catecholamine synthesis and function in the brain. J. Nutr. (2007) 137: S1539-S1547.
(103) Hanazawa T., Kharitonov S.A., Barnes P.J., Increased nitrotyrosine in exhaled breath condensate of patients with asthma. Am. J. Respir. Crit. Care Med. (2000) 162: 1273-1276.
(104) Ulrey C.L., Liu L., Andrews L.G., Tollefsbol T.O., The impact of metabolism on DNA methylation. Hum. Mol. Genet. (2005) 14: 139-147,
(105) http://www.science-autism.org/oxidative.htm (reviewed in 11/12/2013)
(106) Leipnitz G., Seminotti B., Haubrich J., Dalcin M.B., Dalcin K.B., Solano A., de Bortoli G., Rosa R.B., Amaral A.U., Dutra-Filho C.S., Latini A., Wajner M., Evidence that 3-hydroxy-3-methylglutaric acid promotes lipid and protein oxidative damage and reduces the nonenzymatic antioxidant defenses in rat cerebral cortex. J. Neurosci. Res. (2008) 86: 686-693.
(107) Zhang A., Sun H., Wang P., Han Y., Wang X., Modern analytical techniques in metabolomics analysis. Analyst (2012) 137: 293-300
(108) Bouatra S., Aziat F., Mandal R. Cuo A.C., Wilson M.R., Knox C., Bjorndahl T.C., Krishnamurthy R., Saleem F., Liu P., Dame Z.T., Poelzer J., Huynh J., Yallou F.S., Psychogios N., Dong E., Bogumil R., Roehring C., Wishart D.S., The human urine metabolome. Plos One (2013) 8: e73076.
(109) Goodacre R., Vaidyanathan S., Dunn W.B., Harrigan G.G., Kell D.B., Metabolomics by numbers: acquiring and understanding global compound data. Trens. Biotechnol. (2004) 22: 245-252.
(110) Dunn W.B., Broadhurst D.I., Atherton H.J., Goodacre R., Griffin J.L., Systems level studies of mammalian metabolomes: the roles of mass spectrometry and nuclear magnetic resonance spectroscopy. Chem. Soc. Rev. (2011) 40: 387-426
(111) Zhang A., Sun H., Wang X., Power of metabolomics in biomarker discovery and mining mechanisms of obesity. Obes. Rev. (2013) 14: 344-349
(112) Villas-Bôas S.G., Roessner U., Hansen M.A.E.,Smedsgaard J., Nielsen J., Metabolome Analysis: An Introduction. (2007) John Wiley & Sons, Inc., Hoboken, New Jersey, USA
(113) Álvarez-Sanchéz B., Priego-Capote F., de Castro M.D.L., Metabolomics analysis I. Selection of biological samples and practical aspects preceding sample preparation. Trends Anal. Chem. (2010) 29: 111-119
(114) Zhang S., Gowda G.A.N., Ye T., Raftery D., Advances in NMR-based biofluid analysis and metabolite profiling. Analyst (2010) 135: 1490-1498.
(115) Dunn, W. B., Broadhurst, D. I., Atherton, H. J., Goodacre, R., Griffin, J. L. Systems level studies of mammalian metabolomes: the roles of mass spectrometry and nuclear magnetic resonance spectroscopy. Chem. Soc. Rev. (2011) 40: 387–426.
(116) Ma Y., Zhang P., Yang Y., Wang F., Qin H., Metabolomics in the fields of oncology: a review of recent research. Mol. Biol. Rep. (2012) 39: 7505-7511.

(117) Akoto L., Vreuls R.J.J., Irth H., Pel R., Stellaard F. Fatty acid profiling of raw human plasma and whole blood using direct thermal desorption combined with gas chromatography-mass spectrometry. J. Chromatogr. A (2008) 1186: 365–371
(118) Almstetter M.F., Oefner P.J., Dettmer K. Comprehensive two-dimensional gas chromatography in metabolomics. Anal. Bioanal. Chem.(2012) 402: 1993-2013
(119) Perwaiz S., Tuchweber B., Mignault D., Gilat T., Yousef I.M. Determination of bile acids in biological fluids by liquid chromatography-electrospray tandem mass spectrometry. J. Lip. Res. (2001) 42: 114–119.
(120) Junot C., Madalinski G., Tabet J.C., Ezan E., Fourier transform mass spectrometry for metabolome analysis. Analyst (2010) 135: 2203-2219.
(121) Zelena E, Dunn WB, Broadhurst D, Francis-McIntyre S, Carroll KM, Begley P., O'Hagan S., Knowles J.D., Halsall A., Husermet Consortium, Wilson I.D., Kell D.B., Development of a robust and repeatable UPLC-MS method for the long-term metabolomic study of human serum. (2009) Anal. Chem. 81: 1357–1364.
(122) Pasikanti K.K., Ho P.C., Chan E.C.Y., Gas chromatography/mass spectrometry in metabolic profiling of biological fluids. J. Chromatogr. B. (2008) 871: 202-211.
(123) Lei Z., Huhman D.V., Sumner L.W., Mass spectrometry strategies in metabolomics. J. Biol. Chem. (2011) 286: 25435-25442
(124) Mondello L., Tranchida P.Q., Dugo P., Dugo G., Comprehensive two-dimensional gas chromatography-mass spectrometry: A review. J. Chromatogr. B (2008) 871: 202-211
(125) Li X., Xu Z., Lu X., Yang X., Yin P., Kong H., Yu Y., Xu G., Comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry for metabonomics: Biomarker discovery for diabetes mellitus. Anal. Chim. Acta. (2009) 633: 257-262.
(126) Snyder L.R., Hoggard J.C., Montine T.J., Synovec R.E., Development and application of a comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry method for the analysis of L-beta-methylamino-alanine in human tissue. J. Chromatogr. A. (2010) 1217: 4639-4647.
(127) Pasikanti K.K., Norasmara J., Cai S., Mahendran R., Esuvaranathan K., Ho P.C., Chan E.C., Metabolic footprinting of tumorigenic and nontumorigenic uroepithelial cells using two-dimensional gas chromatography time-of-flight mass spectrometry. Anal. Bioanal. Chem. (2010) 398: 1285-1293.
(128) Hartonen M., Mattila I., Ruskeepää A.L., Orešič M., Hyötyläinen T., Characterization of cerebrospinal fluid by comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry. J. Chromatogr. A. (2013) 1293: 142-149.
(129) Kim H.K., Choi Y.H., Verpoote R., NMR-based metabolomic analysis of plants. Nat. Protoc. (2010) 5: 536-549.
(130) Sitole L.J., Williams A.A., Meyer D., Metabonomic analysis of HIV-infected biofluids. Mol Biosyst. (2013) 9:18-28
(131) Duarte I.F., Gil A.M., Metabolic signatures of cancer unveiled by NMR spectroscopy of human biofluids. Prog Nucl Magn Reson Spectrosc. (2012) 62: 51-74
(132) Hassan-Smith G., Wallace G.R., Douglas M.R., Sinclair A.J., The role of metabolomics in neurological disease. J Neuroimmunol. (2012) 248: 48-52
(133) Griffin J.L., Atherton H., Shockcor J., Atzori L., Metabolomics as a tool for cardiac research. Nat Rev Cardiol. (2011) 8: 630-43.
(134) Atzei A., Atzori L., Moretti C., Barberini L., Noto A., Ottonello G., Pusceddu E., Fanos V., Metabolomics in paediatric respiratory diseases and bronchiolitis. J Matern Fetal Neonatal Med. (2011) 2: 59-62.
(135) Vijverberg S.J., Hilvering B., Raaijmakers J.A., Lammers J.W., Maitland-van der Zee A.H., Koenderman L., Clinical utility of asthma biomarkers: from bench to bedside (2013) 7:199-210.
(136) Murugan A., Prys-Picard C., Calhoun W.J., Biomarkers in Asthma. Curr. Opin. Pulm. Med. (2009) 15: 12-18.
(137) Dent A.G., Sutedja T.G., Zimmerman P.V., Exhaled breath analysis for lung cancer. J. Thorac. Dis. (2013) 5: S540-S550
(138) Thomas P.S., Lowe A.J., Samarasinghe P., Lodge C.J., Huang Y., Abramson M.J., Dharmage S.C., Jaffe A., Exhaled breath condensate in pediatric asthma: promising new advance or pouring cold water on a lot of hot air? a systematic review. Pediatr. Pulmonol. (2013) 48: 419-442.
(139) Murata K., Fujimoto K., Kitaguchi Y., Horiuchi T., Kubo K., Honda T., Hydrogen Peroxide Content and pH of Expired Breath Condensate from Patients with Asthma and COPD. COPD (2013) doi:10.3109/15412555.2013.830094

(140) Caballero Balanzá S., Martorell Aragonés A., Cerdá Mir J.C., Belda Ramírez J., Navarro Iváñez R., Navarro Soriano A., Félix Toledo R., Escribano Montaner A., Leukotriene B4 and 8-isoprostane in exhaled breath condensate of children with episodic and persistent asthma. J. Investig .Allergol. Clin.Immunol. (2010) 20: 237-243.

(141) Rosias P., Methodological aspects of exhaled breath condensate collection and analysis. J. Breath Res.(2012) 6: 027102.

(142) van de Kant K.D., van der Sande L.J., Jöbsis Q., van Schayck O.C., Dompeling E . Clinical use of exhaled volatile organic compounds in pulmonary diseases: a systematic review. Resp. Res. (2012) 13: 117-140

(143) Dragonieri S., Schot R., Mertens B.J., Le Cessie S., Gauw S.A., Spanevello A., Resta O., Willard N.P., Vink T.J., Rabe K.F., Bel E.H., Sterk P.J., An electronic nose in the discrimination of patients with asthma and controls. J. Allergy Clin. Immunol (2007), 120: 856-862.

(144) Gahleitner F., Guallar-Hoyas C., Beardsmore C.S., Pandya H.C., Thomas C.P., Metabolomics pilot study to identify volatile organic compound markers of childhood asthma in exhaled breath. Bioanalysis (2013) 5: 2239-2247.

(145) Robroeks C.M., van Berkel J.J., Jöbsis Q., van Schooten F.J., Dallinga J.W., Wouters E.F., Dompeling E., Exhaled volatile organic compounds predict exacerbations of childhood asthma in a 1-year prospective study. Eur. Respir. J. (2013) 42: 98-106.

(146) Ibrahim B., Basanta M., Cadden P., Singh D., Douce D., Woodcock A., Fowler S.J., Non-invasive phenotyping using exhaled volatile organic compounds in asthma, Thorax (2011) 66: 804-809.

(147) Dallinga J.W., Robroeks C.M., van Berkel J.J., Moonen E.J., Godschalk R.W., Jöbsis Q., Dompeling E., Wouters E.F., van Schooten F.J., Volatile organic compounds in exhaled breath as a diagnostic tool for asthma in children. Clin. Exp. Allergy (2010) 40: 68-76.

(148) Misso N.L., Aggarwal S., Phelps S., Beard R., Thompson P.J., Urinary leukotriene E4 and 9 alpha, 11 beta-prostaglandin F concentrations in mild, moderate and severe asthma, and in healthy subjects. Clin. Exp. Allergy (2004) 34: 624-631.

(149) Balgoma D., Larsson J., Rokach J., Lawson J.A., Daham K., Dahlén B., Dahlén S.E., Wheelock C.E., Quantification of lipid mediator compounds in human urine from asthma patients by electrospray ionization mass spectrometry: controlling matrix effects, Anal. Chem. (2013) 85: 7866-7874.

(150) Mattarucchi E., Baraldi E., Guillou C., Metabolomics applied to urine samples in childhood asthma; differentiation between asthma phenotypes and identification of relevant compounds. Biomed. Chromatogr. (2012) 26: 89-94.

(151) Miekisch W., Schubert J.K., Noeldge-Schomburg G.F., Diagnostic potential of breath analysis--focus on volatile organic compounds. Clin. Chim. Acta (2004) 347: 25–39

(152) Marczin N., Yacoub M.H., Disease Markers in Exhaled Breath: Basic Mechanisms and Clinical Applications (2002), IOS Press, Amesterdam, Netherlands

(153) Pauling L., Robinson A.B., Teranishi R., Cary, P., Quantitative analysis of urine vapor and breath by gas-liquid partition chromatography. Proc. Nat. Acad. Sci. USA (1971) 68: 2374–2376

(154) Marczin N., Kharitonov S.A., Yacoub M.H., Barnes P.J., Disease markers in exhaled breath, (2005) 1st Edition, Taylor&Francis, New York, USA

(155) Gilchrist F.J., Razavi C., Webb A.K., Jones A.M., Spaněl P., Smith D., Lenney W., An investigation of suitable bag materials for the collection and storage of breath samples containing hydrogen cyanide. J. Breath Res. (2012) 6: 036004

(156) Thekedar B., Szymczak W., Höllriegl V., Hoeschen C., Oeh U., Investigations on the variability of breath gas sampling using PTR-MS. J. Breath Res. (2009) 3: 027007

(157) Horvath I., de Jongste J.C., European Respiratory Monograph: Exhaled Biomarkers (2010) Chapter 7, European Respiratory Society, Plymouth, UK

(158) Mochalski P., Wzorek B., Sliwka I., Amann A., Suitability of different polymer bags for storage of volatile sulphur compounds relevant to breath analysis. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. (2009) 877: 189-196.

(159) Mochalski, P., King, J., Unterkofler, K., Amann, A., Stability of selected volatile breath constituents in Tedlar, Kynar and Flexfilm sampling bags. Analyst (2013) 138: 1405-1418

(160) Spaněl P., Dryahina K., Rejšková A., Chippendale T.W., Smith D., Breath acetone concentration; biological variability and the influence of diet. Physiol. Meas., (2011) 32: 23-31.

(161) Folesani G., Corradi M., Goldoni M., Manini P., Acampa O., Andreoli R., Bertorelli G., David S., Franchini I., Urea in exhaled breath condensate of uraemics and patients with chronic airway diseases. Acta Biomed. (2008), 79: 79-86.

(162) Waikar S.S., Sabbisetti V.S., Bonventre J.V., Normalization of urinary biomarkers to creatinine during changes in glomerular filtration rate. Kidney Int., (2010) 78: 486-494.
(163) Delanghe J., Speeckaert M., Preanalytical requirements of urinalysis. Biochem. Med. (2014) 24:89-104.
(164) Ryan D., Robards K., Prenzler P.D., Kendall M., Recent and potential developments in the analysis of urine: a review. Anal. Chim. Acta. (2011) 684: 8-20.
(165) Klein M., Preud'Homme H., Bueno M., Pannier F., Study of volatile selenium compounds stability in normal urine: effects of sample handling and storage conditions, J. Anal. At. Spectrom. (2011) 26: 602-607.
(166) Kataoka H., Saito K., Recent advances in SPME techniques in biomedical analysis. J. Pharm. Biomed. Anal. (2011) 54: 926-950.
(167) http://socratic.org/questions/how-does-solid-phase-extraction-differ-from-solvent-extraction (reviewed on 12/07/2015)
(168) Ouyang G., Vuckovic D., Pawliszyn J., Nondestructive Sampling of Living Systems Using in Vivo Solid-Phase Microextraction. Chem. Rev. (2011) 111: 2784-2814.
(169) Arthur C.L., Pawliszyn J., Solid phase microextraction with thermal desorption using fused silica optical fibers. Anal. Chem., (1990) 62: 2145–2148
(170) Grote C., Pawliszyn J., Solid-phase microextraction for the analysis of human breath. Anal. Chem. (1997) 69: 587-596
(171) Lee X.P., Kumazawa T., Sato K., A simple analysis of 5 thinner components in human body fluids by headspace solid-phase microextraction (SPME). Int J Legal Med. (1995) 107: 310-313
(172) Silva C.L., Passos M., Câmara J.S., Solid phase microextraction, mass spectrometry and metabolomic approaches for detection of potential urinary cancer biomarkers--a powerful strategy for breast cancer diagnosis. Talanta (2012) 89: 360-368.
(173) Guadagni R., Miraglia N., Simonelli A., Silvestre A., Lamberti M., Feola D., Acampora A., Sannolo N., Solid-phase microextraction-gas chromatography-mass spectrometry method validation for the determination of endogenous substances: urinary hexanal and heptanal as lung tumor biomarkers. Anal Chim Acta. (2011) 701: 29-36.
(174) Monteleone M., Naccarato A., Sindona G., Tagarelli A., A reliable and simple method for the assay of neuroendocrine tumor markers in human urine by solid-phase microextraction-gas chromatography-triple quadrupole mass spectrometry. Anal. Chim. Acta. (2013) 759: 66-73.
(175) Zimmermann D., Hartmann M., Moyer M.P., Nolte J., Baumbach J.I., Determination of volatile products of human colon cell line metabolism by GC/MS analysis. Metabolomics (2007) 3: 13-17.
(176) Abaffy T., Möller M.G., Riemer D.D., Milikowski C., Defazio R.A., Comparative analysis of volatile metabolomics signals from melanoma and benign skin: a pilot study. Metabolomics (2013) 9: 998-1008.
(177) Ulanowska A, Trawińska E, Sawrycki P, Buszewski B., Chemotherapy control by breath profile with application of SPME-GC/MS method. J. Sep. Sci. (2012) 35: 2908-2913.
(178) Deng C., Zhang J., Yu X., Zhang W., Zhang X., Determination of acetone in human breath by gas chromatography-mass spectrometry and solid-phase microextraction with on-fiber derivatization. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. (2004) 810: 269-275.
(179) Doong R., Chang S., Sun Y., Solid-phase microextraction and headspace solid-phase microextraction for the determination of high molecular-weight polycyclic aromatic hydrocarbons in water and soil samples. J. Chromatogr. Sci., (2000) 38: 528-534.
(180) Pawliszyn J., Solid Phase Microextraction: Theory and Practice (1997) John Wiley & Sons, Inc., New Jersey, USA
(181) Koster E.H.M., Niemeijer I.S., de Jong G.J., Multiple Solid-Phase Microextraction of Drugs from Human Urine, Chromatographia (2002), 55: 69-73.
(182) Pawliszyn J., Handbook of solid phase microextraction (2009), Chemical Industry Press, Beijing, China
(183) Petronilho S., Coimbra M.A., Rocha S.M., A critical review on extraction techniques and gas chromatography based determination of grapevine derived sesquiterpenes. Anal. Chim. Acta (2014) 846: 8-35.
(184) Sneddon J., Masuram S., Richert J.C., Gas chromatography-mass spectrometry-basic principles, instrumentation and selected applications for detection of organic compounds. Analyt. Lett. (2007) 40: 1003-1012.
(185) Theodoridis G., Gika H.G., Wilson I.D., Mass spectrometry-based holistic analytical approaches for compound profiling in systems biology studies. Mass. Spectrom. Rev. (2011) 30: 884-906

(186) Prado C., Marín P., Periago J.F., Application of solid-phase microextraction and gas chromatography–mass spectrometry to the determination of volatile organic compounds in end-exhaled breath samples. J. Chromatogr. A (2003) 1011: 125–134
(187) Dent A.G., Sutedja T.G., Zimmerman P.V., Exhaled breath analysis for lung cancer. J. Thorac. Dis. (2013) 5: S540-S550
(188) Ligor T., Ligor M., Amann A., Ager C., Bachler M., Dzien A., Buszewski B., The analysis of healthy volunteers' exhaled breath by the use of solid-phase microextraction and GC-MS. J. Breath Res. (2008) 2: 046006
(189) Dang N.A., Janssen H.G., Kolk A.H., Rapid diagnosis of TB using GC-MS and chemometrics. Bioanalysis (2013) 5: 3079-3097
(190) Wahl H.G., Hoffmann A., Luft D., Liebich H.M., Analysis of volatile organic compounds in human urine by headspace gas chromatography-mass spectrometry with a multipurpose sampler. J. Chromatogr. A. (1999) 847 :117-125.
(191) Monteiro M., Carvalho M., Henrique R., Jerónimo C., Moreira N., de Lourdes Bastos M., de Pinho P.G., Analysis of volatile human urinary metabolome by solid-phase microextraction in combination with gas chromatography-mass spectrometry for biomarker discovery: application in a pilot study to discriminate patients with renal cell carcinoma. Eur. J. Cancer. (2014) 50: 1993-2002
(192) de Hoffmann, E., Stroobant V., Mass Spectrometry: Principles and Applications (2003) 2nd edition, John Wiley & Sons, Inc., New Jersey, USA
(193) Van Den Dool, H., Kratz, P.D., A generalization of the retention index system including linear temperature programmed gas—liquid partition chromatography J. Chromatography (1963) 11: 463-471.
(194) Mostafa A., Edwards M, Górecki T., Optimization aspects of comprehensive two-dimensional gas chromatography. J. Chromatogr. A (2012) 1255: 38-55.
(195) Chin S-T., Marriott P.J., Multidimensional gas chromatography beyond simple volatiles separation. Chem. Commun., (2014) 50: 8819-8833
(196) Liu Z., Phillips J.B., Comprehensive Two-Dimensional Gas Chromatography using an On-Column Thermal Modulator Interface. J. Chromatogr. Sci., (1991) 29: 227-231.
(197) Otles S., Handbook of Food Analysis Instruments (2009), CRC Press, Taylor&Francis Group, Boca Raton, Florida, USA
(198) Welke J.E., Zini C.A., Comprehensive Two-Dimensional Gas Chromatography for Analysis of Volatile Compounds in Foods and Beverages. J. Braz. Chem. Soc. (2011) 22: 609-622
(199) Cortes H.J., Winniford B., Luong J., Pursch M., Comprehensive two dimensional gas chromatography review. J. Sep. Sci. (2009) 32: 883-904.
(200) Bertsch W., Two-dimensional gas chromatography. Concepts, instrumentation, and applications - Part 1: Fundamentals, conventional two-dimensional gas chromatography, selected applications. J. High Resolut. Chromatogr. (1999) 22: 657-665.
(201) Cordero C., Rubiolo P., Sgorbini B., Galli M., Bicchi C., Comprehensive two-dimensional gas chromatography in the analysis of volatile samples of natural origin: a multidisciplinary approach to evaluate the influence of second dimension column coated with mixed stationary phases on system orthogonality. J. Chromatogr. A. (2006) 1132: 268-279.
(202) Dallüge J., Beens J., Brinkmann U.A.Th., C omprehensive two-dimensional gas chromatography: a powerful and versatile analytical tool., J. Chromatogr. A (2003) 1000:69-108.
(203) Ryan D., Morrison P., Marriott P., Orthogonality considerations in comprehensive two-dimensional gas chromatography. J. Chromatogr. A (2005) 1071: 47-53.
(204) Korytár P., Haglund P., de Boer J., Brinkamn U.A.Th., Comprehensive two-dimensional gas chromatography for the analysis of organohalogenated micro-contaminants. Trends Anal. Chem.(2006) 25: 373-396.
(205) Dallüge J., Vreuls R.J.J., Beens J., Brinkamn U.A.Th., Optimization and characterization of comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC×GC–TOF MS). J. Sep. Sci. (2002) 25: 201-214.
(206) Guilhaus M., Principles and instrumentation in time-of-flight mass spectrometry. J. Mass Spec. (1995) 30: 1519-1532.
(207) Grob R.L., Barry E.B., Modern Practice of Gas Chromatography (2004), 4th Edition, John Wiley & Sons, Inc., New Jersey, USA
(208) Zrostlikova J., Binkley J., Wojtowicz P., Adam T., Application of Comprehensive Two-Dimensional Gas Chromatography with Time-of-Flight Mass Spectrometry in Human Metabolomic Studies. J. Anal. Chem. (2011) 66: 1333-1340.

(209) Phillips M., Cataneo R.N., Chaturvedi A., Kaplan P.D., Libardoni M., Mundada M., Patel U., Zhang X., Detection of an Extended Human Volatome with Comprehensive Two-Dimensional Gas Chromatography Time-of-Flight Mass Spectrometry. Plos One (2013) 8: e75274

(210) Das M.K., Bishwal S.C., Das A., Dabral D., Varshney A., Badireddy V.K., Nanda R., Investigation of Gender-Specific Exhaled Breath Volatome in Humans by GCxGC-TOF-MS. Anal. Chem., (2014) 86: 1229-1237

(211) Kouremenos K.A., Pitt J., Marriott P.J., Metabolic profiling of infant urine using comprehensive two-dimensional gas chromatography: Application to the diagnosis of organic acidurias and biomarker discovery. J. Chromatogr. A (2010) 1217: 104-111.

(212) Pasikanti K.K., Esuvaranathan K., Hong Y., Ho P.C., Mahendran R., Mani L.R.N., Chiong E., Chan E.C.Y., Urinary metabotyping of bladder cancer using two-dimensional gas chromatography time-of-flight mass spectrometry. J. Proteome Res. (2013) 12: 3865–3873

(213) Mitrevski B.S., Wilairat P., Marriott P.J., Comprehensive two-dimensional gas chromatography improves separation and identification of anabolic agents in doping control. J. Chromatogr. A. (2010) 1217: 127-135.

(214) Beckonert O., Keun H.C., Ebbels T.M., Bundy J., Holmes E., Lindon J.C., Nicholson J.K., Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. Nat. Protoc. (2007) 2: 2692-2703.

(215) Pimenta L.P., Kim H.K., Verpoorte R., Choi Y.H., NMR-based metabolomics: a probe to utilize biodiversity. Methods Mol Biol. (2013) 1055: 117-27.

(216)Bertini I., McGreevy K.S., Parigi G., NMR of biomolecules (2012). Wiley-VHC Verlag&Co, Weinheim, Germany

(217) Zaragozá P., Ruiz-Cerdá J.L., Quintás G., Gil S., Costero A.M., León Z., Vivancos J-L., Martínez-Máñez R., Towards the potential use of H-1 NMR spectroscopy in urine samples for prostate cancer detection. Analyst (2014) 139: 3875-3878

(218) Rocha C.M., Barros A.S., Goodfellow B.J., Carreira I.M., Gomes A., Sousa V., Bernardo J., Carvalho L., Gil A.M., Duarte I.F., NMR metabolomics of human lung tumours reveals distinct metabolic signatures for adenocarcinoma and squamous cell carcinoma. Carcinogenesis (2014) doi: 10.1093/carcin/bgu226

(219) Moltu S.J., Sachse D., Blakstad E.W., Strømmen K., Nakstad B., Almaas A.N., Westerberg A.C., Rønnestad A., Brække K., Veierød M.B., Iversen P.O., Rise F., Berg J.P., Drevon C.A., Urinary Compound Profiles in Premature Infants Show Early Postnatal Metabolic Adaptation and Maturation. Nutrients (2014) 6: 1913-1930.

(220) Diaz S.O., Pinto J., Graça G., Duarte I.F., Barros A.S., Galhano E., Pita C., Almeida M. do C., Goodfellow B.J., Carreira I.M., Gil A.M., Metabolic biomarkers of prenatal disorders: an exploratory NMR metabonomics study of second trimester maternal urine and blood plasma. J. Proteome Res. (2011) 10: 3732-42.

(221) Heather L.C., Wang X., West J.A., Griffin J.L., A practical guide to metabolomic profiling as a discovery tool for human heart disease. J. Mol. Cell. Cardiol. (2013) 55: 2-11.

(222) Snowden S., Dahlén S.E., Wheelock C.E., Application of metabolomics approaches to the study of respiratory diseases. Bioanalysis. (2012) 4: 2265-90.

(223) Xu X.H., Huang Y., Wang G., Chen S.D., Metabolomics: a novel approach to identify potential diagnostic biomarkers and pathogenesis in Alzheimer's disease. Neurosci. Bull. (2012) 28: 641-8

(224) Weiss R.H., Kim K., Metabolomics in the study of kidney diseases. Nat. Rev. Nephrol.(2011) 8: 22-33.

(225) Worley B., Powers R., Multivariate Analysis in Metabolomics. Curr. Metabolomics (2013) 1: 92-107.

(226) Goodacre, R., Broadhurst, D., Smilde, A.K., Kristal, B.S., Baker, J.D., Beger, R., Bessant, C., Connor, S., Calmani, G., Craig, A., Ebbels, T., Kell, D.B., Manetti, C., Newton, J., Paternostro, G., Somorjai, R., Sjostrom, M., Trygg, J., Wulfert, F., Proposed minimum reporting standards for data analysis in metabolomics. Metabolomics (2007) 3: 231-241.

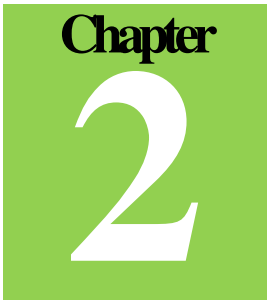
(227) Craig A., Cloarec O., Holmes E., Nicholson J.K., Lindon J.C., Scaling and Normalization Effects in NMR Spectroscopic Metabonomic Data Sets. Anal. Chem. (2006) 78: 2262-2267.

(228) Kohl S., Klein M., Hochrein J., Oefner P., Spang R., Gronwald W., State-of-the art data normalization methods improve NMR-based metabolomic analysis. Metabolomics (2012) 8: 146-160

(229) Wiklund S., Multivariate Data Analysis for Omics (2008) Umetrics, Umeå, Sweden

(230) Boccard J., Veuthey J.L., Rudaz S., Knowledge discovery in metabolomics: An overview of MS data handling. J. Sep. Sci. (2010) 33: 290-304.

(231) Trygg J., Gullberg J., Johansson A.I., Jonsson P., Moritz T., Chemometrics in Metabolomics – An Introduction. Plant Metabolomics (2006) 57: 117-128.
(232) Jonsson, P., Bruce, S. J., Moritz, T., Trygg, J., Sjoestroem, M., Plumb, R., Granger, J., Maibaum, E.,Nicholson, J. K., Holmes, E., Antti, H., Extraction, interpretation and validation of information for comparing samples in metabolic LC/MS data sets.Analyst (2005) 130: 701-707.
(233) Haenlein M., A Beginner's Guide to Partial Least Squares Analysis. Understanding statistics (2004) 3: 283-297.
(234) Barker M., Rayens W., Partial least squares for discrimination. J. Chemometrics (2003) 17: 166-173.
(235) Szymańska E., Saccenti E., Smilde A.K., Westerhuis J.A., Double-check: validation of diagnostic statistics for PLS-DA models in metabolomics studies., Metabolomics (2012) 8: 3-16.
(236) Sahu A., Dalal K., Naglot S., Aggarwal P., Krishna C.M., Serum Based Diagnosis of Asthma Using Raman Spectroscopy: An Early Phase Pilot Study. Plos One (2013) 8: e78921.
(237) Xu X., Liang F., Asymptotic minimax risk of predictive density estimation for non-parametric regression. Bernoulli (2010), 16: 543-560.
(238) Picard R., Cook D., Cross-Validation of Regression Models. J. Amer. Statist. Assoc. (1984) 79: 575-583.
(239) Xu Q.-S., Liang Y.-Z, Monte Carlo cross validation, Chemometr. Intell. Lab. (2001) 56:1-11.
(240) Dubitzky W., Granzow M., Berrar D.P., Fundamentals of Data Mining in Genomics and Proteomics (2007), Springer Verlag Berlin, Heidelberg, Germany
(241) Molinaro,A., Simon,R., Pfeiffer,R. M. Prediction error estimation: a comparison of resampling methods, (2005) Bioinformatics,21: 3301–3307.



CHAPTER 2

EXHALED BREATH AS A SOURCE OF INFORMATION ON ASTHMA

2.1 Overview

The use of odor, namely in breath, remounts to ancient Greece where physicians related odor of a patient's breath and a pathology (e.g., sweet acetonic breath indicating uncontrolled diabetes). Over the past years several reports in exhaled breath have surfaced in the analysis of an array of pathologies that inclusively led to the development of specific equipment for exhaled breath analysis. Exhaled breath is a rich source of information with several compounds from different chemical families being identified. The focus of our study in exhaled breath was ultimately develop a methodology able to differentiate asthmatic children from healthy ones using the exhaled volatile organic compounds or "breath-print" yielding insights into this heterogeneous pathology.

To acomplish this goal a HS-SPME/GC-MS method was developed. First an experimental design was built to select the best conditions for HS-SPME using chemical standards previously reported in exhaled breath, followed by the study of parameters related to breath sampling (containers, cleaning procedures and intra-individual variability). The optimized methodology was then sucessfully applied to a set of 35 asthmatic and 15 healthy children and the first results showed that compounds such as the alkanes/aldehydes (linked to lipid peroxidation) characterized the pathology. To improve the obtained results a high throughput analytical technique, GC×GC–ToFMS was considered in a different set of 32 asthmatic and 27 healthy children. Several hundreds compounds were identified and the model had high classification rate, sensitivity and specificity. A model with a set of 9 compounds was built and discriminated asthmatic from healthy children. The role that EB may have in non-invasive diagnostic method and in monitoring the asthmatic therapy was then accessed by applying the developed method on a new cohort of 49 asthmatic children. The developed models were then tested to demonstrate the true value in the clinical practice in following up the evolution of three children in exarcebation with the prescribed therapy (during 3 months), as well, the follow the Omalizumab therapy effect on one patient with severe asthma throughout 20 months.

2.2 Profiling allergic asthma volatile metabolic patterns using a headspacesolid phase microextraction/gas chromatography based methodology

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Profiling allergic asthma volatile metabolic patterns using a headspace-solid phase microextraction/gas chromatography based methodology

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2.2.1 Abstract

Allergic asthma represents an important public health issue with significant growth over the years, especially in the paediatric population. Exhaled breath is a non-invasive, easily performed and rapid method for obtaining samples from the lower respiratory tract. In the present manuscript, the metabolic volatile profiles of allergic asthma and healthy children were evaluated by HS-SPME/GC–qMS. The lack of studies in breath of allergic asthmatic children by HS-SPME led to the development of an experimental design to optimize SPME parameters. To fulfill this objective, three important HS-SPME experimental parameters that influence the extraction efficiency, namely fibre coating, temperature and time extractions were considered. The selected conditions that promoted higher extraction efficiency corresponding to the higher GC peak areas and number of compounds were: DVB/CAR/PDMS coating fibre, 22ºC and 60 min as the extraction temperature and time, respectively. The suitability of two containers, 1 L Tedlar® bags and BIOVOC®, for breath collection and intra-individual variability were also investigated. The developed methodology was then applied to the analysis of children exhaled breath with allergic asthma (35), from which 13 had also allergic rhinitis, and healthy children (15), allowing to identify 44 volatiles distributed over the chemical families of alkanes (linear and ramified) ketones, aromatic hydrocarbons, aldehydes, acids, among others. Multivariate studies were performed by PLS–DA using a set of 28 selected compounds and discrimination between allergic asthma and healthy children was attained with a classification rate of 88%. The allergic asthma paediatric population was characterized mainly by the compounds linked to oxidative stress, such as alkanes and aldehydes. Furthermore, more detailed information was achieved combining the volatile metabolic data, suggested by PLS–DA model, and clinical data.

2.2.2 Framework

As molecular diagnosis is the next generation of personalized medicine[1] an objective of the purposed work is to contribute for the interdisciplinary link between physicians and biochemists, providing tools for a more efficient and precise diagnosis. Exhaled breath is a rich source, as found out by Pauling in the 1970s, with the identification of 250 metabolites beside nitrogen, oxygen and water vapour[2]. The development of new and more efficient extraction methods, as well as, more sensitive and efficient separation techniques led to a significant progress in this research area. The analysis and characterization of exhaled breath became a new approach with potential to provide valuable information about respiratory and systemic diseases that can lead to a deeper knowledge of human health status, in the identification of disease-related marker compounds and consequently a potential information source regarding the knowledge of these diseases metabolic pathways. Exhaled breath is a non-invasive, easily performed and rapid sampling, either in the gaseous or condensed state.

The lack of a prominent HS-SPME study in allergic asthma exhaled breath led us to develop an experimental design by combining HS-SPME with GC-MS to assess information from allergic asthma metabolomic patterns. To fulfil these objectives three important HS-SPME experimental parameters that influence the extraction efficiency, namely extraction temperature and time, and coating fibre were considered. A full factorial design was applied to determine the best extraction conditions using different chemical standards (hydrocarbons, aldehydes, ketones, aromatic and aliphatic alcohols) reported in literature to be present in exhaled breath[3,4,5]. Some exhaled breath sampling parameters were also optimized. Two different breath sampling containers were tested, Tedlar® gas sampling bags and BIOVOCS® and the washing procedure of the chosen container was optimized. Another important parameter, the intra-individual variability, was checked over a period of three weeks with sampling throughout a single day. After this optimization, the proposed methodology was applied to the analysis of children exhaled breath with allergic asthma and healthy children, used as control.

2.2.3 Experimental

Standards and materials

The reagents used were of analytical grade and from different chemical families: linear hydrocarbons (hexane (99.5%, Fluka, Madrid, Spain), undecane (99.8%, Fluka, Madrid, Spain)), aldehydes (hexanal (98%, Sigma-Aldrich, Madrid, Spain), (*E)*-2-nonenal (95%, Acros Organics, Geel Belgium)), ketones (acetone (99.5%, Fluka, Madrid, Spain), 3-heptanone (97%, Sigma-Aldrich, Madrid, Spain)), and aromatic and aliphatic alcohols (phenol (99%, Merck, New Jersey, USA), 1-hexanol (98%, Sigma-Aldrich, Madrid, Spain)). Absolute ethanol was supplied by Panreac (99.5 %, analytical grade, Barcelone, Spain). Ultra pure water was obtained from a Milli-Q system from Millipore (Milford, MA, USA). Four different fibres from Supelco (Bellefonte, PA, USA) were used: divinylbenzene/carboxen/polydimetylsiloxane (DVB/CAR/PDMS, 50/30 μm), polydimetylsiloxane/divinylbenzene (PDMS/DVB, 65 μm), polydimetylsiloxane (PDMS, 100 μm) and polyacrilate (PA, 85 μm). SPME fibres and SPME manual holder was obtained from Supelco (Bellenfonte, PA, USA). All the fibres were of the same length (1 cm) and conditioned prior to use, as recommended by the manufacturer. 1 L Tedlar® bags (SKC Inc., Eighty Four, PA, USA) and BIOVOCS® (Markes International, Llanstrisant, UK) were used for sampling.

Breath samples

A group of 35 children with allergic asthma, from which 13 had allergic rhinitis, and 15 healthy children volunteered for this study. All parents signed an informed consent for participation in the study. The children with allergic asthma were recruited from the outpatient clinic of the department of paediatric immunoalergology of Hospital D. Pedro (Aveiro, Portugal) whilst the healthy children were recruited at the local facilities that presented no asthma episodes or symptoms. Asthma diagnosis was made based on clinical symptoms and exams (skin prick tests and IgE values). Appropriate therapy was prescribed by the patient's own physician. All allergic asthma population represented a controlled asthma status. The characteristics of the patients and healthy children are presented in Table 2.1. No restrictions were applied regarding drugs or diet and all allergic asthma subjects were sampled in the same room at the local hospital. The study was approved by the local hospital ethics committee.

Table 2.1 Characteristics of the studied population: allergic asthma and healthy children.

	Allergic asthma (*n*=35)	Healthy (*n*=15)
Age in years (mean±SD) / (range)	8.5±2.5 / 4-13	8.0±3.1 / 4-13
Gender (male/female)	13/22	7/8
Pathology		
Allergic Asthma (AA)	22 (63%)	-
Allergic Asthma + Allergic Rhinitis (AA+AR)	13 (37%)	-
Allergens[a]		
Dust mite	19 (54%)	-
Dust mite + cat fur	3 (9%)	-
Dust mite + gramineae	3 (9%)	-
Dust mite + cat fur + gramineae	1 (3%)	-
Information not available	9 (25%)	-
Therapy		
Corticosteroid + leukotriene receptor antagonist	12 (34%)	-
Corticosteroid and anti-histamines	4 (12%)	-
Corticosteroid and bronchodilator	13 (37%)	-
No therapy	6 (17%)	-

[a] Results obtained by prick-tests

Experimental design

Different chemical standards were used to perform the full factorial design,: hexane, acetone, hexanal, 3-heptanone, undecane, 1-hexanol, (*E*)-2-nonenal and phenol. A stock solution of each standard (1 g/L) was prepared in absolute ethanol and made up to volume, and from this a solution of 100 mg/L was set up. A working solution was prepared to yield different concentrations from 0.4 mg/L (undecane) to 1.6 mg/L (hexanal). To reproduce a two-phase system (headspace and coating fibre), as in breath analysis, 50 µL was added to a 120 mL SPME flask and sealed with an aluminium crimp cap with a vial was capped with a PTFE septum (Chromacol, Hertfordshire, UK) and concentrations ranged from 166.7 to 670.0 ng/L. Three important SPME experimental parameters were considered on this study: coating fibre, extraction time and temperature. Four different fibres, DVB/CAR/PDMS, PDMS/DVB, PDMS and PA, were tested and compared. Before daily analysis each fibre was conditioned for 15 min at 250 ºC. Two different temperatures (5 and 22 ºC) and five different extraction times (5, 15, 30, 45 and 60 min) were evaluated.

Breath analysis

Breath sampling containers: The suitability of two breath sampling containers was tested and from the wide range available commercially Tedlar® gas sampling bags and

BIOVOC® were chosen. All tests were made using the exhaled breath of a child with allergic asthma and using the method developed in the experimental design.

Tedlar® gas sampling bags were chosen and due to impurities, derived from the manufacturing process, the washing procedure was verified as the manufacturer suggests. Prior to this step, the purity of the compressed air/nitrogen available in the laboratory was verified and the main conclusion is that the used gases are adequate for this procedure as there is no further contamination by these washing agents (data not shown). The manufacturer recommends that before using Tedlar® bags three nitrogen/compressed air flushes should suffice, however the comparison was made between no flush, 3 and 10 flushes.

Breath sampling: Exhaled breath was collected in 1 L Tedlar® bags with parallel ambient air extraction. Breath samples were obtained while children waited for consultation. Children were asked to cleanse their mouth with water before sampling. Subsequently, children were instructed to inhale and exhaled normally and then exhale deeply into the Tedlar® bag previously holding their breath for 5 seconds. The collection method was successfully done by all volunteers. Each subject provided one sample using a disposable mouthpiece. Before collecting exhaled breath, all bags were thoroughly cleaned to remove residual contaminants by flushing with high purity nitrogen gas allowing the re-use of the Tedlar® bags. The bags were transported to the laboratory and the analysis was performed to a maximum of six hours as recommended by Mochalski *et al.*[6]. On average, the analysis was performed two to three hours after sampling. The bags were storage at 22 °C. Parallel room air sampling was also performed using a large volume syringe to fill a Tedlar bag with room air.

Intra-individual variability evaluation: There have been reports on the intra-individual variation of exhaled breath over a period of time[7]. This is an important parameter to be assessed for the developed methodology to verify if the individual profile is repeatable or consistent over time. Hence, the exhaled breath of a child with allergic asthma was collected over a period of three consecutive weeks, six replicates were performed and analysed in the same day. All sampling was performed in the same room.

HS-SPME general procedure: Breath analysis was performed with the optimized conditions using the DVB/CAR/PDMS fibre in a 22 ºC termostated room for 60 min. Following the extraction procedure, the SPME fibre was retracted from the Tedlar® bag and

inserted in the GC system injection port for 5 min where the compounds were thermally desorbed and transferred to the GC column.

GC-MS analysis

The SPME coating fibre containing the volatile compounds from the standard working solution and exhaled breath was manually introduced into the GC injection port at 250 ºC and kept for 5 min for desorption. The injection port was lined with a 0.75 mm I.D. glass liner. The desorbed volatile compounds were analyzed in an Agilent Technologies 6890N Network gas chromatograph, equipped with a 60m×0.32mm I.D., 0.25 μm film thickness DB-FFAP fused silica capillary column (J&W Scientific, Folsom, CA, USA), connected to an Agilent 5973 quadrupole mass selective detector. Splitless injections were used (5 min). The oven temperature program was set initially to 50 ºC, with a temperature increase of 5 ºC/min to 220 ºC held (6 min). Helium carrier gas had a flow rate of 1.7 mL/min and the column head pressure was 12 psi. The mass spectrometer was operated in the electron impact mode (EI) at 70 eV scanning the range 33–300 *m/z* in a 4.76-s cycle, in a full scan acquisition mode. The identification of the chromatogram peaks was done comparing all mass spectra with the library data system of the GC–MS equipment (NIST 05 MS Library). The spectra were also compared with spectra found in the literature. The identification of each volatile compound was confirmed by comparing its mass spectrum and retention time with those of the pure standard compounds, when available. The GC peak area data were used as an indirect approach to estimate the relative content of each volatile compound. All measurements concerning the chemical standards were made with three replicates and the reproducibility was expressed as Relative Standard Deviation (R.S.D., %). Each breath represents a single sample, and was analysed once. To verify the absence of any carry over, blanks, corresponding to the analysis of the coating fibre not submitted to any extraction procedure, were run between sets of three analyses.

Statistical analysis

Single factor ANOVA was performed in the evaluation of the intra-individual variability over a period of three weeks. A critical value of 0.05 was used as a criterion of significance and all calculations were performed using the Excel® software (Microsoft). PLS is a very important procedure for both regression and classification purposes. Concerning the classification application of PLS, known as PLS-DA[8], the most common approach is to use a

Y matrix containing dummy variables which defines sample memberships to pre-defined groups and allow extracting relevant information/variability that could describe the reasons for the observed patterns (clusters). This methodology allows one to understand which variables (metabolites) contribute the most for the observed separation. The PLS-DA was applied to volatile metabolites compositional data (28) identified by HS-SPME/GC-qMS present in all the cases (50) and for classification purposes were used two groups (healthy and asthma). The classification model complexity (number of latent variables) and classification rate were estimated by internal cross-validation (7 blocks splits).

2.2.4 Results and discussion

Experimental design: HS-SPME optimization

The first stage of this study addressed the HS-SPME parameters optimization that influences the extraction process. Different chemical standards reported in literature[3,9] to be present in exhaled breath were used: hexane, acetone, hexanal, 3-heptanone, undecane, 1-hexanol, (E)-2-nonenal and phenol. Experimental design allows estimating the effect of several variables simultaneously and the complete factorial design enabled the evaluation of three significant factors that greatly influences the vapour pressure and equilibrium of the target compounds in the headspace, therefore affecting the extraction efficiency: fibre coating (DVB/CAR/PDMS, PDMS/DVB, PDMS and PA), temperature (5 and 22ºC) and extraction time (5, 15, 30, 45 and 60min) with a total of 120 runs being performed. The obtained results can be seen in Figure 1, in which each bubble represents the total chromatographic area of the eight standards under study inherent to three different variables (extraction temperature, extraction time and SPME fibre coating). Through the bubble illustration, showed in Figure 2.1, it is easy to evaluate the overall extraction efficiency, since a larger bubble represents a higher total chromatographic area.

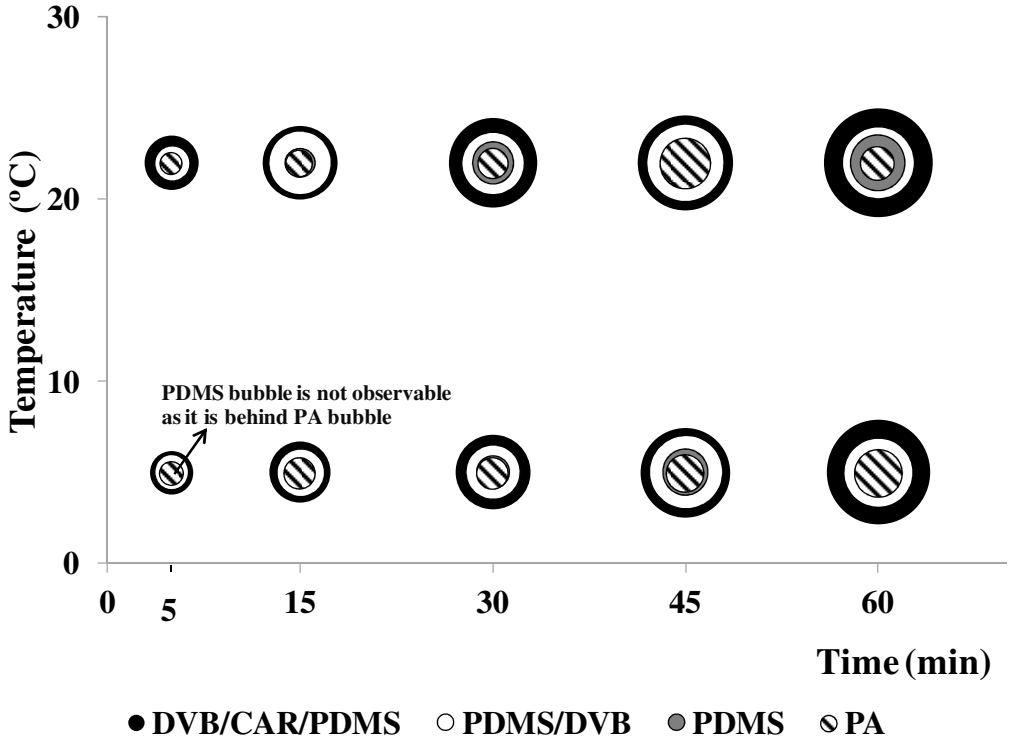

Figure 2.1 - Full factorial design of solid-phase microextraction conditions for GC-MS analysis of exhaled breath.

The comparison was made in terms of total extraction efficiency and reproducibility. The highest extraction efficiency was obtained using the DVB/CAR/PDMS fibre in all studied extraction times and temperatures while PA (22ºC) and PDMS (5ºC) showed the lowest sorptive capacity. The nature of the analytes influences the SPME fibre selection. Previous reports[10,11] on exhaled breath analysis used PDMS because these studies were focused in alkanes and aromatic hydrocarbons. These compounds are non-polar, so it is preferable a PDMS phase. The methodology optimized in the present manuscript is to be used in a wide range of analytes, which explains that DVB/CAR/PDMS fibre obtained the best results, as its stationary phase has a synergistic effect between adsorption and absorption. The mutually synergetic effect of adsorption and absorption of the stationary phase promotes a high retention capacity and, consequently, a higher sensitivity than fibres based on absorption only. Therefore, as DVB/CAR/PDMS presents a wide range capacity of sorbing compounds and it is recommended by the producers in the analysis of compounds with different physicochemical properties within a molecular weight ranging from 40 to 275.

The extraction time was studied by increasing the fibre exposure time from 5 to 60 min. Higher extraction time promoted higher extraction efficiency for all the fibres under

study. Sorption time for the selected standards indicated an extraction time of 60 min. Although this is a long extraction time, maximum sensitivity is desirable, at this particular stage, so that the knowledge of exhaled breath is maximized.

The SPME process is also influenced by temperature. Higher extraction temperature usually increases the analytes release, therefore increasing their concentration in the headspace, due to the enhanced mass transfer (kinetics). Two temperatures were studied: 5ºC and 22ºC (room air temperature). Higher temperatures were avoided because heating these containers could possibly release contaminants or produce artifacts to the headspace, therefore influencing the results. For all fibres and extraction times under study, 22ºC allowed higher extraction efficiency.

The extraction at 22ºC for 60min with the SPME coating fibre DVB/CAR/PDMS was selected for further breath extractions. For the selected conditions, RSD was considered acceptable (10%).

Breath analysis

Beside the HS-SPME optimization for breath analysis there are several factors that have to be considered and optimized. Among the factors, we optimized the type of breath sampling containers used, the cleaning procedure adopted to such containers as well as an important parameter such as intra-individual variability. After these optimization steps, the developed methodology was applied to a set of 50 children (35 with allergic asthma, among these 13 had also allergic rhinitis and 15 healthy children).

Breath sampling containers

There are a wide range of methods for sampling exhaled breath, which can include canisters, cold trapping, adsorbing agents, BIOVOC®, Teflon and Tedlar® gas sampling bags. These containers should be easy to operate, easy to handle and be able to store a sample for a prolonged period of time (in breath sampling the storage time is a major drawback). Cost, re-usability, durability, size and versatility are other important issues to take in consideration when choosing the adequate container. As a result, Tedlar® gas sampling bags and BIOVOC® were chosen for further studies.

The suitability of two sampling containers Tedlar® gas sampling bags and BIOVOC® was investigated using the exhaled breath of a child with allergic asthma with the previously

developed HS-SPME methodology. Taking into consideration the obtained results (Figure 2.2), Tedlar® bags are the best choice for sampling exhaled breath.

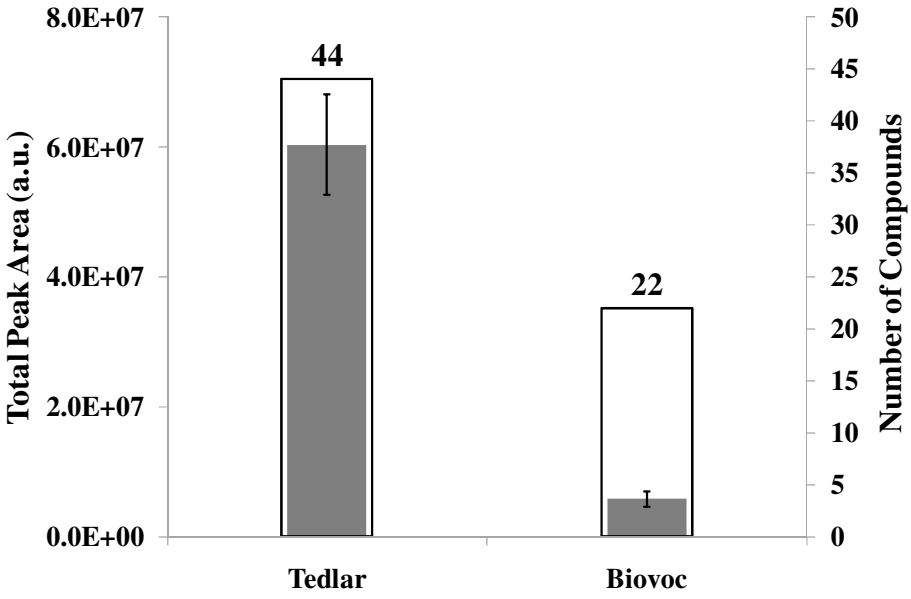

Figure 2.2 - Total peak area and number of compounds comparison between two breath collection containers (Tedlar® gas sampling bags and BIOVOC®). a.u. – arbitrary units.

There was a great difference between both containers with the identification of forty-four compounds for samples stored in Tedlar® bags, while for samples stored in BIOVOC® twenty-two were identified In terms of total peak area, the difference between BIOVOC® and Tedlar® bag was considerable as BIOVOC® total peak area only represented 10% of the obtained by using the Tedlar® bag. For follow-up procedures, the Tedlar® bag was chosen.

Tedlar® bags are frequently used to collect exhaled breath[12] as it fulfils the requisites for sampling and storing this type of samples. Nevertheless, Tedlar® bags have two main disadvantages: (1) impurities derived from the manufacturing process and (2) sample leakage due to adsorption or diffusion through the walls. The manufacturer recommends flushing the bag three times with compressed air or nitrogen before use so that the contaminants are eliminated. The effectiveness of this routine on Tedlar® bags was evaluated from no flush, flushing three and ten times using the previously HS-SPME/GC-MS developed methodology. Blank runs were made, filling the Tedlar® gas sampling bags with compressed air and several pollutants, a total of sixteen compounds, were identified (Table 2.2).

Table 2.2 - Volatiles identified in Tedlar bags and cleansing procedure effects with rising number of nitrogen flushes

Retention time (min.)	Compounds	CAS number	Volatiles identified in Tedlar bags		
			No nitrogen flushes	3 nitrogen flushes	10 nitrogen flushes
Alkanes					
6.34	hexane*	110-54-3	x	x	x
7.20	2,4-dimethyl-heptane	2213-23-2	x	n.d.	n.d.
7.65	4-methyl-octane	2216-34-4	x	n.d.	n.d.
11.40	undecane*	1120-21-4	x	n.d.	n.d.
Carbonyl Compounds					
7.39	acetone*	67-64-1	x	x	n.d.
26.48	benzaldehyde*	100-52-7	x	x	x
21.10	acetic acid*	64-19-7	x	x	x
Aromatic Compounds					
33.77	phenol*	108-95-2	x	x	x
13.56	dMethEthBenz[a]	1075-38-3	x	x	x
14.32	m/z 105, 122, 91	-	x	x	x
Miscellaneous					
8.57	dichloromethane*	75-09-2	x	x	x
10.20	α-pinene*	80-56-8	x	x	x
10.10	chloroform*	67-66-3	x	x	n.d.
25.23	(N,N-dimethyl)-acetamide	127-19-5	x	x	x
35.18	diethyl phthalate	84-66-2	x	x	x
36.71	phthalate isobutyrate	103-28-6	x	x	x

n.d. – not detected
*Compounds confirmed by chemical standards
[a]1-(1,1-dimethylethyl)-3-methyl-benzene – dMethEthBenz

Increasing the number of nitrogen flushes produces a positive result as the number of compounds diminishes. Nevertheless, a more detailed overview of these results is demonstrated taking into consideration two contaminants reported in literature[12], (N,N-dimethyl)acetamide and phenol. There is a significant reduction of the total peak areas with the increase of number of flushes. For (*N,N*-dimethyl)acetamide, the total peak area diminished 74% and 94% whereas for phenol the reductions were of 57% and 99% with three and ten flushes respectively, relatively to the initial composition (no flush) (Figure 2.3). For further analysis, Tedlar® bags were vacuumed and flushed ten times with nitrogen. With these results a standard cleaning protocol for the Tedlar® bags was established for the present study.

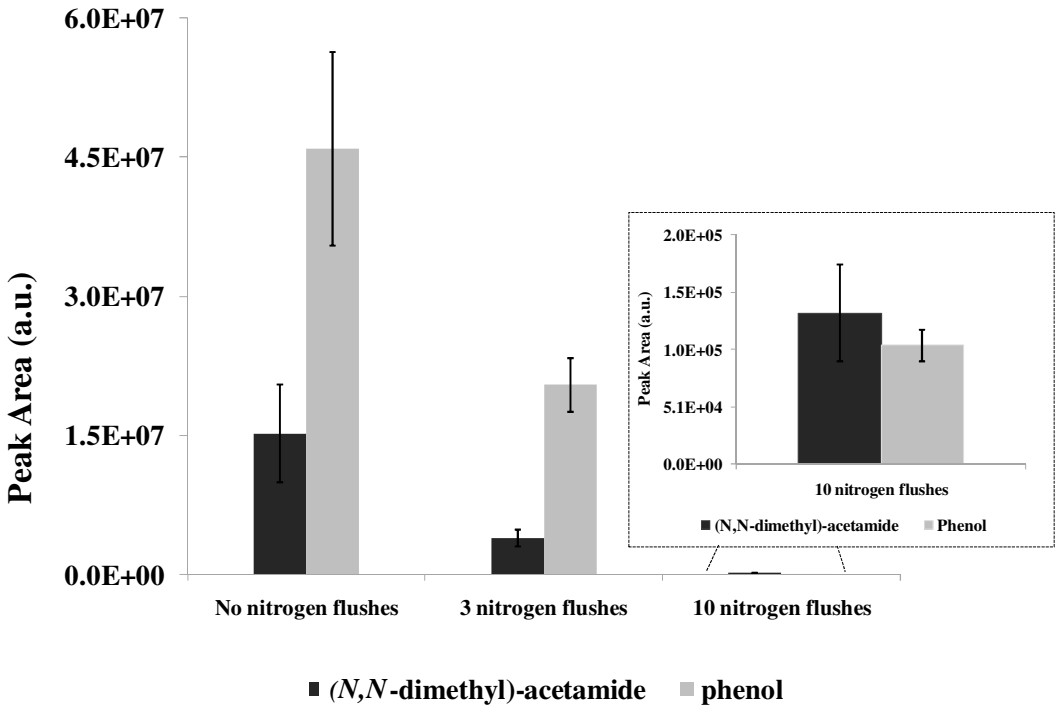

Figure 2.3 - Cleaning procedure effects with rising number of nitrogen flushes for two reported contaminants ((N,N-dimethyl)-acetamide and phenol) in Tedlar bags. a.u. – arbitrary units

Intra-individual variability evaluation

The intra-individual variability was examined, comparing the data per subject from day to day, and throughout each day, by repeating breath sampling for the same child with allergic asthma over a period of 3 weeks with six daily collections (n= 18). This is an important factor in the evaluation of the developed methodology to determine whether a breath sample is dependent on the period of time that it is collected. Figure 2.4 summarizes the results of six selected compounds that represent linear and ramified alkanes and aldehydes.

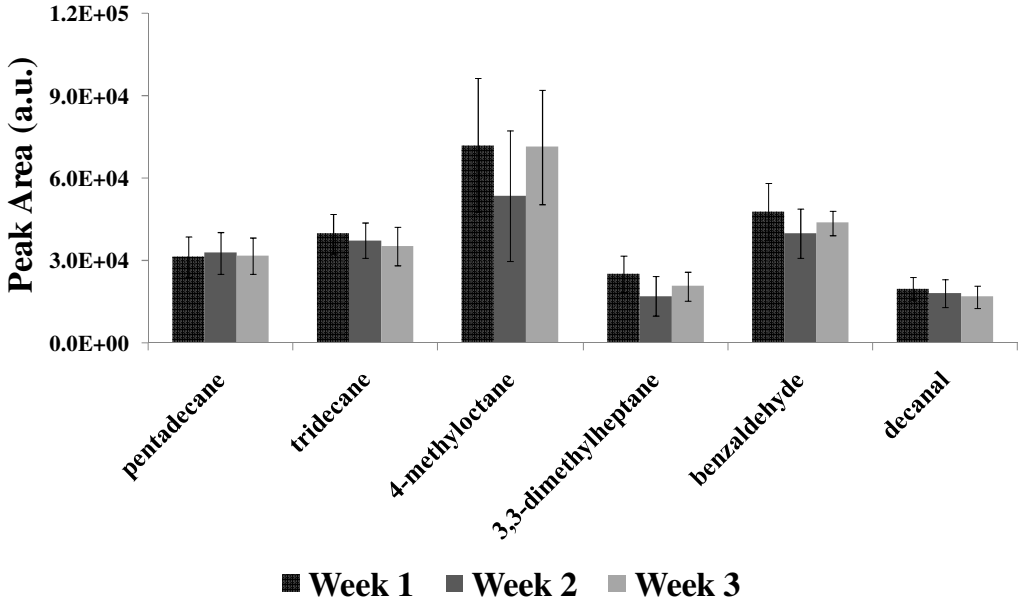

Figure 2.4 - Evaluation of the intra-individual variability over a period of three weeks for selected group of six compounds. – arbitrary units

Single factor ANOVA was performed to evaluate data variability and there were no significant differences between days, and throughout each day, represented by weeks 1, 2 and 3 ($p < 0.05$). However, accordingly to Figure 2.4, there are intra-individual variations between samples over different days of the three weeks as well as in the same day that can be explained by several factors, as for example the circadian rhythm, and diet (sampling were performed throughout the day, before and after meals with different daily diets). It was confirmed that the child asthma status was maintained during this experiment with no occurrence of an asthma crisis. This study was performed with a single subject, and the results obtained are similar to those reported in previous study that has used a larger number of subjects[13].

Exhaled breath analysis

The developed HS/SPME-GC-MS methodology was applied to exhaled children breath samples. Forty-four volatile compounds were identified belonging to different chemical families such as alkanes (linear and ramified), ketones, aromatic hydrocarbons, aldehydes, acids, among others (Table 2.3). The predominant group identified in exhaled breath were the alkanes (26 compounds). The identified compounds are also reported in other studies performed using exhaled breath3[,14, 15].

Table 2.3 - Identified compounds in exhaled breath in both children with allergic asthma and healthy children and obtained from ambient air parallel sampling

Peak Number	R.T. (min.)	Compounds	CAS number	m/z[x]	Breath	Ambient air
		Hydrocarbons				
		Alkanes				
1	6.34	hexane*	110-54-3	**57**[a], 43,41	x	x
3	7.20	2,4-dimethylheptane	2213-23-2	**43**,57,85	x	-
5	7.65	4-methyloctane	2216-34-4	**43**,41,85	x	x
6	8.94	2,2,4-trimethylhexane	16747-26-5	**57**,56,41	x	x
7	9.57	decane*	124-18-5	**43**,57,41	x	-
8	10.19	3,3-dimethylheptane	4032-86-4	**43**,57,71	x	-
10	10.29	2,4-dimethyloctane	4032-94-4	**57**,71,85	x	-
12	11.15	3-ethyl-3-methylheptane	17302-01-1	**71**,43,85	x	-
13	11.27	2,3,7-trimethyldecane	62238-13-5	**43**,71,57	x	-
14	11.40	undecane*	1120-21-4	**71**,43,57	x	-
16	11.61	2,3-dimethyldecane	17312-44-6	**71**,43,85	x	-
19	14.10	dodecane*	112-40-3	**57**,43,71	x	x
21	15.15	3,9-dimethylundecane	17301-31-4	**71**,57,43	x	-
25	16.56	3,6-dimethyldecane	13150-81-7	**71**,43,57	x	-
26	16.57	tridecane*	629.50-5	**57**,43,71	x	-
28	16.82	n.i.	-	**43**,71,57	x	-
29	17.11	n.i.	-	**71**,57,43	x	-
31	17.42	2,5,6-trimethyldecane	62108-23-0	**71**,57,43	x	-
33	19.35	tetradecane*	629-59-4	**57**,43,71	x	x
38	21.64	pentadecane*	629-62-9	**71**,43,85	x	x
39	21.90	n.i.	-	**43**,85,71	x	-
41	22.50	n.i.	-	**57**,71,85	x	-
42	22.73	n.i.	-	**71**,43,57	x	x
47	24.30	hexadecane*	544-76-3	**57**,43,71	x	-
48	25.08	2-methyl-tridecane	1560-96-9	**57**,43,71	x	-
52	25.86	2,6,10-trimethyldodecane	3891-98-3	**57**,71,43	-	x
53	26.24	2-methylpentadecane	1560-93-6	**57,**43,71	x	x
59	28.26	n.i.	-	**57**,71,85	-	x
		Aromatic				
11	10.93	toluene*	108-88-3	**91**,92,65	x	x
18	13.02	p-xylene*	106-42-3	**91**,106,65	x	x
24	16.21	styrene	100-42-5	**104**,103,78	x	-
60	28.72	napthalene*	91-20-3	**128**,120,102	-	x
76	37.23	2,6bmethnapth[b]	24157-81-1	**197**,212,155	-	x
		Ketones				
4	7.39	acetone*	67-64-1	**43**,58,42	x	x
30	17.27	cyclohexanone*	108-94-1	**55**,98,42	-	x
32	18.08	6-methyl-5-hepten-2-one*	110-93-0	**43**,41,69	x	x
54	26.48	acetophenone*	98-86-2	**105**,120,77	x	x
		Monoterpenic compounds				
		Hydrocarbon type				
9	10.20	α-pinene*	80-56-8	**93**,92,91	-	x
17	12.15	α-phellandrene*	99-83-2	**93**,77,79	-	x
20	14.39	limonene*	138-86-3	**68**,67,93	-	x
		Alcohol type				
51	25.61	menthol*	1490-04-6	**71**,95	-	x
		Ketone type				

Peak Number	R.T. (min.)	Compounds	CAS number	m/z[¥]	Breath	Ambient air
63	30.48	neryl acetone*	3879-26-3	**43**,69,107	-	x
		Aldehydes				
15	11.51	hexanal*	66-25-1	**44**,56,41	-	x
34	19.55	nonanal	124-19-6	**43**,57,57	x	x
40	22.24	decanal*	112-31-2	**41**,43,57	x	x
44	23.46	benzaldehyde*	100-52-7	**77**,105,106	x	x
		Acids				
36	21.10	acetic acid*	64-19-7	**43**,45,60	x	x
43	23.20	propanoic acid*	79-09-4	**74**,45,73	x	-
57	27.83	pentanoic acid*	109-52-4	**60**,70,41	-	x
62	30.18	hexanoic acid*	142-62-1	**60**,73,41	-	x
66	32.24	2-ethylhexanoic acid	149-57-5	**88**,73,57	-	x
67	32.40	heptanoic acid*	111-14-8	**60**,73,87	-	x
72	34.49	octanoic acid*	124-07-2	**60**,73,43	-	x
		Miscellaneous				
2	6.48	2-methyl-1,3-butadiene	78-79-5	**67,**68,53	x	-
22	15.27	n.i.	-	**57**,55,43	x	x
23	15.71	n.i.	-	**105**,120,43	x	-
27	16.79	n.i.	-	**105**,120,84	-	x
35	19.68	2-butoxy-ethanol	111-76-2	**57**,45,41	x	x
37	21.45	2,1meox2prop[c]	20324-33-8	**59**,103,43	-	x
45	23.82	n.i.	-	**73**,43,59	-	x
46	23.98	n.i.	-	**57**,82,67	-	x
49	25.19	2etoxeth[d]	111-90-0	**45**,59,72	-	x
50	25.50	phenylethyl acetate	103-45-7	**94**,43,136	x	-
55	26.63	n.i.	-	**71**,57,43	x	x
56	26.77	4-tert-butylcyclohexyl acetate	32210-23-4	**57**,80,73	-	x
58	28.00	benzyl acetate	140-11-4	**108**,91,90	-	x
61	29.19	n.i.	-	**57**,41,71	-	x
64	31.19	benzyl alcohol*	100-51-6	**79**,108,107	-	x
65	31.97	2-phenyldodecane	2719-61-1	**91**,73,122	-	x
68	32.51	1-undecanol*	112-42-5	**55**,43,69	-	x
69	33.19	2-methyl-1-undecanol	10522-26-6	**58**,43,69	-	x
70	33.77	phenol*	87-66-1	**94**,66,65	x	x
71	33.87	isopropyl tetradecanoate	110-27-0	**228**,102,229	-	x
73	35.19	β-ionone*	14901-07-6	**177**,119,135	-	x
74	35.66	2-methyl-β-ionone	127-43-5	**191**,121,105	-	x
75	36.78	β-phenoxyethyl alcohol	9004-78-8	**94**,138,77	-	x

n.i.: not identified
[*]Compounds confirmed by chemical standards
[¥] Identification of highest abundance m/z
[a]Used fragment for area determination
[b] 2,6bmethnapth**:** 2,6-bis(1-methylethyl)naphthalene
[c] 2,1meox2prop**:** 2,1-(2-methoxy-1-methylethoxy)-2-propanol
[d] 2etoxeth**:** 2-(2-ethoxyethoxy)ethanol

Parallel room air sampling was also performed by the reported fact that in the environment there are a whole range of volatiles from several sources, which may influence the obtained results. Room air analysis allowed the identification of fifty-three compounds that belong to several different chemical families: alkanes (linear and ramified ketones, terpenic compounds, aromatic hydrocarbons, aldehydes, acids, among others (Table 2.3).

From these, twenty one compounds were common to children exhaled breath. Compounds that were present in room air with higher areas than in breath were discarded for PLS-DA analysis, as hexane for example, among others.

Multivariate analysis

Twenty-five from the forty-four volatiles were selected for multivariate analysis. This selection was made taking account the compounds that were present in all children with allergic asthma and by disregarding compounds that are known as solvents and contaminations (for example, aromatic hydrocarbons).

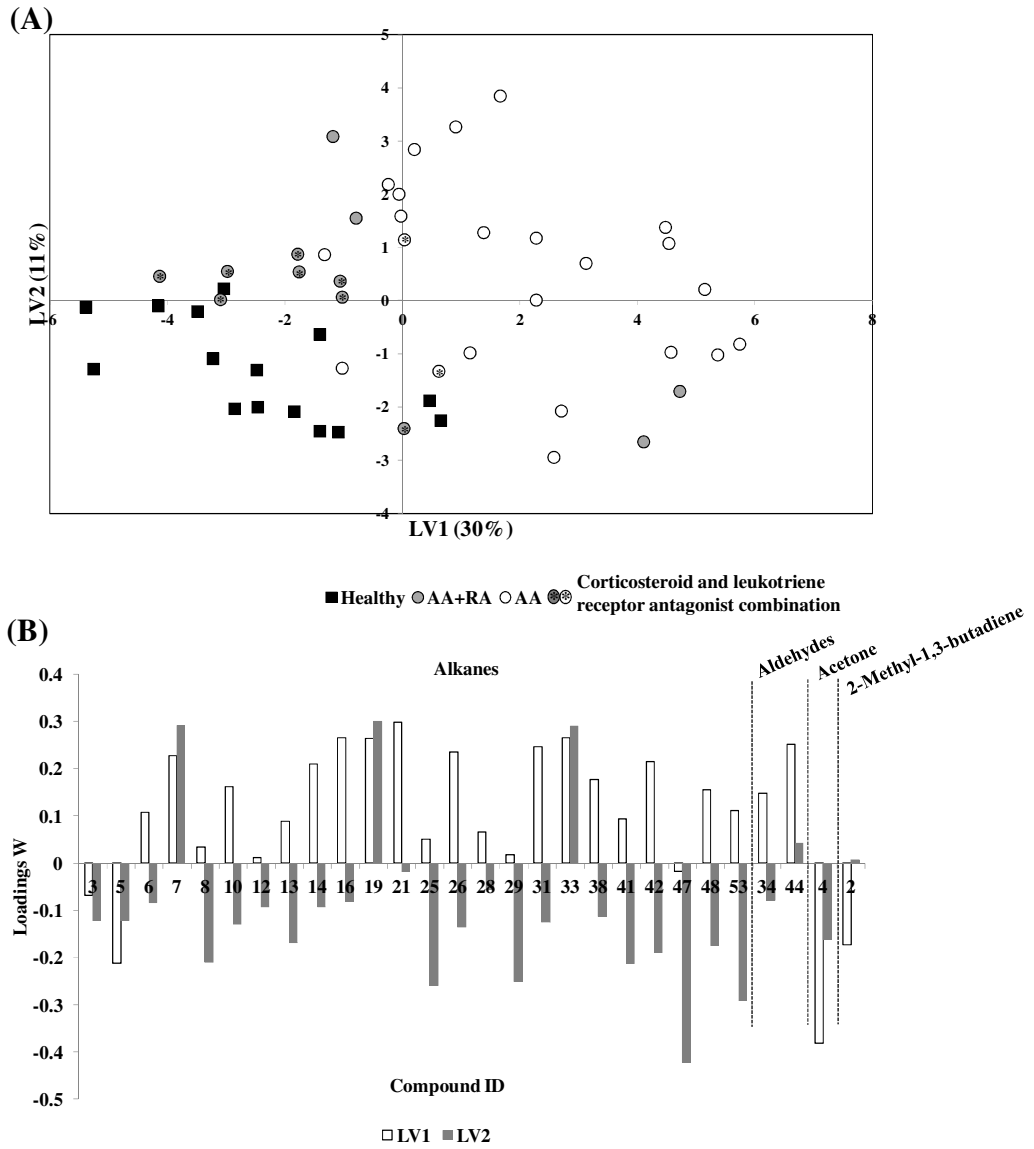

Figure 2.5 – (**A**) PLS-DA LV1xLV2 scores scatter plot and (**B**) loading weights plot of exhaled breath for allergic asthma (AA and AA+RA) and heatlhy children. Peak identification is presented in Table 2.3.

PLS-DA was applied to the GC chromatographic unit variance scaled areas to establish a preliminary classification model and assess the relationships between the compounds and the children under study. Figure 2.5 **A** shows the scores scatter plot of the first two Latent Variables (LV1xLV2), while Figure 2.5 **B** shows the corresponding loading weights plot which establishes the contribution of each volatile compound that promotes the observed discrimination. The classification model was assessed by internal cross-validation (7 blocks) giving a model with 4 LVs with a classification rate of 88%. A limited number of compounds (28) were used to classify children with allergic asthma and healthy children using discriminant analysis, with special highlight to compounds linked to oxidative stress such as alkanes and aldehydes[16]. These compounds were not unique to children with allergic asthma and were also observed in healthy children in greater or lesser abundance as normal human metabolism generates a wide variety of compounds. Airway inflammation plays a central role in the pathophysiology of asthma and during this process a degradation of polyunsaturated fatty acids occurs due to the imbalance between oxidants and antioxidants. This imbalance is explained by the continuous production of ROS caused by an influx of leukocytes characteristic of lung inflammation in asthma. During this process volatile compounds are formed by LPO of ω-3 and ω-6 polyunsaturated fatty acids[17].

From Figure 2.5 **A**, there are two relatively defined clusters with a clear tendency for the healthy group being associated with LV1 and LV2 negative values explained by 2,4-dimethylheptane, 4-methyloctane and acetone, whereas the allergic asthma group is dispersed most probably due to the distinct allergic asthma as well as different disease stages. There are clusters for allergic asthma children for example in LVI and LV2 positive values that seems to have contribution from decane, dodecane and tetradecane. Another region is LV1 negative and LV2 positive values influenced by 2-methyl-1,3-butadiene whereas the allergic asthma children that are clustered in LV1 positive region and LV2 negative values are mainly influenced by 2,2-dimethylhexane, 2,4-dimethyloctane and 2,3,6-trimethyldecane among others. C_{13} alkanes were previously used to discriminate asthma patients from children[13], being more intense in asthmatic ones.

Clinical data such as therapy, spirometry values, IgE values and prick test results were also available. The PLS-DA scores scatter plot was tentatively explained using clinical data. Allergic asthma is a condition associated to airspace inflammation with increasing evidence that LPO may have an important part in the differentiating the composition of exhaled breath between allergic asthma and healthy group. Compounds as the identified alkanes, and that

exerted statistical differences between allergic asthma and healthy group, might be an end-product of LPO but other pathways cannot be excluded. Allergic rhinitis is symptomatic disorder of the nose induced after allergen exposure, an IgE mediated inflammation of the membranes lining of the nose. Both allergic asthma and rhinitis are systemic inflammatory conditions and usually over 80% of asthmatics have rhinitis and 10-40% of patients with rhinitis have asthma. In the studied population, 63% were diagnosed with allergic asthma (AA) whilst 37% with allergic asthma and allergic rhinitis (AA+AR) and from the PLS-DA we denote that AA+AR cases are predominantly in LV1 negative LV2 positive whereas for AA children there is a higher scattering throughout LV1 axis. Both diseases are inflammatory diseases; however inflammation in rhinitis is concentrated in the nose while in allergic asthma occurs in the airways modifying the volatile composition as observed in Figure 2.5 **A**.

Prick tests were also available with 54% of the studied cases being allergic to dust mites, 9% dust mites and gramineae, 9% dust mites and cat fur and 3% to dust mite, gramineae and car fur. Due to a higher number of cases the cases with positive prick tests to dust mites are highlighted in the PLS-DA and from this data we observe a high dispersion through LV1 axis. As for the other possible combinations, a higher number of cases are necessary to exert any conclusions.

Several medication combinations are prescribed by clinicians to control allergic asthma and rhinitis. Inhaled corticosteroids prevent asthma symptoms by blocking the late-phase immune reaction to an allergen, reduce airway hyppersponsiveness and decreases inflammation and inhibit inflammatory cells such as mast cells, eosinophils and basophils. Leukotriene receptor antagonist or leukast are drugs that inhibit the leukotrienes receptors, compounds that are produced by the immune system that cause inflammation in asthma and that constrict airways whereas a histamine antagonist or antihistamine is an agent that inhibits the action of histamine that is released in excess by an allergic reaction. Bronchodilators are used to dilate the bronchi and bronchioles decreasing airway resistance and thereby facilitating airflow. In our population, the three most frequent combinations were: inhaled corticosteroids and leukotriene receptor antagonist, inhaled corticosteroids and anti-histamines or inhaled corticosteroids and bronchodilator. As far as medication effects on breath volatile composition that should reflect the inflammation, we observed in the PLS-DA (highlighted with an asterisk in Figure 2.5 **A** differences between the exhaled breath of children that used a combination between corticosteroid and leukotriene receptor antagonist for asthma control as these individuals presented themselves closer to control subjects than other subjects that were under corticosteroid and anti-histaminic or corticosteroid and

bronchodilator therapy. This occurrence may be explained by the properties of the combination of inhaled corticosteroids and leukotriene receptor antagonist as both decrease inflammation consequently affecting the volatile composition of exhaled breath and thereby inflammation compounds such as linear and ramified alkanes.

2.2.5 Concluding remarks

In this study, we report the development of HS-SPME/GC-MS methodology using a full factorial design. Breath sampling related subjects such as breath sampling containers and their cleaning, as well as an important parameter like intra-individual variability were also determined. The optimization of SPME parameters was done with selected standards representative of different chemical families previously reported in human breath[3,9] and the higher extraction efficiency was obtained with the mixed phase DVB/CAR/PDMS fibre with an extraction of 60min and a temperature of 22ºC.

The comparison between 1 L Tedlar® gas sampling bags and BIOVOC®, for breath collection, showed Tedlar® gas sampling bags to be the most adequate containers for collecting breath samples. The cleaning protocol for the Tedlar® bags was also established and consists of flushing the bag with nitrogen ten times, preceded by vacuum. As for intra-individual variability, results show that there was no statistical difference between sampling exhaled breath within the same day as well as sampling between different weeks, if the asthma status is maintained.

The developed methodology was then successfully applied to breath samples of allergic asthma children and healthy children, allowing the identification of forty-four volatiles from different chemical families, namely: alkanes (linear and ramified) ketones, aromatic hydrocarbons, aldehydes, acids, among others. Multivariate studies were performed (PLS-DA) using a set of 28 selected compounds and the corresponding loadings suggested the compounds that promoted the observed distinction between allergic asthma and healthy children. The allergic asthma population was characterized mainly by the compounds possibly linked to oxidative stress, such as alkanes and aldehydes, which is a characteristic of airway inflammation. Furthermore, more detailed information can be achieved about the asthma metabolic volatile profile by the proposed approach. The advantage of the developed method was observed when the volatile metabolic data processed by PLS-DA was explained using the

clinical data. Further studies are being performed increasing the number of allergic asthma and healthy children.

Although the number of cases studied is relatively small (50), the obtained results generated fundamental information which represents the scientific basis for the definition of a rapid and non-invasive diagnostic tool. The results of this study provided a novel methodological approach to characterize allergic asthma as a function of its metabolomic patterns, which will open new strategies to early diagnosis, therapy monitoring, and understanding the asthma pathogenesis that affects millions around the world.

2.3 Allergic asthma exhaled breath metabolome: A challenge for comprehensive two-dimensional gas chromatography

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Allergic asthma exhaled breath metabolome: A challenge for comprehensive two-dimensional gas chromatography

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2.3.1 Abstract

Allergic asthma represents an important public health issue, most common in the paediatric population, characterized by airway inflammation that may lead to changes in volatiles secreted via the lungs. Thus, exhaled breath has potential to be a matrix with relevant metabolomic information to characterize this disease. Progress in biochemistry, health sciences and related areas depends on instrumental advances, and a high throughput and sensitive equipment such as GC×GC–ToFMS was considered. GC×GC–ToFMS application in the analysis of the exhaled breath of 32 children with allergic asthma, from which 10 had also allergic rhinitis, and 27 healthy children allowed the identification of several hundreds of compounds belonging to different chemical families. Multivariate analysis, using PLS-DA in tandem with MCCV was performed to assess the predictive power and to help the interpretation of recovered compounds possibly linked to oxidative stress, inflammation processes or other cellular processes that may characterize asthma. The results suggest that the model is robust, considering the high classification rate, sensitivity, and specificity. A pattern of six compounds belonging to the alkanes characterized the asthmatic population: nonane, 2,2,4,6,6-pentamethylheptane, decane, 2-methyldecane, dodecane, and tetradecane. To explore future clinical applications, and considering the future role of molecular-based methodologies, a compound set was established to rapid access of information from exhaled breath, reducing the time of data processing, and thus, becoming more expedite method for the clinical purposes.

2.3.2 Framework

Hundreds of different volatiles are present in human breath, and their relative concentrations may alter via the disease[13]. Exhaled breath has been studied using 1D-GC in lung diseases, such as asthma[13], cystic fibrosis[18] and lung cancer[10,19]. Although such approach often provides satisfying analytical results an in-depth chromatogram analysis frequently indicates that some peaks are the result of two or more co-eluting compounds. GC×GC employs two orthogonal mechanisms to separate the constituents of the sample within a single analysis, based on the application of two GC columns coated with different stationary phases, which increases peak capacity as a result of the product of the peak capacity of the two dimensions. For example, a non-polar / polar phase combination (NP / P), connected in series through a modulator interface achieves this goal. For instance, using a cryogenic modulator, the interface samples small (several seconds) portions of the ^{1}D eluate by cryofocusing, and re-injects them into the second column (^{2}D). Each ^{1}D peak is modulated several times, largely preservating the ^{1}D separation. Co-eluting compounds from ^{1}D undergo additional separation on ^{2}D[20] Sensitivity and limits of detection are improved due to focusing of the peak in the modulator and separation of analytes from chemical background[21] compared to 1D-GC. ToFMS brings several advantages such as full mass spectra acquisition at trace level sensitivity and mass spectral continuity, which allows for deconvolution of spectra of co-eluted peaks[22].

Thus, this study aims to obtain a deeper knowledge of allergic asthma based on exhaled breath analysis using a previously developed HS-SPME extraction technique, as well as several other exhaled breath sampling parameters (Chapter 2.1), combined with GC×GC–ToFMS system. The first step was to check the separation potential of GC×GC–ToFMS and sensitivity issues, important parameters in exhaled breath analysis, a complex matrix with several compounds in the micromolar to nanomolar range[23]. Secondly, PLS–DA and MCCV were performed to assess both the predictive power and classification models robustness. Moreover PLS-DA regression vectors were used to help understand metabolic variations important to class discrimination.

2.3.3 Experimental

Standards and materials

Several reagents were used to perform this study: linear alkanes (C_8-C_{20}) in hexane (99.5%, Fluka, Madrid, Spain), linear alkenes (C_8-C_{20}) (98%, Sigma-Aldrich, Madrid, Spain), aldehydes: hexanal (98%, Sigma-Aldrich, Madrid, Spain), (*E*)-2-nonenal (95%, Acros Organics, Geel Belgium), decanal (98%, Sigma-Aldrich, Madrid, Spain), ketones: 3-heptanone (97%, Sigma-Aldrich, Madrid, Spain), 5-methyl-3-heptanone (94%, Sigma-Aldrich, Madrid, Spain), 3-octanone (98%, Sigma-Aldrich, Madrid, Spain), absolute ethanol was supplied by Panreac (99.5 %, analytical grade, Barcelone, Spain). Ultra pure water was obtained from a Milli-Q system from Millipore (Milford, MA, USA).

For the sensitivity studies, a stock solution of each standard (1 g/L) was prepared in absolute ethanol and made up to volume, and from this a solution of 100 mg/L was set up. A working solution was prepared to yield different concentrations and to reproduce a two-phase system (headspace and coating fibre), as in breath analysis, 5 μL was added to a 120 mL SPME flask and sealed with an aluminium crimp cap with a vial was capped with a PTFE septum (Chromacol, Hertfordshire, UK), and concentrations ranged from 20 to 200 $\times 10^3$ pg/L.

The SPME holder for manual sampling and fibre were purchased from Supelco (Aldrich, Bellefonte, PA, USA). The SPME device included a fused silica fibre coating partially cross-linked with 50/30μm divinylbenzene-carboxen-poly(dimethylsiloxane) (DVB/CAR/PDMS). Prior to use, the SPME fibre was conditioned at 270 ºC for 60 min in the GC injector, according to the manufacturer's recommendations. Then, the fibre was daily conditioned for 10 min at 250 ºC.

Samples

A group of 32 children with allergic asthma, from which 10 had allergic asthma and allergic rhinitis, and 27 healthy children volunteered for this study (n=59). The characteristics of the patients and children are presented in Table 2.4. A *naive* patient was also included in this study. This patient was a 9 years old female child that had never taken an asthma drug and was diagnosed by physicians with allergic asthma based on symptoms history and skin prick tests were performed being positive for dust mites. After the first consult, this child was prescribed a combination of anti-histamine and a leukotriene receptor antagonist. The 59 individuals correspond to a total of 69 exhaled breath samples. Usually, each individual

corresponds to one breath sample, except for an allergic asthma child that was collected up to 6 times in different locations/time periods and for the *naive* exhaled breath was collected at four different moments.

Table 2.4 Characteristics of the studied population: allergic asthma and healthy children.

					Allergic asthma	**Healthy**
Age in years (range/median)					4-16/9	3-6/5
Gender (male/female)					18/14	15/12
Pathology						
Allergic Asthma (AA)					22 (69%)	-
Allergic Asthma + Allergic Rhinitis (AA+AR)					10 (31%)	-
Allergens[a]						
Dust mite					18 (56%)	-
Dust mite + gramineae					5 (16%)	-
Gramineae					4 (13%)	-
Dust mite + cat fur + gramineae					2 (6%)	-
Dust mite + cat fur					1 (3%)	-
Gramineae + cat fur					1 (3%)	-
Dust mite + cockroach					1 (3%)	-
Therapy						
Corticostero	Leukotri	Bronchodilato	Anti-	Nasal		
x	x	x	-	-	1 (3%)	-
x	-	x	-	-	9 (28%)	-
x	x	-	-	-	5 (16%)	-
-	x	-	x	x	2 (6%)	-
-	-	x	x	-	5 (16%)	-
-	x	x	-	-	1 (3%)	-
-	-	-	x	x	2 (6%)	-
-	x	-	x	-	1 (3%)	-
No therapy					6 (19%)	-

[a] Results obtained by prick-tests

The allergic asthma population represented a controlled asthma status, with exception of a *naive* child. No restrictions were applied regarding drugs or diet, and each allergic asthma and healthy groups were sampled in two distinct locations (in a total of four collections sites). The study was approved by the hospital ethics committee and the daycare administration.

Breath sampling

The breath sampling parameters were previously optimized (Chapter 2.1). Exhaled breath was collected in 1 L Tedlar® bags. Children were asked to cleanse their mouth with water before sampling. Subsequently, children were instructed to inhale and exhaled normally and then exhale deeply into the Tedlar® bag previously holding their breath for 5s. The collection method was successfully done by all volunteers. Each subject provided one sample using a disposable mouthpiece. Before collecting exhaled breath, all bags were thoroughly cleaned to remove residual contaminants by flushing with high purity nitrogen gas. The bags

were transported to the laboratory and the analysis was performed to a maximum of six hours as recommended by Mochalski *et. al.*[24]. On average, the analysis was performed after two to three hours after sampling. The bags were stored at 22 °C.

HS-SPME methodology

The SPME coating fibre and the experimental parameters were adopted from a methodology previously developed in our laboratory (Chapter 2.1): DVB/CAR/PDMS fibre, and an extraction temperature and time of 22 ºC and 60 min, respectively. Following the extraction procedure, the SPME fibre was retracted from the Tedlar® bag and inserted in the GC system injection port. The HS-SPME methodology was also applied to selected standards to verify the GC×GC sensitivity as previously described in s*tandards and materials section*. Each breath represents a single sample, and was analysed once. To verify the absence of any carry over, blanks (that corresponds to the analysis of the coating fibre not submitted to any extraction procedure and Tedlar® bags) were performed.

GC×GC–ToFMS analysis

After the extraction/concentration step, the SPME coating fibre was manually introduced into the GC×GC–ToFMS injection port at 250 °C. The injection port was lined with a 0.75 mm I.D. splitless glass liner. Splitless injections were used (2 min). The LECO Pegasus 4D (LECO, St. Joseph , MI , USA) GC×GC-ToFMS system consisted of an Agilent GC 7890A gas chromatograph (Agilent Technologies, Inc., Wilmington , DE), with a dual stage jet cryogenic modulator (licensed from Zoex) and a secondary oven, and mass spectrometer equipped with a high resolution ToF analyzer. An HP-5 column (30 m × 0.32 mm I.D., 0.25 µm film thickness, 5% Phenyl-methylpolysiloxane, J&W Scientific Inc., Folsom, CA, USA) was used as ^{1}D column and a DB-FFAP (0.79 m × 0.25 mm I.D., 0.25 µm film thickness, nitroterephthalic acid modified polyethylene glycol, J&W Scientific Inc., Folsom, CA, USA) was used as ^{2}D column. The carrier gas was helium at a constant flow rate of 2.50 mL/min. The GC×GC–ToFMS injection port was at 250 °C. The primary oven temperature program was: initial temperature 35 ºC (hold 1 min), raised to 40 ºC (1 ºC/min), and finally rose to 220 ºC (7 ºC/min) and hold for 1 min. The secondary oven temperature program was 15 ºC offset above the primary oven. The MS transfer line temperature was 250 ºC and the MS source temperature was 250 ºC. A 6 s modulation time with a 30 ºC secondary

oven temperature offset (above primary oven) was chosen to be a suitable compromise as it maintained the ^{1}D separation, maximized the ^{2}D resolution, and avoiding wrap-around effect (the elution time of a pulsed solute exceeds the modulation period) for compounds that were late to elute from the ^{2}D. Ideally, all peaks must be detected before the subsequent re-injection and, hence, $^{2}t_{R}$ must be equal or less than the modulation period[25,26]. The ToFMS was operated at a spectrum storage rate of 125 spectra/s. The mass spectrometer was operated in the EI mode at 70 eV using a range of *m/z* 35-350 and the detector voltage was -1695 V. Total ion chromatograms (TIC) were processed using the automated data processing software ChromaToF (LECO) at signal-to-noise threshold of 80. Contour plots were used to evaluate the separation general quality and for manual peak identification. In order to identify the different compounds, the mass spectrum of each compound detected was compared to those in mass spectral libraries of one home-made (using standards) and two commercial databases (Wiley 275 and US National Institute of Science and Technology (NIST) V. 2.0 - Mainlib and Replib). The identification was also supported by experimentally determining the retention index (RI) values that were compared, when available, with values reported in literature for chromatographic columns similar to that used as the ^{1}D column and whenever available compared to RI values obtained by GC×GC[27-62]. For determination of RI values a C_8-C_{20} *n*-alkanes series was used, calculated according to the Van den Dool and Kratz equation[63]. The majority (> 90%) of the identified compounds presented similarity matches > 850. The GC×GC area data was used as an approach to estimate the relative content of each volatile component of exhaled breath.

Multivariate Analysis

A full dataset comprises 134 metabolites belonging to selected chemical families. A sub-set of 23 metabolites was also established by the compounds simultaneously identified by GC×GC–ToFMS, and those previously reported in Chapter 2.1 (indicated in Table 2.5). PLS is a widely used procedure for both regression and classification purposes. Concerning the classification application of PLS, PLS-DA8, the most common approach is to use a **Y** matrix containing dummy variables which defines sample memberships to pre-defined groups and allow extracting relevant information/variability that could describe the reasons for the observed patterns (clusters). This methodology allows one to understand which variables (metabolites) contribute the most for the observed separation. Each sample was mean normalized and UV (unit variance) scaled which is a data pre-treatment process that gives to

variables the same weight. The PLS-DA was applied to volatile metabolites (both datasets: 23 and 134 metabolites) tentatively identified by HS-SPME/GC×GC-ToFMS in all exhaled breath samples (69) and for classification purposes two groups were used (healthy and asthma).The classification model complexity (number of latent variables) of the full dataset (134 metabolites) was computed, as well as classification rate and Q^2 were estimated by cross-validation (7 blocks splits). Model robustness was assessed using MCCV with 1000 iterations. For each of the 1000 randomly generated classification models, the number of Latent Variables (LV), the Q^2 (expressing the cross-validated explained variability), and the confusion matrix was computed. The selection of model complexity was based on the most frequent list of model properties that maximizes the predictive power (i.e., lower LV and higher Q^2). The sensitivity and the specificity of the model were then depicted from the confusion matrix resulting into a ROC map to further assess the results significance. Then, the same procedure was applied using permuted class membership. Sensitivity is calculated from the ratio between true positives (allergic asthma samples correctly predicted) and the total number of modelled breath samples, whereas specificity is determined from the ratio between true negatives (healthy samples correctly predicted) and the total number of modelled control GC×GC data.

2.3.4 Results and Discussion

The previous study reported the development of an HS-SPME/GC–MS methodology, as well as the optimization of important breath sampling parameters and its application to a group of children with allergic asthma and children. To increase the information obtained on exhaled breath, the HS-SPME technique was applied to exhaled breath of a different population (n= 59) using a powerful tool such as the GC×GC–ToFMS, that relatively to 1D GC analysis is more sensitive, has higher chromatographic resolution and a structured chromatogram is obtained, three relevant advantages.

Structured chromatogram and sensitivity

GC×GC has proven to be a powerful technique in the analysis of complex samples and to detect trace components[64,65]. Automated processing of HS-SPME/GC×GC–ToFMS data was used to tentatively identify all peaks in the GC×GC chromatogram contour plots with signal-to-noise threshold > 80. The peak finding routine based on deconvolution method

allowed to identify ca. 350 compounds per sample comprising several chemical families: linear and ramified alkanes, cycloalkanes, alkenes, aldehydes, ketones, aromatic compounds, terpenoids and esters. One hundred and thirty four compounds belonging to linear, ramified and cycloalkanes, alkenes, aldehydes, ketones and a group of miscellaneous compounds, were selected for further studies. The remaining compounds were considered as possible contaminants, as for example, aromatic compounds from environmental cumulative exposure[66], whereas terpenoids and esters can have its origin in ingested foods[67]. Otherwise, the linear, ramified and cycloalkanes, alkenes, aldehydes and ketones have been reported to be associated to several biochemical processes that may occur in humans[68].

The total number of compounds detected in allergic asthma exhaled breath substantially increased with the use of the GC×GC–ToFMS, approximately 8 times, when compared to the obtained results by 1D GC-MS. By 1D GC-MS a total of 44 compounds were identified whereas by GC×GC–ToFMS ca. 350 compounds were tentatively identified. For example, considering the alkanes, alkenes, aldehydes and ketones, the number of detected compounds increased by 66%, 96%, 67% and 56%, respectively.

The compounds included in the selected dataset were tentatively identified based on comparison of their mass spectra to home-made and commercial databases, and by comparison of the RIs calculated (RI_{calc}) with the values reported in the literature (RI_{lit}) for 5% phenylpolysilphenylene-siloxane (or equivalent) column (Table 2.5). A range between 1 and 30 was obtained for RI_{cal} compared to the RI_{lit} reported in the literature ($|RI_{calc}-RI_{lit}|$) for 1D-GC with 5%-phenyl-methylpolysiloxane GC column or equivalent. This difference in RI is considered minimal (on average lower than 0.5%), and is well justified if one takes into account that: (i) the literature data is obtained from a large range of GC stationary phases (several commercial GC columns are composed of 5% phenylpolysilphenylene-siloxane or equivalent stationary phases), and (ii) the literature values were determined in a 1D-GC separation system, and the modulation causes some inaccuracy in first dimension retention time[65].

Table 2.5 - List of volatile compounds identified by GC×GC –ToFMS in exhaled breath of allergic asthma and healthy children

Peak #	^1t_R [a] (s)	^2t_R [a] (s)	Compounds	CAS Number	RI_{calc} [b]	$RI_{lit.}$ [c] (GC)	$RI_{lit.}$ [d] (GC × GC)
			Hydrocarbons				
			Alkanes				
1	138	0.48	hexane	110-54-3	600	600	600
4	210	0.52	2,4-dimethylhexane	589-43-5	727	736	729
7	252	0.54	octane	111-65-9	800	800	800
11	276	0.55	2,2,4-trimethylhexane [e]	921-47-1	817	810	-*
12	288	0.55	2,4-dimethylheptane [e]	2213-23-2	817	820	822
13	318	0.56	4-ethyl-2-methylhexane	3074-75-7	831	833	-
15	366	0.58	alkane isomer (m/z 43, 57, 85)	-	853	-	-
20	468	0.56	nonane	111-84-2	900	900	900
22	504	0.55	alkane isomer (m/z 43, 57, 85)	-	919	-	-
23	516	0.55	2,4-dimethyloctane [e]	15869-93-9	925	924	-
24	528	0.56	3-ethyl-3-methylheptane [e]	17302-01-1	932	-	942
26	540	0.55	2,6-dimethyloctane	2051-30-1	938	936	933
28	552	0.55	3-ethyl-2-methylheptane	14676-29-0	944	-	942
30	558	0.55	alkane isomer (m/z 57, 43, 71)	-	947	-	-
31	564	0.55	alkane isomer (m/z 43, 57, 71)	-	950	-	-
33	576	0.56	4-ethyloctane	15869-86-0	957	956	-
36	582	0.55	4-methylnonane	17301-94-9	960	962	956
38	588	0.54	alkane isomer (m/z 57, 43, 85)	-	963	-	-
40	594	0.55	alkane isomer (m/z 57, 43, 71)	-	966	-	-
42	600	0.55	2-methylnonane	871-83-0	969	970	-
43	606	0.55	alkane isomer (m/z 57, 43, 41)	-	972	-	-
45	612	0.55	3-methylnonane [e]	5911-04-6	975	976	-
52	636	0.54	2,2,4,6,6-pentamethylheptane	13475-82-6	988	997	-
58	660	0.56	decane [e]	124-18-5	1000	1000	1000
60	672	0.54	alkane isomer (m/z 57, 41, 71)	-	1008	-	-
61	720	0.54	3,9-dimethylnonane [e]	17302-32-8	1039	1038	
64	756	0.54	2-methyldecane [e]	17301-30-3	1062	1063	-
66	768	0.54	alkane isomer (m/z 57, 43, 85)	-	1070	-	-
68	774	0.55	3-methyldecane [e]	13151-34-3	1073	1073	-
72	786	0.55	alkane isomer (m/z 57, 43, 71)	-	1081	-	-
74	798	0.55	2-methyldecane [e]	6975-98-0	1089	1073	-
76	804	0.55	alkane isomer (m/z 43, 71, 57)	-	1093	-	-
81	822	0.55	undecane [e]	1120-21-4	1100	1100	1100
84	864	0.56	2,3-dimethyldecane [e]	1632-71-9	1135	1118	-
86	876	0.56	alkane isomer (m/z 57, 43, 71)	-	1144	-	-
87	894	0.55	5-methylundecane [e]	1632-70-8	1157	1154	-
89	906	0.57	alkane isomer (m/z 57, 43, 71)	-	1166	-	-
90	912	0.57	3,9-dimethylundecane [e]	7045-71-8	1170	1165	-
95	948	0.57	dodecane [e]	112-40-3	1200	1200	1200
96	960	0.58	alkane isomer (m/z 57, 71, 43)	-	1206	-	-
99	966	0.57	alkane isomer (m/z 57, 43, 71)	-	1211	-	-
100	972	0.56	2,5,6-trimethyldecane [e]	17301-28-9	1216	1206	-
101	978	0.57	alkane isomer (m/z 57, 43, 71)	-	1221	-	-
104	996	0.56	alkane isomer (m/z 57, 43, 71)	-	1236	-	-

Peak #	^1t_R [a] (s)	^2t_R [a] (s)	Compounds	CAS Number	RI_{calc} [b]	$RI_{lit.}$ [c] (GC)	$RI_{lit.}$ [d] (GC × GC)
106	1002	0.57	alkane isomer (m/z 57, 43, 71)	-	1241	-	-
107	1008	0.57	6-methyldodecane	6044-71-9	1246	1253	-
108	1020	0.57	alkane isomer (m/z 43, 57, 71)	-	1256	-	-
110	1044	0.55	alkane isomer (m/z 57, 43, 71)	-	1276	-	-
111	1050	0.55	4-ethylundecane	17312-59-3	1281	-	-
112	1056	0.57	alkane isomer (m/z 57, 43, 71)	-	1286	-	-
113	1062	0.55	alkane isomer (m/z 57, 71, 43)	-	1291	-	-
115	1068	0.56	alkane isomer (m/z 43, 57, 71)	-	1296	-	-
117	1074	0.57	tridecane [e]	629-50-5	1300	1300	1300
120	1092	0.57	2,2-dimethyldodecane	49598-54-1	1316	1315	-
121	1104	0.57	alkane isomer (m/z 57, 43, 71)	-	1327	-	-
122	1110	0.57	alkane isomer (m/z 57, 43, 71)	-	1332	-	-
123	1116	0.57	alkane isomer (m/z 57, 71, 43)	-	1337	-	-
124	1128	0.57	3-ethyl-3-methylundecane [e]	-	1348	1347	-
125	1152	0.60	2-methyltridecane [e]	6418-41-3	1369	1371	-
126	1158	0.59	alkane isomer (m/z 57, 43, 71)	-	1374	-	-
127	1170	0.61	alkane isomer (m/z 57, 43, 71)	-	1385	-	-
128	1188	0.61	tetradecane [e]	629-59-4	1400	1400	1400
130	1284	0.58	alkane isomer (m/z 57, 43, 85)	-	1489	-	-
131	1290	0.58	6,6-diethyldodecane	-	1495	1498	-
132	1296	0.64	pentadecane [e]	629-62-9	1500	1500	1500
133	1302	0.59	5-ethyl-5-methyltridecane	-	1507	1511	-
134	1338	0.61	3-ethyl-3-methyltridecane	-	1544	1549	-
			Alkenes				
10	252	0.60	3-octene	592-98-3	803	800	-
14	330	0.61	2,4-dimethyl-1-heptene	19549-87-2	836	842	-
17	450	0.61	1-nonene	124-11-8	892	889	-
27	546	0.57	alkene isomer (m/z 55, 41, 69)	-	941	-	-
29	552	0.57	3-methyl-1-nonene	2980-41-4	944	944	-
32	570	0.59	3,4-diethyl-2-hexene	19550-82-4	957	-	-
37	582	0.59	alkene isomer (m/z 69,41,56)	-	960	-	-
44	606	0.59	alkene isomer (m/z 69,41,56)	-	972	-	-
46	612	059	7-methyl-1-nonene	2980-71-4	975	960	-
56	648	0.59	4-decene	19398-89-1	994	-	994
65	762	0.55	alkene isomer (m/z 55, 69, 41)	-	1066	-	-
67	768	0.56	alkene isomer (m/z 55, 69, 41)	-	1070	-	-
70	780	0.56	(Z)-2-decene	20348-51-0	1077	1072	-
75	798	0.56	alkene isomer (m/z 55, 69, 41)	-	1089	-	-
79	810	0.55	alkene isomer (m/z 69, 55, 41)	-	1097	-	-
83	828	0.57	alkene isomer (m/z 55, 69, 41)	-	1109	-	-
85	870	0.59	2-methyl-1-undecene	18516-37-5	1140	1144	-
92	918	0.60	(E)-5-methyl-4-undecene	41851-94-9	1174	-	-
93	924	0.60	(Z)-5-methyl-5-undecene	57024-93-8	1179	-	-
94	942	0.60	1-dodecene	112-41-4	1192	1192	-
97	960	0.62	alkene isomer (m/z 55, 69, 41)	-	1206	-	-
102	978	0.59	alkene isomer (m/z 55, 69, 43)	-	1221	-	-
114	1062	0.61	1-tridecene	2437-56-1	1291	1292	-

Peak #	^1t_R [a] (s)	^2t_R [a] (s)	Compounds	CAS Number	RI_{calc} [b]	$RI_{lit.}$ [c] (GC)	$RI_{lit.}$ [d] (GC × GC)
			Cyclic				
5	240	0.57	1,2,4-trimethylcyclopentane	930-57-4	780	779	-
8	252	0.58	1,4-dimethylcyclohexane	589-90-2	800	-	806
25	534	0.60	propylcyclohexane	2040-95-1	935	929	-
34	576	0.59	1,1,2,3-tetramethylcyclohexane	6783-92-2	953	958	-
47	618	0.60	2-ethyl-1,3-dimethylcyclohexane	7045-67-2	978	-	-
48	624	0.59	1-methyl-3-propylcyclohexane	4291-80-9	982	-	-
49	630	0.58	1-methyl-3-(2-methylpropyl)cyclopentane	29053-04-1	985	-	-
53	642	0.59	ethylpropylcyclopentane	54111-97-6	991	-	-
57	654	0.59	1-methyl-2-propylcyclohexane	4291-79-6	997	-	-
63	732	0.57	hexylcyclopentane	1003-19-6	1047	-	-
73	792	0.57	1,4-dimethylcycloctane	13151-98-9	1085	-	-
77	804	0.59	1-ethyl-2-propycylohexane	62238-33-9	1093	-	-
105	996	0.62	hexylcyclohexane	4292-75-5	1236	1237	-
116	1068	0.65	1-hexyl-3-methylcyclohexane	591-48-0	1296	-	-
118	1080	0.60	1-butyl-2-propylcyclopentane	62199-50-2	1306	-	-
			Ketones				
2	138	0.79	2-butanone	78-93-3	601	602	-
6	240	1.16	2-hexanone	591-78-6	781	790	-
16	438	1.19	3-heptanone	106-35-4	887	885	884
18	450	1.29	ketone isomer (m/z 43, 58, 71)	-	892	-	-
19	450	2.07	ciclohexanone	108-94-1	893	895	-
54	642	1.10	3-octanone	106-68-3	991	989	-
55	642	1.33	6-methyl-5-hepten-2-one	110-93-0	991	989	-
78	804	1.03	2-nonanone	821-55-6	1093	1093	-
80	810	1.02	ketone isomer (m/z 43, 58, 71)	-	1097	-	-
			Miscellaneous				
50	630	2.13	1-octen-3-ol	3391-86-4	985	986	-
62	720	1.78	2-ethyl-1-hexanol	104-46-7	1040	1026	-
69	774	0.99	2-nonen-1-ol	22104-79-6	1074	1105	-
71	780	1.86	1-octanol	111-87-5	1078	1068	-
91	912	0.97	2-decen-1-ol	22104-80-9	1170	-	-
51	630	3.78	aniline	62-53-3	986	971	-
41	594	1.83	dimethyl trisulfide	58-80-8	967	972	-
103	978	3.73	benzothiazole	95-16-9	1223	1227	-

*–information not available

[a] – Retention times in seconds (s) for first (^1t_R) and second (^2t_R) dimensions.

[b] – RI: retention index obtained through the modulated chromatogram.

[c] – RI: retention index reported in the literature for one dimensional GC with a 5%-Phenyl-methylpolysiloxane GC column or equivalent[17-19,21-46,48-52].

[d] – RI: retention index reported in the literature for a comprehensive GC×GC system with Equity-5 for the first dimension[20,47].

[e] – Set of 23 compounds previously reported in the GC-MS study related to allergic asthma that was used in Figure 2.9.

The most reliable way to confirm the identification of each compound is based on authentic standard co-injection, which in several cases is economically prohibitive, and often unachievable in the time available for analysis[69], or are not commercially available. Thus, GC×GC is an ideal technique for the analysis of complex mixtures where compounds of similar chemical structure are grouped into distinct patterns in the 2D chromatographic plane providing useful information on both their boiling point and polarity (if NP / P set of columns was used), and relationships of structured retentions have proved especially useful for compound identification[70]. Figure 2.6 demonstrates the structured chromatogram.

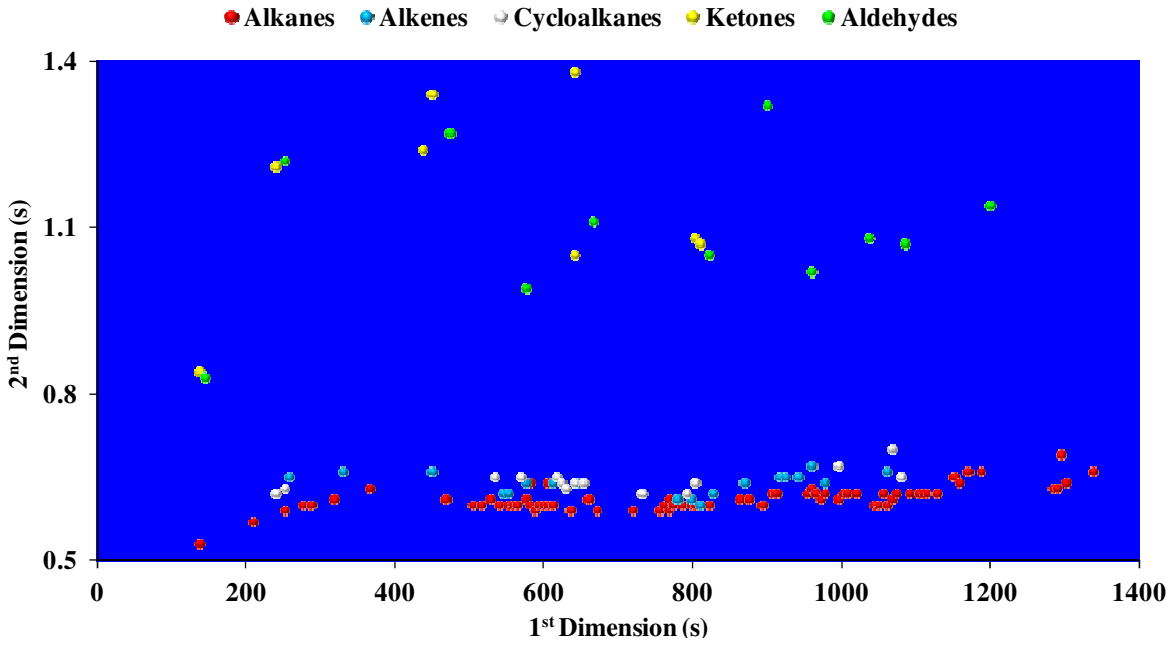


Figure 2.6 - Peak apex plot of the alkanes (linear, ramified and cyclic), alkenes, aldehydes and ketones identified using allergic asthma exhaled breath sample

A chromatographic space with higher peak density, ranging between $^{2}t_R$ 0.45 and 1.45s, was chosen, and a peak apex plot was depicted regarding the alkanes, alkenes, aldehydes and ketones to better visualise the attained structured chromatogram (Figure 2.6).

The components of each chemical group were dispersed through the peak apex plot according to their volatility (^{1}D) and polarity (^{2}D) obtained by a combination of NP/P columns. For the selected chemical families, as expected, it was observed that the decrease in volatility (high $^{1}t_R$) is mainly related to the increase in the number of carbons. The structured 2D chromatographic profile was observed within each chemical family based on the properties and positions of their functional groups. Globally, based on the functional group of the chemical families under study, the $^{2}t_R$ values increase as follows: alkanes < alkenes ~

cycloalkanes < ketones ~ aldehydes. This information can be also confirmed in Table 2.5. Alkanes have the lowest polarity ($^2t_R \cong$ 0.48–0.64 s), followed by alkenes (^2t_R $^2t_R \cong$ 0.57–0.62s) cycloalkanes ($^2t_R \cong$ 0.57–0.65 s), ketones ($^2t_R \cong$0.79–1.33 s), and aldehydes ($^2t_R \cong$0.78–3.58 s). This information is especially useful for classifying unidentified compounds.

A further advantage of a comprehensive chromatographic system can be verified, as compounds with similar boiling points that could co-elute in a 1D system, as for example 4-ethyloctane (**33**), 1,1,2,3-tetramethylcyclohexane (**34**) and 2-ethylhexanal (**35**), are able to be separated using the comprehensive chromatographic system (Figure 2.7). These compounds have similar volatility, the same ^1t_R of 576 s but present different polarities, and as a consequence they were separated by the second column (^2t_R of 0.56, 0.59 and 0.92 s, respectively).

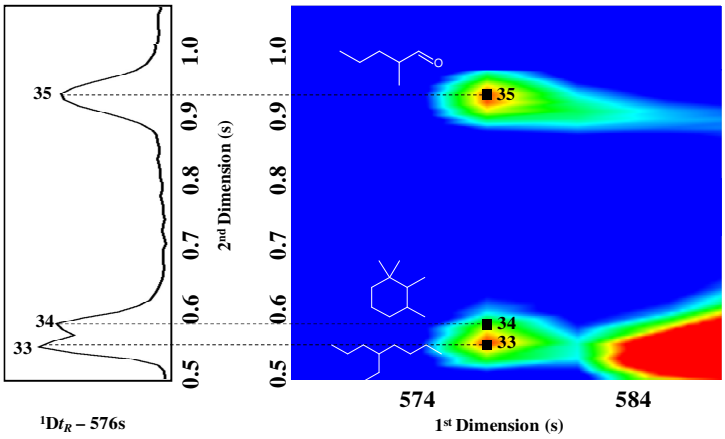


Figure 2.7 - Blow-up of a part of total ion GC×GC chromatogram contour plot obtained from an allergic asthma exhaled breath showing the corresponding ramified alkane, cycloalkane and ramified aldehyde: 4-ethyloctane (**33**), 1,1,2,3-tetramethylcyclohexane (**34**) and 2-ethylhexanal (**35**), respectively

Different concentrations, ranging from nmolar to μmolar have been reported for volatile breath components[71,72], so an important issue is the sensitivity of the used equipment. Consequently, the GC×GC–ToFMS sensitivity was verified, and for this purpose a standard solution comprising standards pertaining to the previously selected families (alkanes, alkenes, aldehydes and ketones) was used, whose concentration, for instance, varied between 20 pg/L to 200 ng/L. The standards from the tested compound families were detected at the level under study. For demonstration purposes, 1-dodecene (**94**) and dodecane (**95**), showed in Figure 2.8, were detected at pg/L and ng/L levels. The studied range was lower than the reported values to verify that this equipment is able to detect compounds at this concentration

level, which could be relevant to identify target compounds that could be important for asthma metabolomic studies.

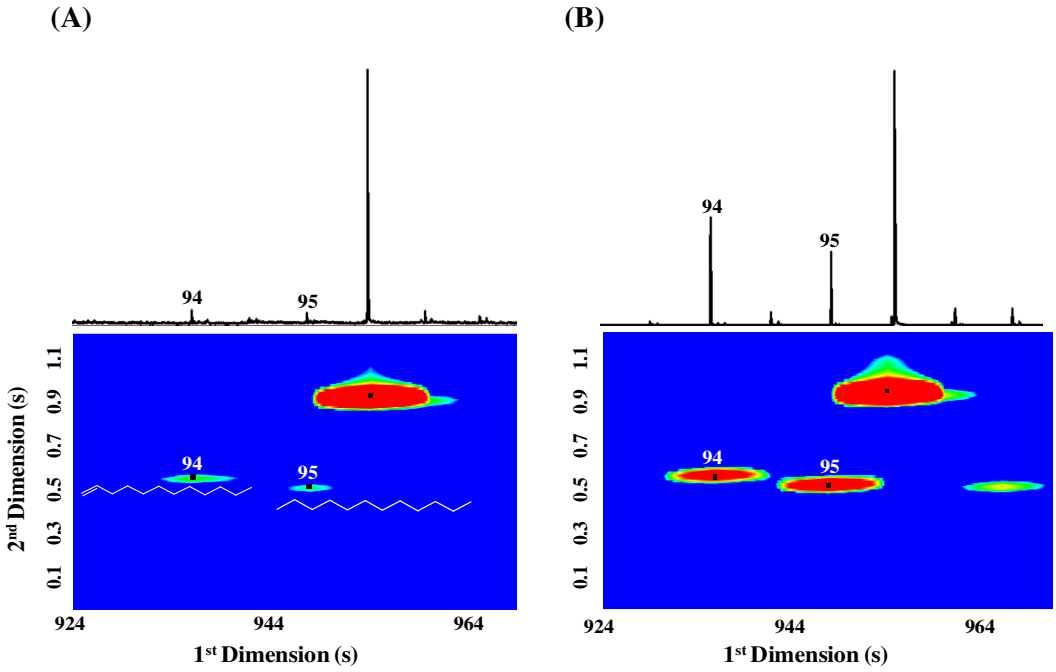


Figure 2.8 - Total ion GC×GC chromatogram and corresponding contour plots of 1-dodecene (**94**) and dodecane (**95**) varying the concentration from 20 **(A)** to 200×10^3 pg/L **(B)**

Multivariate analysis in the establishment of asthma "breath-print"

In the study performed with GC-MS, 28 compounds, from a total of 44, were selected and distinction was achieved with two relatively defined clusters between the healthy and the allergic asthma groups. As a first approach, using a different allergic asthma and healthy children population, from the 28 compounds identified by GC-MS, 23 were also identified by GC×GC–ToFMS and selected for multivariate analysis (indicated in Table 2.5) to verify the results obtained in the previous study. PLS-DA was applied to the GC×GC chromatographic unit variance scaled areas to establish a preliminary classification model and assess the relationships between the compounds and the samples under study (Figure 2.9).

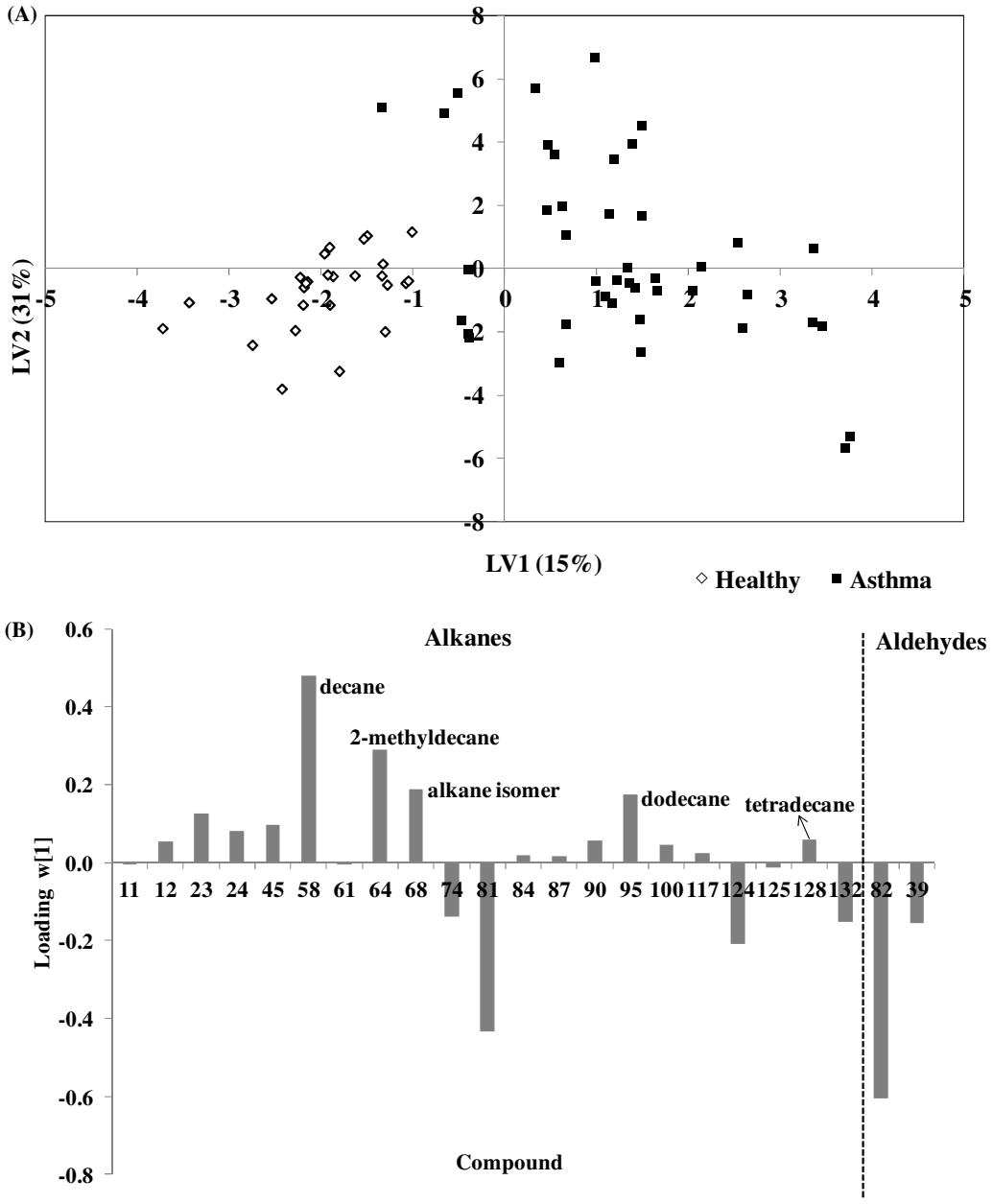


Figure 2.9 - PLS-DA LV1×LV2 scores scatter plot **(A)** and LV1 loading weights plot **(B)** of exhaled breath for allergic asthma and healthy children using a sub-set of 23 compounds identified by GC×GC –ToFMS, and previously reported in a study related to allergic asthma[3] (peak attribution shown in Table 2.5)

Figure 2.9 **A** shows that there are two defined clusters with the healthy group being mainly associated to LV1 negative values and the allergic asthma group to LV1 positive values. From the previous study, the allergic asthma group was mainly characterized by decane, dodecane and tetradecane, which was confirmed with this new set of children (Figure

2.9 **B**). However a clear distinction was sought, thus PLS-DA was applied to the full dataset of 134 compounds identified by GC×GC–ToFMS. The results obtained are shown in Figure 2.10.

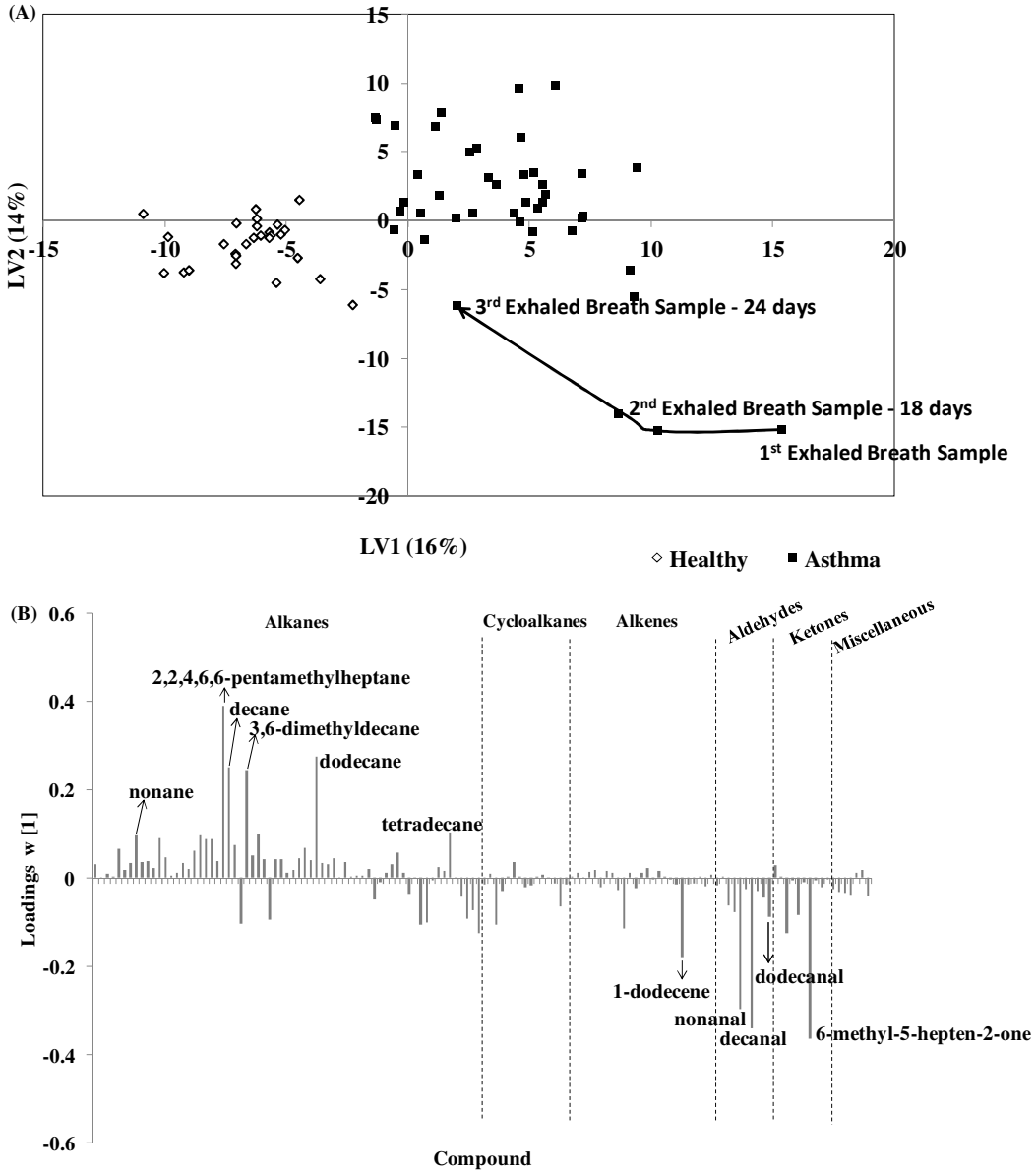


Figure 2.10 - PLS-DA LV1×LV2 scores scatter plot **(A)** and LV1 loading weights plot **(B)** showing the potentiality of exhaled breath using the full dataset of 134 compounds identified by GC×GC –ToFMS for allergic asthma and healthy children discrimination..

Figure 2.10 **A** that presents the scores scatter plot of LV1×LV2, while Figure 2.10 **B** (corresponding LV1 loading weights plot) establishes the contribution of each volatile compound that promotes the observed distinction.

Scores scatter plot (Figure 2.10 **A**) shows that the healthy group is associated to LV1 negative values whereas the allergic asthma group is linked to positive LV1 values. As observed in Figure 2.10 **B**, nonane, 2,2,4,6,6-pentamethylheptane, decane, 2-methyldecane, dodecane, and tetradecane are related to the allergic asthma group. The healthy group is mainly characterized by 6-methyl-5-hepten-2-one, 1-dodecene, nonanal, decanal, and dodecanal. Comparing these results to the previous study, there was an increase in the number of compounds that characterize allergic asthma and healthy samples. Interestingly the healthy children are characterized mainly by aldehydes and the asthmatic children are characterized by alkanes, namely those that arise from the corresponding aldehydes. Hence a pattern seems to be noticeable that mainly involves these two chemical families. The behaviour shown in the healthy group has also been reported by Corradi *et al.*[73] as nonanal had lower values in exhaled breath condensate of children with exacerbated asthma (before and after treatment) when compared to healthy. The behaviour of the remaining aldehydes compounds that have higher weight in the healthy group has not been previously described.

According to MCCV statistics, the PLS-DA model had a classification rate of 98% and showed 96% sensitivity (≅4% allergic asthma children being misclassified as healthy children) and 95% specificity (≅5% of false positives). The most frequent Q^2 value was around 0.9 (Figure 2.11), with a large prevalence of values in the range of 0.8-1. These results suggest that confounding factors, such as, ambient air, gender or age seems to have no significance in the distinction power.

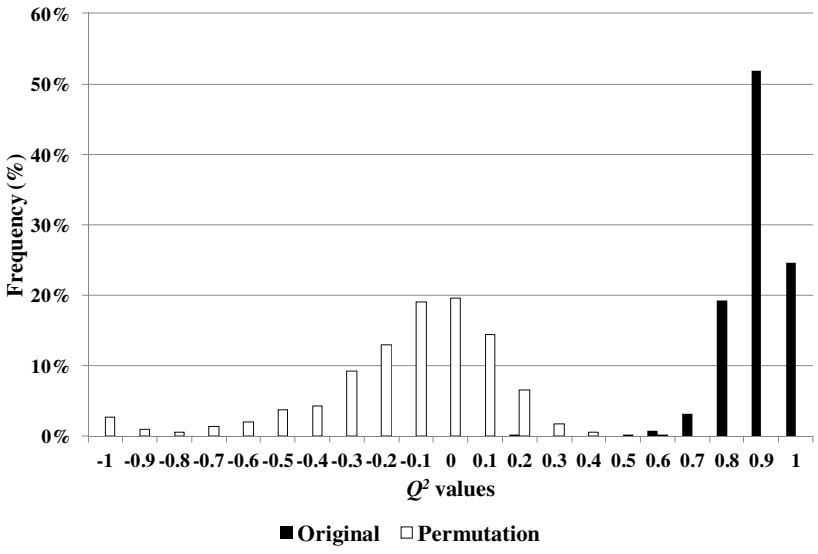


Figure 2.11 - Q^2 values distribution of the original and permuted Monte-Carlo Cross Validation for PLS-DA of exhaled breath of full dataset (134 compounds)

A relevant aspect brought by the results is that from the 67 identified alkanes 19 are methylated, which corresponds to 28% of this chemical family and from these, two methylated compounds, as for example 2,2,4,6,6-pentamethylheptane and 2-methyldecane have a major contribution in the observed distinction. The methylated alkanes family have also been previously reported as these may well be important in asthma characterization[13,74]. These compounds also have an important role in diseases, in which oxidative stress apparently may be involved, but to other extents, and with different consequences than asthma, such as lung and breast cancer, as well as in lung cancer cell lines[75,76,77]. These compounds have been reported in literature in other exhaled breath studies associated to pathological states of the lungs, but as individual markers. For example, 2,2,4,6,6-pentamethylheptane and decane were identified and compared by Poli *et al.*[78] in exhaled breath of patients with non-small lung cancer (NSLC), chronic obstructive pulmonary disease (COPD), smokers and controls and their concentrations were higher for NSLC, COPD and smokers when compared to controls. Dodecane has been proposed as lung cancer markers[79,80].

These results evidence that overall, for the allergic asthma group, there is a greater weight of the alkanes confirming the GC-MS study whereas aldehydes have a major importance in the characterization of the healthy group. Alkanes, in the sequence of oxidation reactions, are end-compounds that have been associated to oxidative stress and inflammation processes[81] and the hypothesis formed is that these compounds indicate that the oxidative state is at a higher extent in asthmatics when compared to healthy children leading to the obtained differences and consequently the alkanes can be associated to allergic asthma. These particular compounds can be formed in the inflammatory response induced by the immune system that leads to the production of activated leukocytes causing the cells to uptake oxygen releasing reactive oxygen species which can damage lung tissue contributing to elevated oxidative stress in asthma[82] and there is evidence that alkanes can arise as products of LPO of unsaturated fats[83]. Lipid metabolism and oxidative metabolism in the mitochondria have been reported recently to be altered in urine of asthma patients[84] and a conjecture could be made that this alteration shown in urine can also be noticed in exhaled breath, considering the alkanes as a measurement of lipid and oxidative metabolism that characterize the allergic asthma group.

"Breath-print" exploration as a potential aid to the clinical practice

Therapy monitoring is one of the challenges of actual medicine as patients may or may not follow treatment as prescribed by the physician. To test the hypothesis that a change

would occur in the exhaled breath composition with the intake of the prescribed medication, a naive patient (patient that had never taken an asthma drug) was recruited. This naive patient was diagnosed by physician as having allergic asthma, and exhaled breath was collected previous medication intake and three other moments after intake. The medication that was prescribed was the combination of anti-histamine and a leukotriene receptor antagonist. Anti-histamines are drugs that inhibit the action of histamine whereas leukotriene receptor antagonist inhibits leukotrienes that are compounds produced by the immune system that cause inflammation. This therapy combination directed to block the effects of histamine and leukotriene mediators was performed as it is shown that it is better than stand-alone therapy[85]. The behaviour of a naive child was monitored throughout 24 days (Figure 2.10 **A**, marked by the path trajectory). Initially and after 18 days after the intake of the prescribed drugs, the naive individual remains in LV1 positive values but far from the remaining controlled subjects. There is an evolution throughout LV1 axis with treatment administration explained by the area reduction of nonane, 2,2,4,6,6-pentamethylheptane, decane, 2-methyldecane, and tetradecane. Considering that asthma crisis is mainly characterized by inflammation, which is accompanied by oxidative stress and subsequently LPO, and that alkanes are evidence of these biochemical processes, the observed decrease may be due to a lesser inflammation state of this subject leading to lower areas of these compounds. Clinically, the child in the initial stages was in a crisis situation almost in a daily basis and throughout the 24 days there was a significant improvement of the asthma status control.

As verified, the obtained "breath-print" allowed the distinction between allergic asthmatic and healthy children which could be helpful in understanding this pathology through a better insight into the metabolic pathways that may be associated to this condition. Nevertheless, for clinical purposes, and having in mind the future of molecular diagnosis, a smaller set of compounds is necessary to allow a rapid use of exhaled breath for complementary purposes in diagnosis, to follow the disease status and/or the medication effect. For this intent, and taking into consideration the previous assertion that a pattern of alkanes and aldehydes clearly defines both populations under study, just 9 compounds (nonane, 2,2,4,6,6-pentamethylheptane, decane, 2-methyldecane, dodecane, tetradecane, nonanal, decanal, and dodecanal) were selected for a new PLS-DA model. The results are shown in Figure 2.12 and the chosen pattern was able to discriminate both groups showing the exhaled breath testing is a tool that can be used as non-invasive diagnostic method for allergic asthma.

A remarkable result observed in Figure 2.12 is shown by the path of the naive child (**a** through **d** - four breath sampling), which suggest the mitigation of asthma symptoms following drug intake. This suggests the application of exhaled breath analysis, not only for metabolomic profiling of allergic asthma, but also in clinical practice as a possible surrogate to the invasive diagnosis tests performed actually.

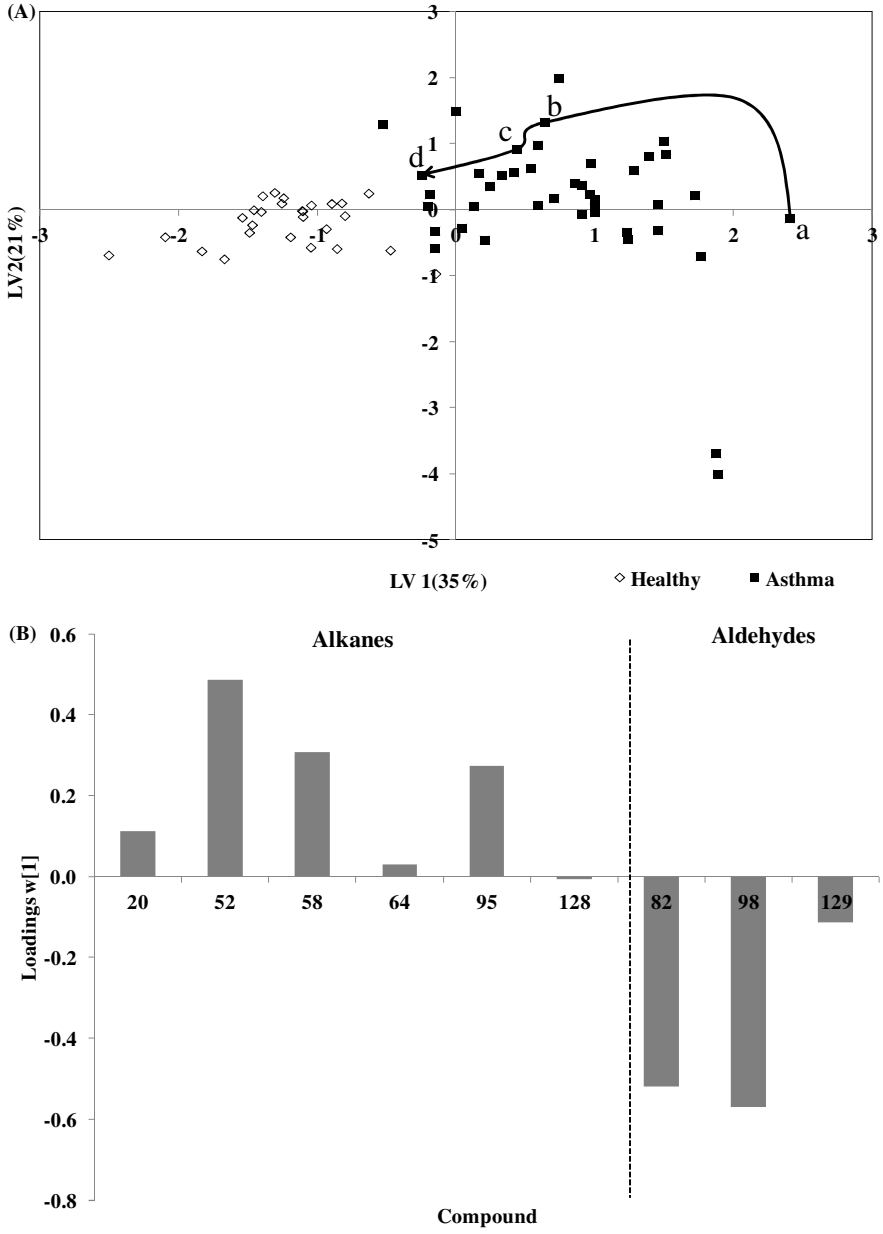


Figure 2.12 - PLS-DA LV1×LV2 scores scatter plot (A) showing the potentiality of using a sub-set of 9 compounds of exhaled breath in discrminating allergic asthma and healthy children and the corresponding LV1 loading weights plot (B): nonane (**20**), 2,2,4,6,6-pentamethylheptane (**52**), decane (**58**), 2-methyldecane (**64**), dodecane (**95**), tetradecane (**128**), nonanal (**82**), decanal (**98**), dodecanal (**129**). Path of the naive child - **a** through **d** - four breath sampling

To assess both the predictive power and classification model robustness, MCCV was also performed, using similar conditions to the previous test. According to MCCV statistics, the PLS-DA model had a classification rate of 96% and showed 98% sensitivity (≅ 2% allergic asthma children being misclassified as healthy) and 93% specificity (≅7% of false positives). The most frequent Q^2 value was around 0.8 (Figure 2.13), with a large prevalence of values in the range of 0.7-0.9. Classification rate and specificity were slightly lower than those obtain for the full dataset, but, still, remained high. The sensitivity was improved. These results suggest that the model is robust, even using this set of 9 compounds, reducing the time of data processing, and thus, becoming more expedite method for the clinical purposes.

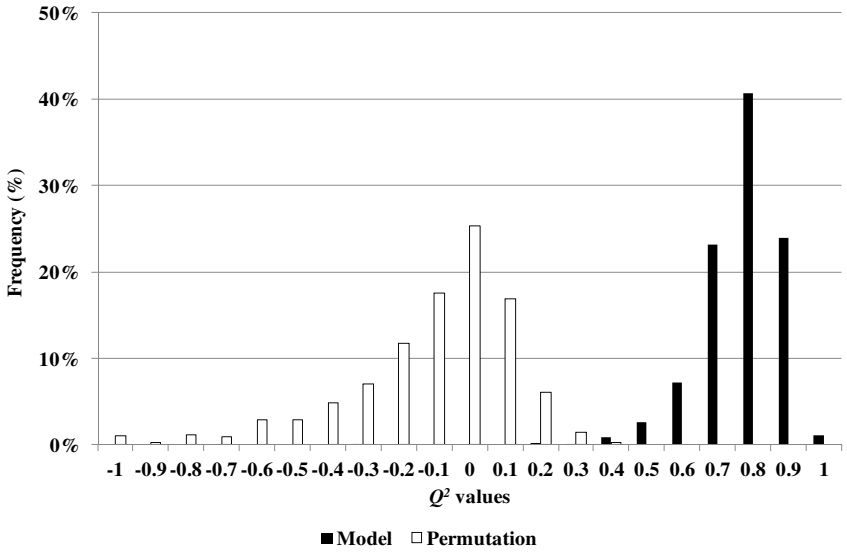


Figure 2.13 - Q^2 values distribution of the original and permuted Monte-Carlo Cross Validation for PLS-DA of exhaled breath of sub-set of 9 compounds: nonane, 2,2,4,6,6-pentamethylheptane, decane, 2-methyldecane, dodecane, tetradecane, nonanal, decanal, and dodecanal

2.3.5 Concluding remarks

In this study, the development of the first HS–SPME/GC×GC–ToFMS methodology was reported for the analysis of exhaled breath of allergic asthma children and the advantages of comprehensive chromatography was explored in issues such as the structured chromatogram and sensitivity. The structured 2D chromatogram that arose from ^{1}D volatility and ^{2}D polarity was shown and sensitivity was assessed. A well-defined chromatographic space was obtained with the resulting structured chromatogram, which can aid posterior

exhaled breath analysis for example in the identification of otherwise unknown compounds. Subsequently, the potentiality of the GC×GC–ToFMS was verified in exhaled breath samples from allergic asthma and healthy children.

The methodology allowed the identification of several hundred compounds belonging to different chemical families (linear and ramified alkanes, cycloalkanes, alkenes, aldehydes, ketones, aromatic compounds, terpenoids and esters). Multivariate analysis was performed by PLS-DA to a group of selected compounds pertaining to alkanes, alkenes, aldehydes, and ketones and the GC×GC–ToFMS showed to be advantageous as distinction between both groups was attained and a high classification rate was achieved. The obtained "breath-print" allowed the discrimination between allergic asthmatic and healthy children, providing insights into the metabolic pathways that may be associated to allergic asthma. In general, a pattern of six compounds pertaining to the alkanes characterized the asthmatic population: 2-methyldecane, nonane, 2,2,4,6,6-pentamethylheptane, decane, dodecane, and tetradecane. Otherwise, a set of aldehydes (nonanal, decanal, and dodecanal) characterizes the healthy population. Thus, a smaller set of 9 compounds comprising alkanes and aldehydes was chosen to verify the potential clinical usefulness of exhaled breath for allergic asthma evaluation and the obtained results are very satisfactory as, with this set, distinction was obtained. It was also confirmed that it is also possible to follow through the effects of medication.

Exhaled breath metabolome presents itself as a challenge, and in our opinion, GC×GC–ToFMS offers advantages that were verified in the present study that corresponded to the challenge. This new methodological approach to characterize allergic asthma as a function of its metabolomic patterns will enhance the possibility of further allergic asthma pathways knowledge. It also provides with an easier methodology combined with a non-invasive sampling for allergic respiratory disease assessment, regarding diagnostic, prognostic and treatment follow-up. Further studies with a larger population are necessary to confirm these findings.

2.5 The unexplored potential of exhaled breath analysis in the aid of asthma clinical management

2.5.1 Abstract

Molecular diagnosis is the epitome of personalized medicine where physicians and biochemists play a crucial role in the development of tools for a more rational and objective disease monitoring and screening. The main goal of this research study was to use of previously developed breath based metabolomic models in asthma disease control, diagnosis and monitoring using a new cohort of children with asthma, attending on the secondary health care consultations. The ability of using these exhaled breath models in a clinical scenario was tentatively performed to monitor the clinical outcome of three children in an exacerbation condition, and in addition, to follow the Omalizumab therapy effect on a patient with severe asthma. SPME combined with GC×GC-ToFMS was used to recover the breath volatile metabolomic signature. A supervised multivariate analysis technique was used to extract signal features, to tentatively characterize the disease evolution and to predict the outcome. MCCV was applied to the PLS-DA models to evaluate the sensitivity and specificity levels and to assess classification model robustness. The prediction tool using the reported models was successful based on classification rate, sensitivity and specificity values (≥ 93%). Aldehydes and alkanes, end-products of lipid peroxidation, known to be involved in asthma oxidative stress, seems to be increased in exacerbation state compared to stable state. Furthermore, Omalizumab therapy promoted changes in the patient exhaled breath profile over time, namely with the decrease of nonane, 2,2,4,6,6-pentamethylheptane, decane, 2-methyldecane, dodecane, and tetradecane. Exhaled breath sampling combined with comprehensive two-dimensional gas chromatography and multivariate feature extraction, represents a non-invasive and rapid tool allowing the molecular data recovering, useful to monitor the disease status as a part of the medical treatment or even its interruption, i.e., the adherence to medication.

2.5.2 Framework

Exhaled breath is a realistic matrix to be studied in asthma as it contains metabolites whose relative concentrations may be altered by the disease[81] echoing the metabolic activity within the airways. The study of exhaled breath, known as *breathomics*, is an emerging research field focused in health. Usually, method development is crucial and it finds a pattern of metabolites related to the abnormal metabolic processes that occurs with the studied disease[86]. The volatile analysis of exhaled breath analysis has captured interest in clinical practice87, since it comprises thousands of volatile organic metabolites that are expelled with each breath one exhale, making it a high potential non-invasive source for medical purposes in lung related diseases, such as asthma, among others[74]. The exhaled breath volatile composition has been relevant in this field shown by many studies that have been reported on exhaled breath of asthmatic patients[13].

The aim was to explore the potential of exhaled breath analysis in the aid of asthma clinical management (diagnosis and monitoring) using non-invasive sampling methodologies developed in our laboratory that combines the SPME with a high throughput technology such as GC×GC-ToFMS in tandem with PLS-DA. Firstly, the applicability of the previous developed breath metabolomics based models was done using a new cohort of asthmatic children (n=49) attending on the secondary health care consultations. Also, the ability of using these models of exhaled breath in a medical scenario was tentatively done to gauge the clinical improvement of three children in an exacerbation condition, and to follow the Omalizumab therapy effect on one patient with severe asthma. MCCV was applied to the PLS-DA models to evaluate the sensitivity and specificity levels and to assess classification model robustness.

2.5.3 Material and methods

Patients clinical history and samples collection schedule

A group of 49 children (4-18 years) with allergic asthma, from which 25 had allergic asthma and allergic rhinitis, volunteered for this study. The 49 individuals correspond to a total of 74 exhaled breath samples. The general traits of the patients are presented in Table 2.6.

Table 2.6 - Traits of the allergic asthma population.

					Allergic asthma (*n=49*)
Age and Gender					
Age in years (range/median)					4-18/8
Gender (male/female)					28/21
Pathology					
Allergic Asthma					24 (49%)
Allergic Asthma + Allergic Rhinitis					25 (51%)
Allergens[a]					
Dust mite					26 (53%)
Dust mite + gramineae					11 (23%)
Gramineae					3 (6%)
Dust mite + cat fur + gramineae					2 (4%)
Dust mite + dog fur					1 (2%)
Dust mite + eucalyptus					1 (2%)
Dust mite + platan leaves					1 (2%)
Dust mite + olive tree pollen					1 (2%)
Gramineae + cat fur + dog fur					1 (2%)
Birch pollen					1 (2%)
Gramineae + weeds					1 (2%)
Therapy					
CCS	LTRA	BRC	ATH	NCCS	
x	x	x	x	x	1 (2%)
x	x	x	-	-	2 (4%)
x	-	x	-	-	5 (10%)
x	x	-	-	x	1 (2%)
x	-	x	-	x	1 (2%)
-	x	-	-	-	1 (2%)
x	-	-	-	-	4 (8%)
x	x	-	-	-	7 (15%)
x	-	-	x	-	1 (2%)
-	-	-	x	-	1 (2%)
-	x	-	x	x	4 (8%)
-	-	x	-	x	1 (2%)
-	-	-	x	x	8 (17%)
-	x	-	x	-	3 (6%)
-	x	-	-	x	3 (6%)
No therapy					6 (12%)

[a] Results obtained by prick-tests

Usually, each individual corresponds to one breath sample; nevertheless, some volunteers had some pertinent asthma conditions that were further studied, and hence, for that reason were sampled in different moments: *i)* all patient were analysed in asthma stable state, *ii)* three patients were analysed in stable state and in asthma exacerbation condition, and *iii)* Omalizumab therapy adherence was follow-up just for one patient.

The group of three patients studied under stable and exacerbation condition included a 5 years old male Caucasian child that had never taken an asthma drug and was diagnosed by physicians with allergic asthma and rhinitis based on symptoms history and skin prick tests were performed being positive for dust mites and gramineae. After the first consult, this child was prescribed a combination of corticosteroids anti-histamine, a nasal corticosteroid and a bronchodilator (when in exacerbation). A 7 years old female Caucasian child diagnosed with allergic asthma with skin prick tests positive for dust mites, cat and dog fur and using a combination of corticosteroids and bronchodilator (when in exacerbation) was recruited to follow the disease status throughout 3 months, from a exacerbation situation to a controlled asthma status. For this patient the effect of nebulisation (salbutamol) was also verified with two exhaled breath samples being collected before and after the nebulisation procedure. Also, a child whose exhaled breath was collected over a three year period was a 12 years old female Caucasian diagnosed with allergic asthma and rhinitis. The allergen, determined by skin prick tests and confirmed by allergen-specific Immunoglobulin E blood test, that this child is sensitized to are dust mites and the prescribed medication is a combination of leukotriene receptor antagonist, corticosteroids and bronchodilator (when in exarcebation).

For the Omalizumab study, a recruited patient was an 18 years old female white Caucasian diagnosed by physicians with severe persistent asthma and rhinitis. Skin prick tests were performed and showed sensitization to dust mites (*Dermatophagoides farinae*), storage mites (*Lepidoglyphus destructor*) and grass pollens (*Dactylis glomerata, Festuca elatior, Loliumperenne, Phleum pratense, Poa pratensis*). Appropriate therapy was prescribed associating inhaled fluticasone/salmeterol (500/100 μg/daily), and as rescue therapy salbutamol (400 to 1200 μg/daily). To control rhinitis symptoms 5 mg desloratadine/daily budesonide nasal spray and 64 μg/daily was prescribed. As the severe asthma symptoms persisted, although subcutaneous specific immunotherapy and corticosteroids in higher doses were performed, 600 mg of Omalizumab was initiated and administered fortnightly via subcutaneous in the deltoid region of both arms and both anterior thighs. In this case, exhaled breath collection (two samples each time) started 4 months after the Omalizumab therapy initiation. Omalizumab therapy was performed in accordance to physician prescription, i.e.,

twice a month, until the first six months and half. After that period until the last collection (20^{th} month), the therapy was performed intermittently due to the fact that the patient was not compliant, and only performed 13 out of 26 treatments. Each child was doing their specific therapy and was in different clinical conditions. Also, the time of day at which samples were collected was variable (depending on the moment of the medical consultation) and there were no dietary or pharmacologic restrictions, as these conditions are more representative of the reality demonstrating the applicability of the developed methodologies in the clinical context.

Breath sampling and analysis

Exhaled breath was collected in 1 L Tedlar® bags. The breath sampling parameters are reported in Chapter 2.1. At each sampling moment, the patient was asked to cleanse her mouth with water before sampling. Subsequently, the patient was instructed to inhale and exhaled normally and then exhale deeply using a disposable mouthpiece into the Tedlar® bag previously holding her breath for *ca.* 5s. As breath samples were not CO_2-controlled, each breath represents a single mixed expiratory sample, and was analysed once. The SPME fibre (DVB/CAR/PDMS) was introduced in the Tedlar® sampling bag for 60 min, at 22 ºC to extract breath metabolites. Following the extraction procedure, the SPME fibre was retracted from the bag and inserted into the GC×GC-ToFMS injection port. The instrumental details were described in Chapter 2.2. Briefly, GC×GC–ToFMS LECO Pegasus 4D (LECO, St. Joseph, MI, USA) consists of an Agilent GC 7890A gas chromatograph (Agilent Technologies, Inc., Wilmington, DE), with a dual stage jet cryogenic modulator (licensed from Zoex) and a secondary oven, and mass spectrometer equipped with a high resolution ToF analyzer. An HP-5 column (30 m × 0.32 mm I.D., 0.25 µm film thickness, 5% Phenyl-methylpolysiloxane, J&W Scientific Inc., Folsom, CA, USA) was used as ^{1}D column (first dimension) and a DB-FFAP (0.79 m × 0.25 mm I.D., 0.25 µm film thickness, nitroterephthalic acid modified polyethylene glycol, J&W Scientific Inc., Folsom, CA, USA) was used as ^{2}D column (second dimension). The carrier gas was helium at a constant flow rate of 2.50 mL/min. The GC×GC–ToFMS injection port was at 250 °C. The primary oven temperature program was: initial temperature 35 ºC (hold 1 min), raised to 40 ºC (1 ºC/min), and finally rose to 220 ºC (7 ºC/min) and hold for 1 min. The secondary oven temperature program was 15 ºC offset above the primary oven. The MS transfer line temperature was 250 ºC and the MS source temperature was 250 ºC. A 6 s modulation time with a 30 ºC secondary oven temperature offset (above secondary oven) was chosen. The ToFMS was operated at a spectrum storage rate of 125 spectra/s. The mass

spectrometer was operated in the EI mode at 70 eV using a range of *m/z* 35-350 and the detector voltage was -1695 V. The GC×GC area data was used as an approach to estimate the relative content of each volatile component of exhaled breath.

Multivariate Analysis

PLS-DA[8] is a widely used procedure for classification purposes, where the MCCV framework was used to assess the predictive power and classification models robustness. The PLS-DA models previously developed (Chapter 2.2) for distinction between asthmatic and healthy children were used as starting-point for prediction purposes in this study. The MCCV had a classification rate of 96%, and showed 98% of sensitivity (~ 2% allergic asthma children being misclassified as controls) and 93% of specificity ~7% of false positives). For the projection purposes, a dataset comprising 130 metabolites (Table S1 in *Appendix*) and a data set of 9 metabolites (nonane, 2,2,4,6,6-pentamethylheptane, decane, 2-methyldecane, dodecane, tetradecane, nonanal, decanal, and dodecanal), previously established in Chapter 2.2 as relevant for asthma diagnosis were used. The projections were performed for both datasets 130 and 9 metabolites tentatively identified by HS-SPME/GC×GC-ToFMS, using the previously developed and validated PLS-DA models (Chapter 2.4), by applying the regression models to new dataset of 62 asthmatic children breath samples (corresponding to 49 individuals) to obtain the response variable. Each sample was mean normalized and UV (unit variance) scaled.

Model robustness was assessed using MCCV with 500 iterations. 60 % of the data comprised the calibration set whilst the remaining 40% were the validation set. Then, each iteration, with randomly changed composition of the calibration and validation sets, performed internal cross validation of the calibration set using seven blocks and prediction of class membership for samples in the validation set. For each of the 500 randomly generated classification models, the number of LVs, the Q^2 (expressing the cross-validated explained variability), and the confusion matrix was computed. The selection of model complexity was based on the most frequent list of model properties that maximizes the predictive power (i.e., lower LV and higher Q^2). The sensitivity and the specificity of the model were then depicted from the confusion matrix resulting into a ROC space to further assess the results significance. Then, the same procedure was applied using permuted class membership. Sensitivity is calculated from the ratio between true positives (asthma samples correctly

predicted) and the total number of modelled breath samples, whereas specificity is determined from the ratio between true negatives (control samples correctly predicted) and the total number of modelled control GC×GC data.

2.5.4 Results and Discussion

PLS-DA model prediction: applicability of developed breath metabolomics based model

Taking into consideration the previous developed breath metabolomics based model in chapter 2.4, a new group of children with asthma (coded as Asthma Projection) was studied and the robustness of the developed classification model was confirmed (Figure 2.14 **A**).

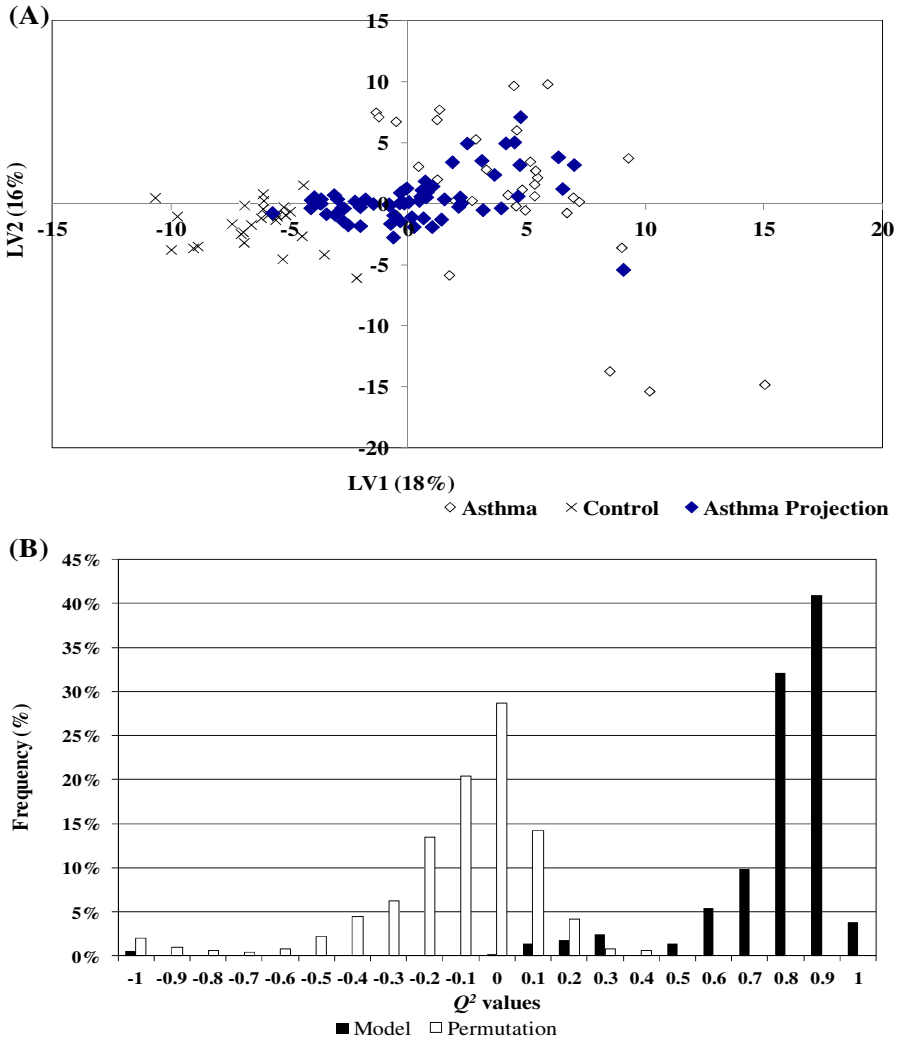


Figure 2.14 – (**A**) Projection of new exhaled breath dataset using the breath metabolomics based model of chapter 2.4 for asthmatic and healthy children and (**B**) validation of the obtained results Q^2 values distribution of the original and permuted MCCV (1000 permutations).

Using a full dataset, scores scatter plot (Figure 2.14 **A**) shows that the healthy group is associated to LV1 negative values whereas the asthma group is mainly linked to positive LV1 values. The new set of 49 asthmatic children were projected mainly along the LV1 positive values, close to model asthma children population.

The validation of PLS-DA models has been thoroughly studied to verify the quality of the obtained discrimination models[88]. Due to the large number of variables, the chance of false correlations and the risk of overfit are high[89]. Several diagnostic tools have been developed, among them cross validation, such as MCCV, first reported by Cook[90] and tested for chemometric purposes by Xu and Liang[91]. MCCV can avoid an unnecessary large model and therefore decreases the risk of over-fitting for the calibration model. MCCV splits the data into a learning set or a test set and the model developed on the learning set and the error evaluated in the test set. The test set estimates are averaged over the learning-testing random splits and each case only appears in the learning set or the test set, but not in both[92]. MCCV is used in assessing the robustness and accuracy of PLS-DA models being used in different fields[93,94,95]. It has been reported that MCCV is a valuable and effective tool in estimating model complexity[91]. According to MCCV statistics for projection analysis (figure 1b), the PLS-DA model had a classification rate of 98%, showed 100% sensitivity (no asthmatic children being misclassified as controls) 93% specificity (~7% of false positives) The prediction error measure Q^2 is based on the evaluation of the error between the predicted categorical variable ŷ and the known y. It focuses on how well the class label can be predicted from new data depending on class separation but also on within class variability[88]. The most frequent Q^2 value in MCCV statistics was around 0.9 whilst the model Q^2 was 0.8. In spite the addition of new asthmatic exhaled breath samples, the classification rate and most frequent Q^2 values were the same and the sensitivity increased by 4%. However, the specificity decreased by 2% when compared with the previously developed model. Despite these asthmatic children presented a stable asthma status, this group is relatively heterogeneous since it contains patients with severe and non-severe asthma, also different therapies were considered, which may explain the dispersion of the data along LV1.

Using the sub-set of 9 compounds reported in chapter 2.4 as relevant for asthma diagnosis, it was observed that the asthmatic children were projected mainly in LV1 positive (Figure 2.15 **A**), close to model asthma children population. According to MCCV statistics for projection analysis (Figure 2.15 **B**), the PLS-DA model had a classification rate of 96% and showed 96% sensitivity (~ 4% allergic asthma children being misclassified as controls) and

96% specificity (~4% of false positives). The most frequent Q^2 value was around 0.7 with a large prevalence of values in the range of 0.6-0.8 whilst the model Q^2 was 0.7. Comparing these results with the previously reported method we observed that the classification rate was identical, a decrease in sensitivity by 2% and an increase of 3% in specificity. The most frequent Q^2 value decreased as the previously reported value was 0.8. The high predictive power and robustness of these results and those obtained for the previous data projection (Figure 2.15 **B**) suggest that confounding factors, such as, ambient air, gender or age seems to have a low or even no influence in the discrimination power.

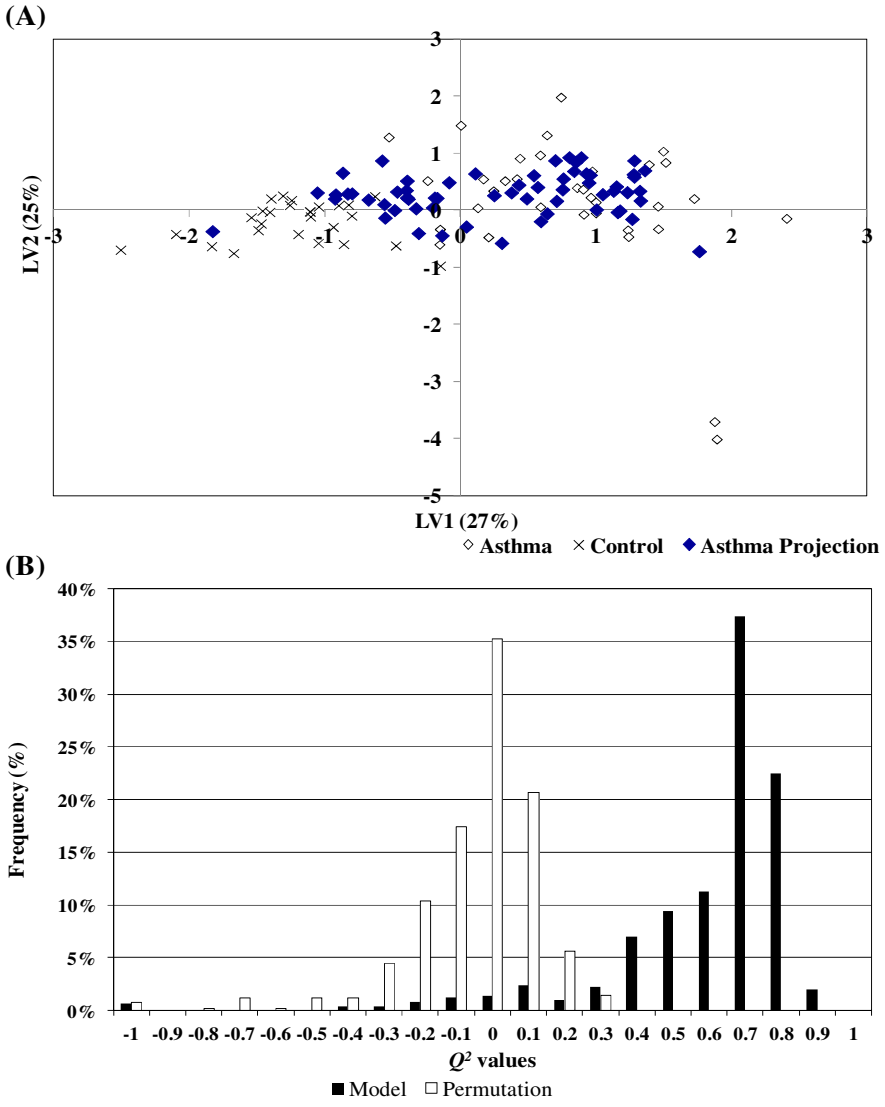


Figure 2.15 – (**A**) Projection of new exhaled breath dataset using the breath metabolomics based model of chapter 2.4 using a sub-set of 9 compounds (nonane, 2,2,4,6,6-pentamethylheptane, decane, 2-methyldecane, dodecane, tetradecane, nonanal, decanal, and dodecanal) and (B) validation of the obtained results Q^2 values distribution of the original and permuted MCCV (1000 permutations).

Also, Figures 2.16 **A** and 2.16 **B** show that the main factor that explains the classes discrimination is the disease versus healthy condition, and within each class, individuals with different age or breath collected at different location are represented, indicating that these possible confounding factors seem not be relevant.

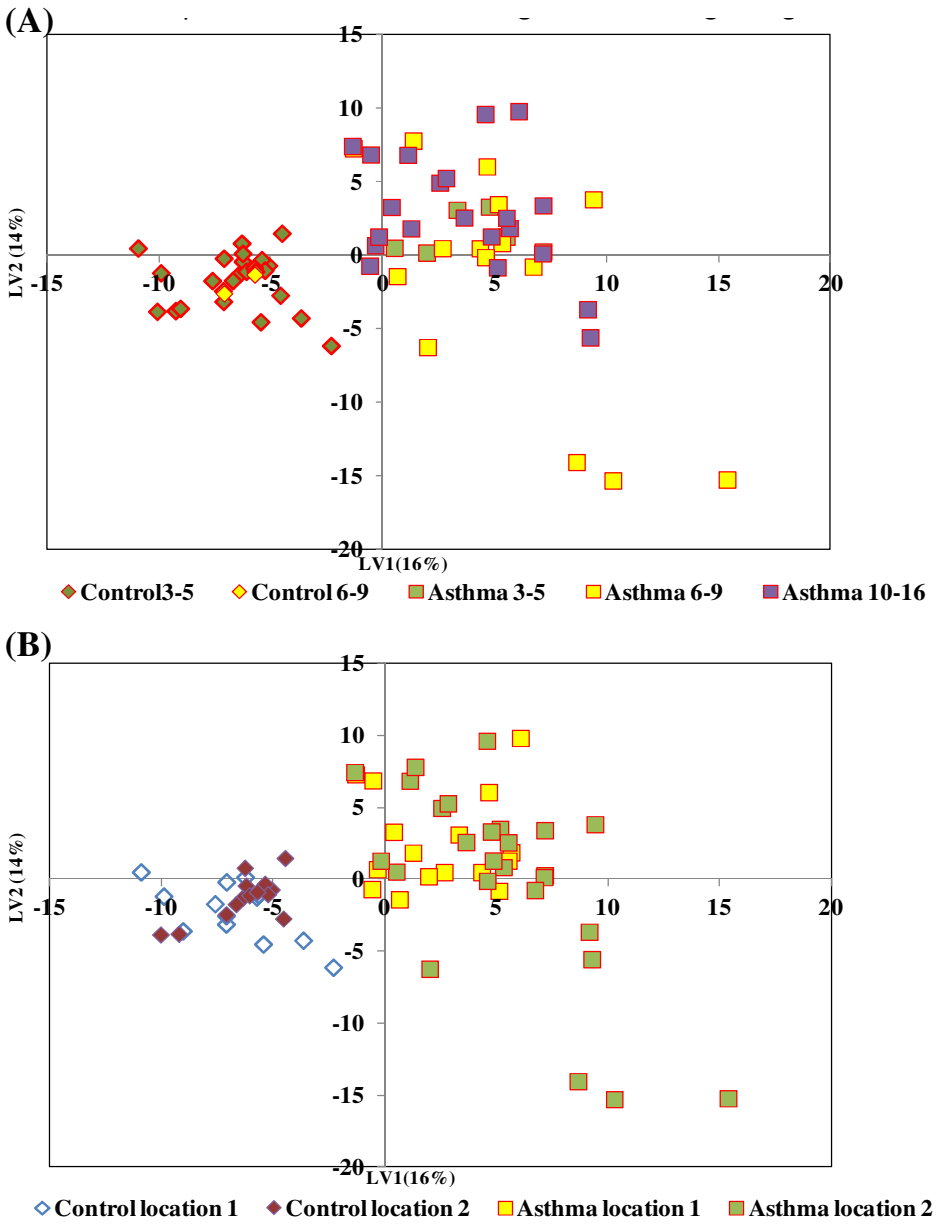


Figure 2.16- PLS-DA LV1×LV2 scores scatter plot of exhaled breath for asthmatic and healthy children using a data set with 130 compounds coloured according to **(A)** age range (3 to 5 years old, 6 to 9 years old and 10 to 16 years old) and (**B**) according to exhaled breath collection site demonstrating no influence on the discrimination. Figures based on metabolomic PLS-DA based models previously developed.

Asthma clinical management through breath analysis: exacerbation condition

The ability of the developed models was tested regarding a relevant condition for the asthma clinical management, i.e., the disease state evaluation (stable state versus exacerbation condition). Exacerbation condition may promote several lung damages, and the evaluation and understanding of the alterations associated represent a significant improvement in the asthma management. Breath from an asthmatic child (IND) was collected throughout three years, corresponding to 10 breath samples, and Figure 2.17 **A** represents the volatile composition of six alkanes, that characterize asthma population, on those 10 sampling moments. These data were normalized against the median of the corresponding data of the healthy group. Figure 2.17 **B** depicts the plot of the predict Y-value (data from model plus 10 breath samples projection) obtained from built PLS-DA model, against the reference Y-value (group classification, 0 for healthy and 1 for asthmatic population).

According to Figure 2.17 **A**, slight variations were observed throughout this period of time. Relevant changes may be noticed when the child was in exacerbation, highlighting mainly the alterations on the relative content of nonane, decane and 2-methyldecane. Nevertheless when observing the predicted Y values from figure 2.17 **B** we can verify that these breath samples are still included in the asthma group, suggesting that despite the intra-individual variability observed, the asthma pathology superimposes the intra-individual variability that might occur due to all external factors that cannot be controlled. These results also showed that alkane levels increased in exacerbation condition compared to control state, indicating the inflammation and lipid peroxidation are associated to this condition, as previously reported by Loureiro *et al.* [96].

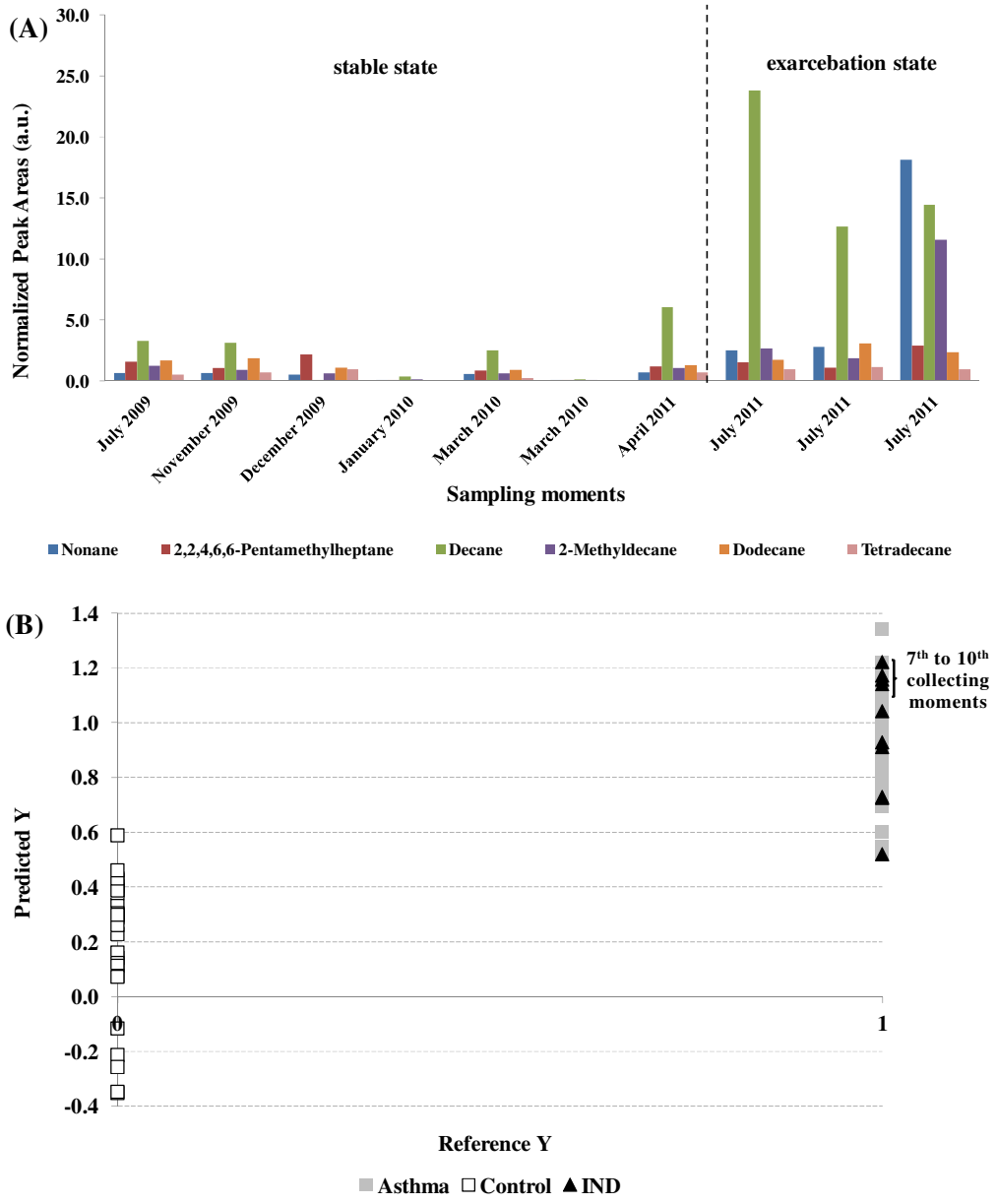


Figure 2.17 – (**A**) Alkane variations for 10 breath samples collected for three years in the same asthmatic child based on the built PLS-DA model with two LVs and (**B**) predicted Y value against reference Y value behavior. a.u. arbitrary units.

To verify the usefulness of the methodology under study, two other case-studies were evaluated. Breath samples of two children in exacerbation (E1 and E2) were sampled in different sampling moments. Stable state (S1 and S2) exhaled breaths were also collected. As verified in Figure 2.18, both patients showed an evolution along LV1 axis from an asthmatic volatile profile toward the healthy group.

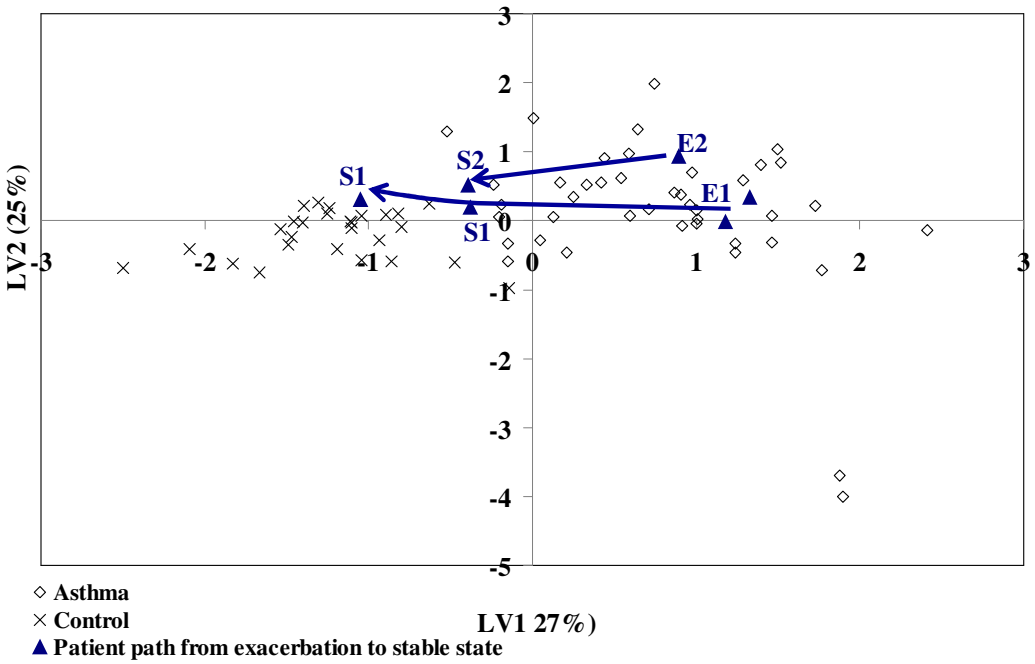


Figure 2.18 - PLS-DA LV1×LV2 scores scatter plot of exhaled breath for asthmatic and healthy children using the 9 compounds subset and projection of new exhaled breath dataset (state sate and exacerbation conditions for two patients). The path of two children in exacerbation condition (**E**) and stable state (**S**) is shown, through different sampling times. E1 and E2 are the initial exhaled breath samples: S1 exhaled breath was collected twice: 1 month and half and 3 months and nine days after the initial samples. S2 was collected 3 months and half later after the initial sampling

The volatile composition behaviour was accompanied by the clinical improvement of both patients with treatment administration. Considering the breath profile, it was observed a relative area reduction of the alkanes. E1 exhaled breath was collected twice (separated by an hour from each other) in the first consult as a nebulisation procedure (only using salbutamol) was performed. It was found that, although from a clinical stand-point there was a slight improvement, the breath samples had similar composition leading to the conclusion that although the improvement of the patient clinical status, the biochemical processes inherent to asthma change slowly and herein there are no differences in the exhaled breath composition that was collected.

Omalizumab therapy adherence: Evaluation of breath sampling relation and patient's clinical status

Patients with severe persistent asthma are at high risk of serious exacerbations and asthma-related mortality and treatment with Omalizumab has shown a significant reduction of

asthma exacerbations and emergency visits[97]. The recruited patient was an 18 years old white Caucasian female diagnosed by physicians with severe persistent asthma and rhinitis. Omalizumab therapy was proposed, as the patient severe asthma status was persistent and respiratory function deterioration was observed. The response to this therapy was assessed by symptoms severity and frequency, such as exacerbations, emergency room visit/unscheduled consultations, medication increase and school absenteeism. Figure 2.19 **A** shows the Omalizumab therapy monthly frequency monitoring and Figure 2.19 **B** represents the PLS-DA LV1×LV2 scores scatter plot of exhaled breath for asthmatic and healthy children highlighting the path of the severe asthma patient throughout the therapy.

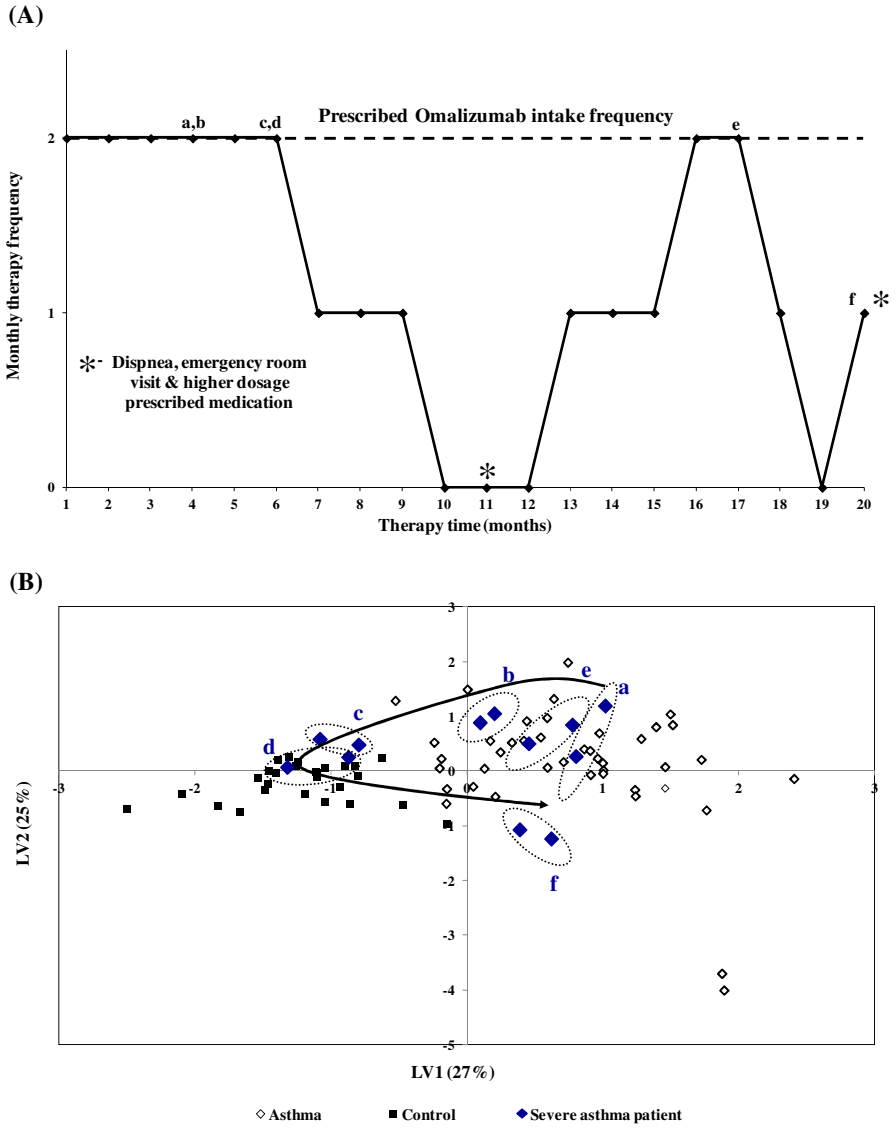


Figure 2.19 - (A) Omalizumab therapy monthly frequency monitoring and **(B)** PLS-DA LV1×LV2 scores scatter plot of exhaled breath for asthmatic and healthy children using a sub-set of 9 compounds. Path of the severe asthma patient is highlighted - A through F sampling moments: 4 months (**a**), 4 months and half (**b**), 6 months (**c**), 6 months and half (**d**), 17 months (**e**), and 20 months (**f**) after Omalizumab initial treatment

There was a significant improvement in the clinical status of the patient by the second month of Omalizumab therapy and there was no need to use the prescribed corticosteroid medication, β–2-agonist, and the patient did not attend the emergency room. However, there was an irregular adherence to Omalizumab therapy from the sixth month and half, and for the subsequent two months whilst between the tenth and twelfth month no therapy was performed (Figure 2.19 **A**). During this period, there was a progressive deterioration of health status with daily episodes of breathlessness, and the prescribed medication had to be used in higher dosages (preventive β–2-agonist and an inhaled corticosteroid). Also, during this period the patient had to go to the emergency room for treatment. The same behaviour was observed in the nineteenth month, with the patient not adhering to treatment and this reflected in a deterioration of the health status and an emergency room visit on the twentieth month (Figure 2.19 **A**.

The patient was in the twentieth month of Omalizumab therapy, and the irregularity in which therapy is performed, affects the outcome with progressive deterioration of the patient health status with subsequent aggravation in symptoms severity and frequency. In the periods that the patient adhered to medication there was clinical improvement without nocturnal exacerbation or effort dyspnea, an improvement of rhinitis symptoms and from the respiratory point of view there is a slight functional improvement.

The hypothesis was to verify if the effect of the therapy on the clinical status could be followed through exhaled breath analysis. Using the developed model reported in Chapter 2.4 that included the screening of 9 compounds associated to oxidative stress, a prediction was made and a interesting result is observed by the path of the patient (Figure 2.19 **B**, a through f – six breath sampling), in which Omalizumab intake can be followed throughout LV1 axis. For the first four and half months, this patient had an asthmatic volatile profile and then after six months of therapy her volatile profile converged towards a control volatile signature. This behaviour was also accompanied by a clinical improvement throughout this period. The tendency along LV1 axis with treatment administration can be explained by the relative area reduction of all alkanes, with a higher prevalence for nonane, decane and 2-methyldecane. These alkanes are an indication of oxidative stress and subsequently lipid peroxidation and the decrease may be due to a reduced inflammation state of this subject leading to lower relative amount of these compounds. After seventeen and twenty months of treatment (Figure 2.19 **B**, e and f), a pathway relapse was observed in the patient behaviour (irregular adherence to Omalizumab therapy). The patient missed several treatments; however it was possible to confirm this behaviour based on the exhaled breath composition. The alkanes signal

increased, mostly by dodecane. According to LV1, the patient distanced herself from the healthy group (Figure 2.19 **B**) and from a clinical point-of-view the patient health status deteriorated. These results show the potential applicability of exhaled breath in the clinical practice for drug follow-up and health status monitoring showing inflammatory changes that current physiologic or functional tests might not always reflect.

2.5.5 Concluding remarks

Multivariate analysis of exhaled breath sampling combined with two-dimensional gas chromatography based methodology represents a non-invasive and rapid (ca. 2 hours) tool allowing the recovery of molecular data, useful for diagnosis and monitoring asthma. The results obtained using the breath metabolomics based models reported in Chapter 2.4 confirmed the usefulness of this methodology. Breath metabolic composition was highly altered in asthmatic population compared to healthy ones used as control. Also, relevant changes may be noticed in the exacerbation condition compared to the stable state, and the Omalizumab therapy also promoted modifications on breath metabolomics. Indeed, the alkanes and aldehydes profile indicate an oxidative state and have been found consistently altered in asthmatic population. Within this population, in the exacerbated state compared to the stable condition the behaviour is similar. The prospect to follow-up the individual response was very interesting, as it unlocks potentialities as the therapy evaluation and adjusting. For instance, it was observed for the child with severe asthma that only the fortnightly Omalizumab therapy intake promoted the decrease of inflammation and oxidative stress level. The clinical observations are in accordance to the breath alkane pattern.

Although requiring validation using a much larger sample cohort, these results clearly show the potential of this methodology for future clinical assessments, allowing timely therapeutic approaches and/or future antioxidant therapies to prevent the broad damaging effects associated to asthma. The obtained results support the up-rising issue of personalized medicine and the implementation of this methodology in a hospital laboratory, as well as, the use of exhaled breath in clinical settings could be easily achieved.

2.6 References

(1) Lim M. D., Dickherber A., Before you analyze a human specimen, think quality, variability, and bias. Anal. Chem. (2011) 83 8-13.

(2) Pauling L., Robinson A.B., Teranishi R., Cary P., Quantitative analysis of urine vapor and breath by gas-liquid partition chromatography. Proc. Natl. Acad. Sci. (1971) 68: 2374-2376.

(3) Ligor T., Ligor M., Amann A., Ager C., Bachler M., Dzien A., Buszewski B., The analysis of healthy volunteers' exhaled breath by the use of solid-phase microextraction and GC-MS. J. Breath Res. (2008) 2: 046006.

(4) Schwarz K., Filipaik W., Amann A., J. Breath Res., Determining concentration patterns of volatile compounds in exhaled breath by PTR-MS. (2009) 3: 027002.

(5) Mashir A., Dweik R.A., Exhaled breath analysis: The new interface between medicine and engineering. Adv. Powder Technol. (2009) 20: 420-425.

(6) Mochalski P., Wzorek B., Sliwka I., Amann A., Suitability of different polymer bags for storage of volatile sulphur compounds relevant to breath analysis. J. Chromatogr. B, (2009) 877: 189-196.

(7) Van Berkel J.J., Dallinga J.W., Moller G-M., Godschalk R.W.L., Moonen E., Wouters E.F.M., Van Schooten F.J., Development of accurate classification method based on the analysis of volatile organic compounds from human exhaled air. J. Chromatogr. B. (2008) 861: 101-107.

(8) Eriksson, L., Johansson, E., Kettaneh, N., Wold, N., Wold, S., Multi and Megavariate Data Analysis, Principles and Applications (2001) Umetrics AB Umea, Sweden.

(9) Song G., Qin T., Liu H., Xu G.B., Pan Y.P., Xiong F.X., Gu K.S., Sun G.P., Chen Z.D., Quantitative breath analysis of volatile organic compounds of lung cancer patients. (2010) Lung Cancer 67: 227-231

(10) Gaspar E.M., Lucena A.F., da Costa J. D., das Neves H. C., Organic metabolites in exhaled human breath--a multivariate approach for identification of biomarkers in lung disorders. J. Chromatogr. A, (2009) 1216: 2749-2756.

(11) Yu H., Xu L., Wang P., Solid phase microextraction for analysis of alkanes and aromatic hydrocarbons in human breath. J. Chromatogr. B, (2005) 826: 69 -74.

(12) Steeghs M., Cristescu S., Harren F.J., The suitability of Tedlar bags for breath sampling in medical diagnostic research. Physiol. Meas. (2007) 28: 73-84.

(13) Dallinga J.W., Robroeks C.M., Van Berkel J.J., Moonen E. J.C., Godschalk R.W.L., Jobsis Q., Dompeling E., Wouters E.F.M., Van Schooten F.J., Volatile organic compounds in exhaled breath as a diagnostic tool for asthma in children. Clin. Exp. Allergy., (2009) 40: 68-76

(14) Filipiak W., Sponring A., Filipiak A., Ager C., Schubert J., Miekisch W., Amann A., Troppmair J., TD-GC-MS analysis of volatile compounds of human lung cancer and normal cells in vitro. Cancer Epidemiol Biomarkers Prev. (2010) 19: 182-195.

(15) Bajtarevic A., Ager C., Pienz M., Klieber M., Schwarz K., Ligor M., Ligor T., Filipiak W., Denz H., Fiegl M., Hilbe W., Weiss W., Lukas P., Jamnig H., Hackl M., Haidenberger A., Buszewski B., Miekisch W., Schubert J., Amann A., Noninvasive detection of lung cancer by analysis of exhaled breath. BMC Cancer(2009) 9: 348

(16) Phillips M., Cataneo R.N., Ditkoff B.A., Fisher P., Greenberg J., Gunawardena R., Kwon C.S., Rahbari-Oskoui F., Wong C., Volatile markers of breast cancer in the breath. The Breast Journal (2003) 9: 184-191.

(17) Roebroeks C.M., van Berkel J.J., Dallinga J., Jobsis Q., Zimmermann L., Hendriks H., Wouters M., Grinten C., Kant K., Schooten F., Dompeling E., Metabolomics Of Volatile Organic Compounds In Cystic Fibrosis Patients and Controls. Ped. Res. (2010) 68: 75-80

(18) Barker M., Hengst M., Schmid J., Buers H-J., Mittermaier B., Klemp D., Koppmann R., Volatile organic compounds in the exhaled breath of young patients with cystic fibrosis. Eur. Respir. J. (2006) 27: 929-936.

(19) Horváth I., Lázár Z., Gyulai N., Kollai M., Losonczy G., Exhaled biomarkers in lung cancer Eur. Respir. J. (2009) 34: 261-275.

(20) Górecki T., Panić O., Oldridge N., Recent advances in comprehensive two-dimensional gas chromatography (GC × GC). J. Liq. Chromatogr. Rel. Tech. (2006) 29: 1077-1104.

(21) Marriott P., Shellie R., Principles and applications of comprehensive two-dimensional gas chromatography. Trend. Anal. Chem. (2002) 21: 573-583.

(22) Shellie R., Marriott P.J., Morrison P., Concepts and preliminary observations on the triple-dimensional analysis of complex volatile samples by using GC× GC-TOFMS. Anal. Chem. (2001) 73: 336-1344.

(23) Marczin N., Kharitonov S.A., Yacoub M.H., Barnes P.J., Disease Markers in Exhaled Breath. (2005) Taylor&Francis, New York, pp 220.

(24) Mochalski P., Wzorek B., Sliwka I., Amann A., Suitability of different polymer bags for storage of volatile sulphur compounds relevant to breath analysis. J. Chromatogr. B (2009) 877: 189-196.
(25) Dallüge J., Beens J., Brinkman U.A.T., Comprehensive two-dimensional gas chromatography: a powerful and versatile analytical tool. J. Chromatogr. A 1000 (2003) 69-108.
(26) Mondello L., Tranchida P.Q., Dugo P., Dugo G., Comprehensive two-dimensional gas chromatography-mass spectrometry: a review. Mass Spectrom. Rev. (2008) 27: 101-124.
(27) Engel E., Baty C., LeCorre D., Souchon I., Martin N., Flavor-active compounds potentially implicated in cooked cauliflower acceptance. J. Agric. Food Chem. (2002) 50: 6459-6467.
(28) Ciccioli P., Brancaleoni E., Cecinato A., Sparapani R., Frattoni M., Identification and determination of biogenic and anthropogenic volatile organic compounds in forest areas of northern and southern Europe and a remote site of the Himalaya region by high-resolution gas chromatography-mass spectrometry J. Chromatogr. A (1993) 643: 55-69.
(29) Wu S., Zorn H., Krings U., Berger R.G., Volatiles from submerged and surface-cultured beefsteak fungus, Fistulina hepatica. Flavour Fragr. J. (2007) 22: 53-60.
(30) Xu X., van Stee L.L.P., Williams J., Beens J., Adahchour M., Vreuls R.J.J., Brinkman U.A.T., Comprehensive two-dimensional gas chromatography (GC × GC) measurements of volatile organic compounds in the atmosphere. Atmos. Chem. Phys. (2003) 3: 665-682.
(31) Quijano C.E., Salamanca G., Pino J.A., Aroma volatile constituents of Colombian varieties of mango (Mangifera indica L.). Flavour Fragr. J. (2007) 22: 401-406
(32) Courtois E.A., Paine C.E., Blandinieres P.A., Stien D., Bessiere J., Houel E., Baraloto C., Chave J., Diversity of the volatile organic compounds emitted by 55 species of tropical trees: a survey in French Guiana. J. Chem. Ecol. (2009) 35: 1349-1362.
(33) Ramarathnam N., Rubin L.J., Diosady L.L., Studies on meat flavor. 3. A novel method for trapping volatile components from uncured and cured pork, J. Agric Food Chem. (1993) 41: 933-938.
(34) Ramarathnam N., Rubin L.J., Diosady L.L., Studies on meat flavor. 4. Fractionation, characterization, and quantitation of volatiles from uncured and cured beef and chicken. J. Agric. Food Chem. (1993) 41: 939-945.
(35) Alissandrakis E., Kibaris A.C., Tarantilis P.A., Polissiou P.C., Flavour compounds of Greek cotton honey. J. Sci. Food Agric. (2005) 85: 1444-1452.
(36) Zaikin V.G., Borisov R.S., Chromatographic-mass spectrometric analysis of Fischer-Tropsch synthesis products J. Anal. Chem. (2002) 57: 544-551.
(37) Pino J.A., Mesa J., Muñoz Y., Martí M.P., Marbot R., Volatile components from mango (Mangifera indica L.) cultivars. J. Agric. Food Chem. (2005) 53: 2213-2223.
(38) Flamini G., Tebano M., Cioni P.L., Bagci Y., Dural H., Ertugrul K., Savran A., A multivariate statistical approach to Centaurea classification using essential oil composition data of some species from Turkey Pl. Syst. Evol. (2006) 261: 217-228.
(39) Wang Z., Fingas M., Li K., Fractionation of a Light Crude Oil and Identification and Quantitation of Aliphatic, Aromatic, and Biomarker Compounds by GC-FID and GC-MS, Part II J. Chromatogr. Sci (1994) 32: 361-382.
(40) Macku C., Shibamoto T., Volatile sulfur-containing compounds generated from the thermal interaction of corn oil and cysteine. J. Agric. Food Chem. (1991) 11: 1987-1989.
(41) Zhang X., Ding L., Sun Z., Song L., Sun T., Study on quantitative structure-retention relationships for hydrocarbons in FCC gasoline. Chromatographia (2009) 70: 511-518.
(42) Jarunrattanasri A., Theerakulkait C., Cadwallader K.R., Aroma components of acid-hydrolyzed vegetable protein made by partial hydrolysis of rice bran protein. J. Agric. Food Chem. (2007) 55: 3044-3050.
(43) Hazzit M., Baaliouamer A., Faleiro M.L., Miguel M.G., Composition of the essential oils of Thymus and Origanum species from Algeria and their antioxidant and antimicrobial activities. J. Agric. Food Chem. (2006) 54: 6314-6321.
(44) Rostad C.E., Pereira W.E., Kovats and Lee retention indices determined by gas chromatography/mass spectrometry for organic compounds of environmental interest. J. Hi. Res. Chromatogra. & Chromatogr. Comm. (1986) 9: 328-334.
(45) Insausti K., Goñi V., Petri E., Gorraiz C., Beriain M.J., Effect of weight at slaughter on the volatile compounds of cooked beef from Spanish cattle breeds. Meat Sci. (2005) 70: 83-90.
(46) Flamini G., Cioni L., Morelli I., Essential oils of Galeopsis pubescens and G. tetrahit from Tuscany (Italy) Flavour Fragr. J. (2004) 19: 327-329.
(47) Singer R.B., Flach A., Koehler S., Marsaioli A.J., Amaral M.C.E., Sexual Mimicry in Mormolyca ringens (Lindl.) Schltr. (Orchidaceae: Maxillariinae). Ann. Bot. Rome (2004) 93: 755-762.

(48) Maccioni S., Baldini R., Cioni P., Tebano M., Flamini G., In vivo volatiles emission and essential oils from different organs and pollen of Cistus albidus from Caprione (Eastern Liguria, Italy). Flavour Fragr. J. (2007) 22: 61-65.
(49) Gómez E., Ledbetter C.A., Hartsell P.L., Volatile compounds in apricot, plum, and their interspecific hybrids. J. Agric. Food. Chem. (1993) 41: 1669-1676.
(50) Zeng Y.X., Zhao C.X., Liang Y.Z., Yang H., Fang H.Z., Yi L.Z., Zeng Z.D., Comparative analysis of volatile components from Clematis species growing in China. Anal. Chim. Acta (2007) 595: 328-339.
(51) Berdague J.L., Denoyer C., Wuéré J.L., Sermon E., Volatile compounds of dry-cured ham. J. Agric. Food Chem. (1991) 39: 1257-1261.
(52) Asuming W.A., Beauchamp P.S., Descalzo J.T., Dev B.C., Dev V., Frost S., Ma C.W., Essential oil composition of four Lomatium Raf. species and their chemotaxonomy. Biochem. Syst. Ecol. (2005) 33: 17-26.
(53) Celik S., Gokturk R.S., Flamini G., Cioni P.L., Morelli I., Essential oils of Phlomis leucophracta, Phlomis chimerae and Phlomis grandiflora var. grandiflora from Turkey. Biochem. Syst. Ecol. (2005) 33: 617-623.
(54) Flamini G., Tebano M., Cioni P.L., Volatile emission patterns of different plant organs and pollen of citrus limon. Anal. Chim. Acta (2007) 589: 120-124.
(55) Widmer H., Gas chromatographic identification of hydrocarbons using retention indices. J. Gas Chromatogr. (1967) 5: 506-510.
(56) Quijano C.E., Salamanca G., Pino J.A., Aroma volatile constituents of Colombian varieties of mango (Mangifera indica L.). Flavour Fragr. J. (2007) 22: 401-406.
(57) Kallio M., Jussila M., Rissanen T., Antilla P., Hartonen K., Reissel A., Vreuis R., Adahchour M., Hyotylainen T., Comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry in the identification of organic compounds in atmospheric aerosols from coniferous forest. J. Chromatogr. A (2006) 1125: 234-243.
(58) Lai W.C., Song C., Temperature-programmed retention indices for gc and gc-ms analysis of coal-and petroleum-derived liquid fuels. Fuel (1995) 37: 1436-1451.
(59) Rembold H., Wallner P., Nitz S., Kollmannsberger H., Drawert F., Volatile components of chickpea (Cicer arietinum L.) seed. J. Agric. Food Chem. (1989) 37: 659-662.
(60) Alissandrakis E., Tarantilis P.A., Harizanis P.C., Polissiou M., Comparison of the volatile composition in thyme honeys from several origins in Greece. J. Agric. Food Chem. (2007) 55: 8152-8157.
(61) Flamini G., Cioni P., Morelli I., Composition of the essential oils and in vivo emission of volatiles of four Lamium species from Italy: L. purpureum, L. hybridum, L. bifidum and L. amplexicaule, Food Chem. (2005) 91: 63-68.
(62) Kenig F., Simons D.J.H., Crich D., Cowen J.P., Ventura G.T., Rehbein-Khalily T., Structure and distribution of branched aliphatic alkanes with quaternary carbon atoms in Cenomanian and Turonian black shales of Pasquia Hills (Saskatchewan, Canada). Org. Geochem. (2005) 36: 117-138.
(63) Van den Dool H., Kratz P.D., J. Chromatogr. A generalization of the retention index system including linear temperature programmed gas—liquid partition chromatography (1963) 11: 463-471.
(64) Perestrelo R., Barros A.S., Câmara J.S., Rocha S.M., In-depth search focused on furans, lactones, volatile phenols, and acetals as potential age markers of Madeira wines by comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry combined with solid phase microextraction. J. Agric. Food Chem. (2011) 59: 3186-3204.
(65) Silva I., Rocha S. M., Coimbra M.A., J. Marriott, Headspace solid-phase microextraction combined with comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry for the determination of volatile compounds from marine salt. Chromatogr. A (2010) 1217: 5511-5521.
(66) Pleil J.D., Stigiel M.A., Sobus J.R., Tabucchi S., Ghio A.J., Madden M.C., Cumulative exposure assessment for trace-level polycyclic aromatic hydrocarbons (PAHs) using human blood and plasma analysis. J. Chromatogr. B (2010) 878: 1753–1760.
(67) Beauchamp J., Kirsch F., Buettner A., Real-time breath gas analysis for pharmacokinetics: monitoring exhaled breath by on-line proton-transfer-reaction mass spectrometry after ingestion of eucalyptol-containing capsules J. Breath Res. (2010) 4: 026006.
(68) Miekisch W., Schubert J., Noeldge-Schomburg G., Diagnostic potential of breath analysis--focus on volatile organic compounds. Anal Chim. Acta (2004) 347: 25–39.
(69) von Mühlen C., Marriott P.J., Retention indices in comprehensive two-dimensional gas chromatography. Anal Bioanal. Chem. (2011) 401: 2351–2360.
(70) Tran T.C., Logan G.A., Grosjean E., Ryan D., Marriott P.J., Use of comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry for the characterization of biodegradation and unresolved complex mixtures in petroleum. Geochim. Cosmochim. Ac. (2010) 74: 6468–6484.

(71) Marczin N., Kharitonov S.A., Yacoub M.H., Barnes P.J., Disease Markers in Exhaled Breath. (2005) Taylor&Francis, New York, pp 220.
(72) Rudnicka J., Kowalkowski T., Ligor T., Buszewski B., Determination of volatile organic compounds as biomarkers of lung cancer by SPME-GC-TOF/MS and chemometrics. J. Chromatogr. B (2011) 879: 3360–3366.
(73) Corradi M., Folesani G., Andreoli R., Manini P., Bodini A., Piacentini G., Carraro S., Zanconato S., Baraldi E., Aldehydes in exhaled breath condensate of patients with chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. (2003) 167: 395–399.
(74) Ibrahim B., Basanta M., Cadden P., Singh D., Douce D. Woodcock A. , Fowler S.J., Non-invasive phenotyping using exhaled volatile organic compounds in asthma. Thorax (2011) 66: 804–809.
(75) Ligor M., Ligor T., Bajtarevic A., Ager C., Pienz M., Klieber M., Denz H., Fiegl M., Hilbe W., Weiss W., Lukas P., Jamnig H., Hackl M., Buszewski B., Miekisch W., Schubert J., Amann A., Determination of volatile organic compounds appearing in exhaled breath of lung cancer patients by solid phase microextraction and gas chromatography mass spectrometry. Clin. Chem. Lab. Med. (2009) 47: 550–560.
(76) Filipiak W., Sponring A., Filipiak A., Ager C., Schubert J. Miekisch W., Amann A., Troppmair J., TD-GC-MS Analysis of Volatile Compounds of Human Lung Cancer and Normal Cells In vitro Cancer Epidemiol. Biomarkers Prev. (2010) 19: 182–195.
(77) Bajtarevic A., Ager C., Pienz M., Klieber M., Schwarz K., Ligor M., Ligor T., Filipiak W., Denz H., Fiegl M., Hilbe W., Weiss W., Lukas P., Jamnig H., Hackl M., Haidenberger A., Buszewski B., Miekisch W., Schubert J., Amann A., Noninvasive detection of lung cancer by analysis of exhaled breath BMC Cancer (2009) 9: 348–364.
(78) Poli D., Carbognani P., Corradi M., Goldoni M., Acampa O., Balbi B., Bianchi L., Rusca M., Mutti A., Exhaled volatile organic compounds in patients with non-small cell lung cancer: cross sectional and nested short-term follow-up study. Resp. Res. (2005) 6: 71–81.
(79) Chen X., Xu F., Wang Y., Pan Y., Lu D., Wang P., Ying K., Chen E., Zhang W., A study of the volatile organic compounds exhaled by lung cancer cells in vitro for breath diagnosis. Cancer (2007) 110: 835–844.
(80) Yu H., Xu L., Wang P., Solid phase microextraction for analysis of alkanes and aromatic hydrocarbons in human breath. J. Chromatogr. B (2005) 826: 69–74.
(81) Kharitonov S.A., Barnes P.J., Exhaled markers of pulmonary disease. Am. J. Respir. Crit. Care Med. (2001) 163: 1693–1722.
(82) MacNee W., Oxidative stress and lung inflammation in airways disease. Eur. J. Pharmacol. (2001) 429: 195–207.
(83) Aghdassi E., Allard J., Breath alkanes as a marker of oxidative stress in different clinical conditions. Free Radical Biol. Med. (2000) 28: 880–888.
(84) Saude E., Skappak C., Regush S., Cook K., Ben-Zvi A., Becker A., Moqbel R., Skyes B., Rowe B., Adamko D., Metabolomic profiling of asthma: Diagnostic utility of urine nuclear magnetic resonance spectroscopy. J. Allergy Clin. Immunol. (2011) 127: 757–764.
(85) Roquet A., Dahlen B., Kumlin M., Ihre E., Anstren G., Binks S., Dahlen S.E., Combined antagonism of leukotriene and histamine produces a predominant inhibition of allergen-induced early and late phase airway obstruction in asthmatics. Am. J. Respir. Crit. Care Med. (1997) 155: 1856–1863.
(86) Smolinska A., Hauschild A.-C., Fijten R.R.R., Dallinga J.W., Baumbach J., Schooten F.J.V., Current breathomics—a review on data pre-processing techniques and machine learning in metabolomics breath analysis. (2014) J. Breath Res. 8: 027105-
(87) van de Kant K.D., van der Sande L.J., Jöbsis Q., van Schayck O.C., Dompeling E . Clinical use of exhaled volatile organic compounds in pulmonary diseases: a systematic review. Resp. Res. (2012) 13:117-140.
(88) Westerhuis J.A., Hoefsloot H.C.J., Smit S., Vis D.J., Smilde A.K., van Velzen E.J.J., van Duijnhoven J.P.M., van Dorsten F.A., Assessment of PLSDA cross validation. Metabolomics (2008) 4: 81-89.
(89) Rubingh C.M., Bijlsma S., Derks E.P., Bobeldijk I., Verheij R., Kochhar S., Smilde A.K., Assessing the performance of statistical validation tools for megavariate metabolomics data. Metabolomics (2006) 2: 53-61.
(90) Picard R., Cook D., Cross-Validation of Regression Models. J Amer Statist Assoc (1984) 79: 575-583.
(91) Xu Q-S., Liang Y.-Z., Monte Carlo cross validation. Chemometr. Intell. Lab. (2001) 56 1-11.
(92) Dubitzky W., Granzow M., Berrar D.P., Fundamentals of Data Mining in Genomics and Proteomics. (2007) Springer Verlag Berlin, Heidelberg, Germany.
(93) Rocha C.M., Carrola J, Barros AS, Gil AM, Goodfellow BJ, Carreira IM, Bernardo J, Gomes A, Sousa V, Carvalho L, Duarte IF Metabolic signatures of lung cancer in biofluids: NMR-based metabonomics of blood plasma J Proteome Res (2011) 10: 4314-4324.

(94) Pereira A.C., Reis M.S., Saraiva P.M., Marques J.C., Analysis and assessment of Madeira wine ageing over an extended time period through GC-MS and chemometric analysis. Anal Chim Acta (2010) 660: 8-21

(95) Graca G. , Moreira A.S. , Correia A.J.V., Goodfellow B.J., Barros A.S., Duarte I.F., Carreira I.M., Galhano E., Pita C., Almeida M.D., Gil A.M., Mid-infrared (MIR) metabolic fingerprinting of amniotic fluid: A possible avenue for early diagnosis of prenatal disorders? Anal. Chim. Acta (2013) 764: 24-31.

(96) Loureiro C.C, Gomes J., Barros A.S., Gil A.M., Carrola J., Duarte I.F., Bousquet J., Bom A.T., Rocha S.M., Urine metabolomics applied to characterize exacerbation in asthmatic patients. J. Allergy Clin. Immunol. (2014) 133: 261-263.

(97) Bousquet J., Cabrera P., Berkman N., Buhl R., Holgate S., Wenzel S., Fox H., Hedgecock S., Blogg M., Cioppa G.D., The effect of treatment with Omalizumab, an anti-IgE antibody on asthma exacerbations and emergency medical visits in patients with severe persistent asthma. Allergy (2005) 60: 302-308.

Chapter 3

CHAPTER 3

EXPLORATION OF ASTHMA URINE METABOLOME

3.1 Overview

Urine is an easily obtained matrix in large volumes when compared to other biofluids and in the last years this matrix has been increasingly studied. The identified compounds may be end products of normal and pathological cellular processes or compounds of environmental exposure. Urinary metabolomics is still in a developmental phase moving from discovey towards a method validation phase that will lead this matrix to be a considerable part of diagnosis, treatment evaluation and prognosis. The goal of our study was to ascertain the volatile and non-volatile urinary metabolomic changes caused by the asthma pathology.

With this in mind, in-lab developed and reported method GC×GC-ToFMS was used to ascertain the overall urine volatile metabolome. The volatile urinary of 26 asthma patients and 10 healthy children was obtained with c.a. 200 compounds being identified. From these compounds, 78 belonging to the alcohols, aldehydes, alkanes, alkenes and ketones were selected as these metabolites have been linked to lipid peroxidation, consequently connected to oxidative stress that has been related to asthma. A heat map, a quick and simple visualization tool, of the selected compounds was built and it was possible to verify that the levels of the 78 compounds in the urine of the asthmatic children were higher in the majority of the selected compounds. MVA analysis was then performed, namely the supervised method PLS-DA, and discrimination was attained. The developed models were also validated using MCCV. It was possible to verify that, with the exception of 9 metabolites, most of metabolited characterized the asthma population.

The use of ^{1}H NMR in analysing urine of asthmatics has been already proven and reported in literature[1]. Nevertheless, and to complement the volatile metabolome results, urine analysis of 92 asthmatic children and 50 healthy children by ^{1}H NMR was performed. The goal was to ascertain the urinary metabolomic changes thus revealing the compounds involved in central metabolic pathways affected by the asthma pathology and in addition develop a method that could be used in the clinical practice. Before performing the analysis three normalization procedures and metabolite selections were tested. It was possible to sucessfully differentiate asthma patients from heatlhy children with compounds that are involved in fulcral metabolic pathways such as tricarboxylic acid (TCA) cycle, histidine metabolism, lactic acidosis, modification of free tyrosine residues after eosinophil stimulation and DNA damage.

3.2 Asthma volatile urinary metabolome uncovered by comprehensive two-dimensional gas chromatography-time of flight mass spectrometry

3.2.1 Abstract

The use of a powerful and high throughput analytical technique, such as GC×GC–ToFMS, in the evaluation of a biological system provides an array of bicohemical information otherwise unobtainable. This tool combined with SPME was considered to explore the urinary volatile metabolomic profile of asthma yielding c.a. 200 compounds that belonged to different chemical families. A targeted analysis was then performed to assess the impact provoked, comparing asthma individuals with healthy ones, on lipid peroxidation due to the oxidative stress levels. Five biochemically relevant families used as a lipid peroxidation index (alcohols, aldehydes, alkanes, alkenes and ketones), in a total of 78 compounds, were carefully selected. A heat map was built to quickly compare the urine volatile metabolome of the asthmatics versus the healthy and it was possible to verify that the levels of urine volatile compounds of the asthmatics was higher in the majority (88%) of the selected compounds. MVA tools were applied and it was found out that from the 78 compounds only 9 compounds (octane, tridecane, 3-methylhexane, 2,2,4,6,6-pentamethylheptane, 6-methyldodecane, 3-ethyl-3-methylundecane, 3-methy-1-heptene and 2,4-dimethyl-1-heptene) characterized the healthy population whilst the remaining characterized the asthmatic population. MCCV was performed to assess the predictive power. The results suggest that the model is robust, considering the high classification rate (97.3%), sensitivity (98.7%), and specificity (93.9%). Beside the development of statistical models that were able to discriminate asthmatic children from healthy ones, urine analysis by GC×GC–ToFMS allowed to use compounds that are reportedly linked to lipid peroxidation, thus revealing the impact of oxidative stress on asthma. These results showed the possibility to obtain this knowledge as the compound levels related to lipid peroxidation were predominantly increased for the asthma population.

3.2.2 Framework

Metabolomics is the global unbiased analysis of all the small-molecule metabolites within a biological system under a given set of conditions. These methods offer the potential for a holistic approach to clinical medicine, as well as improving disease diagnosis and understanding of pathological mechanisms[2]. It is an emerging field useful in the detection of metabolic profiles of the pathologies under study and pathology exploration by metabolomics has evolved throughout the years driven by the technological advances in instrumentation, data analysis software and in the development of metabolite databases. Asthma diagnosis metabolomic studies have been carried out to provide new tools for diagnosis purposes, disease management and knowledge on molecular alterations.

When compared with other biofluids, urine has several advantages as it can be obtained in large quantities non-invasively and usually less pre-treatment is required. Urine metabolome is potentially vast including all endogenous and exogenous metabolites that might be established in a wide dynamic concentration range. Asthma is a lung condition, that could alter the metabolism of cells outside the lung leading to a different metabolome that could be potentially measured in urine. Several cellular pathways can be studied through this matrix, assuming that there is no significant renal disease, making urine as an accurate reflection of a person's metabolic health and disease[3].

The metabolomic urinary profile of 26 children with allergic asthma (from which 12 with allergic rhinitis) and 10 healthy children was explored using the SPME/GC×GC–ToFMS methodology developed by Rocha *et al.*[4]. Urine volatile metabolome in asthma, to the best of our knowledge, has not been previously described. Relevant biochemical families (alcohols, aldehydes, alkanes, alkenes and ketones) were selected as these metabolites are generated though the interaction of ROS with fatty acids (in a known series of reactions known as lipid peroxidation) measuring the increased oxidative stress in asthma[5,6]. PLS-DA was performed in order to obtain the metabolites responsible for class discrimination. MCCV was applied to the PLS-DA models to evaluate the sensitivity and specificity levels and to assess classification model robustness.

3.2.3 Material and Methods

Subjects

Twenty-six patients with allergic asthma (from which 12 with allergic rhinitis) and 10 healthy control subjects were enrolled in this study following informed consent and approval of the study protocol by the Hospital Infante D. Pedro E.P.E (Aveiro, Portugal) and the healthy population consisted of children of the faculty staff. The characteristics of the patients and controls are presented in Table 3.1.

Table 3.1 - Characteristics of the studied population: allergic asthma patients and healthy controls.

	Allergic asthma (n=26)	**Control** (n=10)
Age (mean±SD)	8.8±3.4	9.7±3.3
Gender (male/female)	16/10	5/5
Pathology		
Allergic Asthma	14 (54%)	-
Allergic Asthma + Allergic Rhinitis	12 (46%)	-
Allergens[a]		
Dust mite	10 (38%)	-
Dust mite + gramineae	7 (27%)	-
Gramineae	4 (15%)	-
Dust mite + cat fur	2 (8%)	-
Dust mite + cat fur + dog fur	2 (8%)	-
Dust mite + olive tree	1(4%)	-
Therapy		
Corticosteroid + bronchodilator	6 (22%)	-
Corticosteroid + leukotriene receptor antagonist + bronchodilator	4 (15%)	-
Leukotriene receptor antagonist + bronchodilator + nasal corticosteroid	4 (15%)	-
Nasal corticosteroid and antihistamine	3 (12%)	-
Leukotriene receptor antagonist + bronchodilator	3 (12%)	-
Corticosteroid + leukotriene receptor antagonist	2 (8%)	-
Nasal corticosteroid and bronchodilator	1 (4%)	-
Corticosteroid	1 (4%)	-
No therapy	**2 (8%)**	**-**

[a] Results obtained by prick-tests

All parents signed an informed consent for participation in the study. The children with allergic asthma were recruited from the outpatient clinic of the Immunoalergology Department of the Hospital Infante D. Pedro E.P.E (Aveiro, Portugal). Asthma diagnosis was made based on clinical symptoms and exams (skin prick tests and IgE values, among other specific tests). Appropriate therapy was prescribed by the patient's own physician. The allergic asthma population represented a controlled asthma status. No restrictions were applied regarding drugs or diet. Each individual (either patient or healthy volunteer) provided

a urine sample in a sterile cup. Then, aliquots of approximately 1 mL were transferred into vials, frozen and stored at -80 °C, until analysis.

Urine analysis using SPME/GC×GC–ToFMS

The SPME experimental parameters and the GC×GC–ToFMS conditions have been previously reported by Rocha *et al.*4. Briefly, 500 µL of urine (pH adjusted to 2.0), 0.1g NaCl and a stirring bar (0.5×0.1 mm) were placed into a 5 mL glass vial and the vial was capped with a PTFE septum and a screw cap (Chromacol, Hertfordshire, UK). The DVB/CAR/PDMS fibre was used with an extraction temperature and time of 40.0±0.1 ºC and 30 min, respectively, with constant stirring of 500 rpm. Three independent replicates of each sample were performed. After the extraction/concentration step, the SPME coating fibre was manually introduced into the GC×GC−ToFMS LECO Pegasus 4D (LECO, St. Joseph, MI, USA), consisted of an Agilent GC 7890A gas chromatograph equipped with a dual stage jet cryogenic modulator (licensed from Zoex) a secondary oven and an high-speed time-of-flight mass spectrometer detector. Most (> 90%) of the identified compounds presented similarity matches > 850. The DTIC (Deconvoluted Total Ion Current) GC×GC area data was used as an approach to estimate the relative content of each volatile metabolite of urine.

Data Processing

The dataset comprised 78 metabolites belonging to selected chemical families: alcohols, aldehydes, alkanes, alkenes and ketones. Scaling procedures divide each variable by a factor removing the dependence on concentration and magnitude of the metabolites[7]. Each sample was range-scaled between 0 and 1, as this scaling procedure proved to be the most adequate in urine analysis as Weber *et al.*[8] and Van den Berg7 demonstrated. Mean normalization procedure was then applied to the 78 selected metabolites, by dividing each row of data matrix by its average. A heat map was then built to visualise the quantitative data on two axes using R statistical software[9]. On the *x-axis* the samples are represented whereas in the *y-axis* the normalized areas of the 78 metabolites of the selected chemical families. The colours are coded to reflect quantitation with higher levels corresponding to red whilst lower levels are blue.

Partial Least Squares (PLS) is a widely used procedure for both regression and classification purposes. PLS-DA[10], commonly uses a **Y** matrix containing dummy variables which defines sample memberships to pre-defined groups and this allows to extract relevant

information/variability that could describe the reasons for the observed patterns (clusters) allowing to verify which metabolites contribute for the observed separation.

Then, PLS-DA was applied to the selected 78 volatile scaled and normalized metabolites tentatively identified by GC×GC-ToFMS in all urine samples.

For classification purposes two groups were used, healthy subjects and asthma patients. A random internal cross validation method has been computed with 20 segments and 6 samples per segment. The data is divided into the calibration set containing all samples used to compute the model components, using X- and Y-values. The test set that contains the remaining samples for which X-values are fed into the model once a new component has been computed. Their predicted Y-values are then compared to the observed Y-values, yielding a prediction residual. MCCV was first reported by Picard and Cook[11] and has proven to be assymptotically consistent and is characterized by leaving out a major part of samples at a time for validation instead of leaving out one sample at a time[12]. Model robustness was assessed using MCCV with 500 iterations. The classification model complexity (number of LVs) of the dataset (78 metabolites) was computed, as well as classification rate and Q^2 were estimated by cross-validation (7 blocks splits). For each of the 500 randomly generated classification models, the number of LV, the Q^2 and the confusion matrix were computed. The selection of model complexity was based on the most frequent list of model properties that maximizes the predictive power (i.e., lower LV and higher Q^2). The sensitivity and the specificity of the model were then depicted from the confusion matrix resulting into a ROC map to further assess the results significance. Then, the same procedure was applied using permuted class membership. Sensitivity is calculated from the ratio between true positives (allergic asthma samples correctly predicted) and the total number of modelled breath samples, whereas specificity is determined from the ratio between true negatives (control samples correctly predicted) and the total number of modelled control GC×GC data.

3.2.4 Results and discussion

Urine volatile composition provided by HS-SPME/GC×GC–ToFMS

The urinary volatile metabolome was attained using a high throughput and powerful technique, GC×GC-ToFMS that is an established technique in the analysis of complex samples due to its enhanced separation capacity, sensitivity, peak resolution and reproducibility[13,14,15]. A non-polar/polar (NP/P) column combination was used and the HS-

SPME/GC×GC–ToFMS data was automatically processed using in the identification of all peaks in the GC×GC chromatogram contour plots with signal-to-noise threshold > 50. The peak finding routine based on deconvolution method allowed to identify several hundred compounds. A manual inspection of the mass spectra was performed combined with the use of retention index and compared with literature values for similar chromatographic columns. Table 3.2 shows a list of ca. 200 volatile compounds belonging to different chemical families including acids, alcohols, aldehydes, alkanes, alkenes, esters, furan compounds, ketones, lactones, norisoprenoids, sulphur compounds and terpenic compounds.

Table 3.2 - Urinary compounds dataset tentatively identified by GC×GC–ToFMS from allergic asthma and healthy children

Peak #	^1t_R [a] (s)	^2t_R [a] (s)	Compounds	CAS Number	RI_{calc} [b]	$RI_{lit.}$ [c] (GC)	$RI_{lit.}$ [d] (GC × GC)
			Acids				
1	678	5.81	hexanoic acid	142-62-1	1000	990	-
2	876	5.91	ethylhexanoic acid	149-57-5	1132	1128	-
3	1062	5.46	nonanoic acid	112-05-0	1276	1280	-
4	1176	4.56	decanoic acid	334-48-5	1372	1380	-
5	1380	3.52	dodecanoic acid	143-07-7	1568	1571	-
6	1566	2.90	tetradecanoic acid	544-63-8	1763	1780	-
7	1656	2.62	pentadecanoic acid	1002-84-2	1867	1866	-
8	1740	2.43	hexadecanoic acid	57-10-3	1967	1964	-
			Alcohols				
15	186	2.34	3-methyl-3-buten-1-ol	763-32-6	655	667	-
12	192	1.85	2-methyl-1-butanol	137-32-6	677	677	-
9	234	2.33	1-pentanol	71-41-0	741	741	760
13	402	1.27	2-methyl-2-propanol	78-93-1	853	853	-
14	744	1.47	2-ethyl-1-hexanol	104-46-7	1040	1026	-
16	792	0.76	2-octen-1-ol	22104-79-6	1074	1060	-
10	798	1.56	1-octanol	111-87-5	1078	1068	1069
17	936	0.79	2-nonen-1-ol	22104-80-9	1170	1171	-
11	942	1.37	1-nonanol	143-08-8	1170	1175	1171
			Aldehydes				
12	126	0.55	pentanal	110-62-3	685	697	690
13	192	1.06	2-methyl-2-butenal	497-03-0	697	753	-
14	276	0.96	hexanal	66-25-1	801	802	800
15	396	1.60	*(E)*-2-hexenal	6728-26-3	872	857	851
16	504	0.98	heptanal	111-71-7	904	904	-
17	600	0.92	2-ethylhexanal	123-05-7	957	955	954
18	690	0.86	octanal	124-13-0	1005	1003	1003
19	774	1.10	*(E)*-2-octenal	2548-87-0	1074	1056	1058
20	846	0.81	nonanal	124-19-6	1105	1106	1103
21	918	1.04	*(E)*-2-nonenal	18829-56-6	1162	1164	1168

Peak #	^1t_R [a] (s)	^2t_R [a] (s)	Compounds	CAS Number	RI_{calc} [b]	$RI_{lit.}$ [c] (GC)	$RI_{lit.}$ [d] (GC × GC)
23	1104	0.85	undecanal	112-44-7	1311	1310	1309
24	1218	0.81	dodecanal	112-54-9	1412	1410	-
25	1326	0.85	tridecanal	10486-19-8	1513	1511	-
26	1428	0.82	tetradecanal	124-25-4	1613	1611	-
27	1524	0.80	pentadecanal	09/11/2765	1714	1711	-
28	1614	0.85	hexadecanal	629-80-1	1815	1819	-
			Hydrocarbons				
			Alkanes				
29	90	0.37	3-methylpentane	107-83-5		560	558
30	150	0.40	3-methylhexane	589-34-4	640	671	672
31	222	0.43	C_7 isomer (*m/z* 57, 43, 71)	-	759	-	-
32	276	0.40	octane	111-65-9	800	800	800
33	318	0.46	4-ethyl-2-methylhexane	3074-75-7	831	833	-
34	498	0.42	nonane	111-84-2	900	900	900
35	624	0.45	3-methylnonane	06/04/5911	975	976	971
36	666	0.44	2,2,4,6,6-pentamethylheptane	13475-82-6	988	997	997
37	684	0.42	decane	124-18-5	1000	1000	1000
38	720	0.45	3,9-dimethylnonane	17302-32-8	1039	1038	-
39	840	0.42	undecane	1120-21-4	1100	1100	1100
40	876	0.45	C_{11} isomer (*m/z* 57, 43, 71)	-	1144	-	-
41	930	0.46	2-methylundecane	7045-71-8	1193	1164	1165
42	972	0.43	dodecane	112-40-3	1200	1200	1200
43	1020	0.45	6-methyldodecane	6044-71-9	1256	1253	-
44	1098	0.44	tridecane	629-50-5	1300	1300	1300
45	1128	0.46	3-ethyl-3-methylundecane	-	1348	1347	-
46	1212	0.45	tetradecane	629-59-4	1400	1400	1400
47	1314	0.45	pentadecane	629-62-9	1500	1500	-
48	1416	0.46	hexadecane	544-76-3	1600	1600	-
49	1512	0.47	heptadecane	629-78-7	1700	1700	-
50	1608	0.46	octadecane	593-45-3	1800	1800	-
			Alkenes				
94	210	0.44	3-methyl-1-heptene	3769-23-1	727	743	748
89	258	0.47	1-octene	592-98-3	803	800	790
90	366	0.50	2,4-dimethyl-1-heptene	19549-87-2	853	855	-
92	672	0.49	4-decene	19398-89-1	1008	991	993
93	756	0.48	4-methyl-2-decene	74630-30-1	1062	-	-
94	810	0.47	C_{11} isomer (*m/z* 69, 55, 41)	-	1097	-	-
95	966	0.49	1-dodecene	112-41-4	1211	1192	-
96	1116	0.48	7-methyl-1-undecene	74630-42-5	1337	-	-
97	1200	0.53	1-tetradecene	1120-36-1	1412	1392	-
98	1308	0.56	1-pentadecene	13360-61-7	1511	1481	-
			Cyclic				

Peak #	^1t_R [a] (s)	^2t_R [a] (s)	Compounds	CAS Number	RI_{calc} [b]	$RI_{lit.}$ [c] (GC)	$RI_{lit.}$ [d] (GC × GC)
51	594	0.48	1,1,2,3-tetramethylcyclohexane	6783-92-2	966	958	-
52	828	0.48	1-hexyl-3-methylcyclopentane	61142-68-5	1109	-	-
			Aromatics				
53	126	0.59	benzene	71-43-2	628	648	660
54	222	0.82	toluene	108-88-3	768	762	770
55	402	1.03	ethylbenzene	100-41-4	857	858	868
56	420	1.04	*p*-xylene	106-42-3	865	864	-
57	474	1.07	*o*-xylene	95-47-6	890	893	-
58	540	0.86	isopropylbenzene	98-82-8	923	922	-
59	594	0.86	propylbenzene	103-65-1	952	952	959
60	642	0.91	*o*-ethyltoluene	611-14-3	978	973	-
62	666	0.92	1,2,4-trimethylbenzene	9563-6	991	988	-
63	702	0.87	1-allyl-4-methylbenzene	3333-13-9	1012	1033	-
64	714	0.98	1,2,3-trimethylbenzene	526-73-8	1020	1020	-
65	762	0.78	m-propyltoluene	1074-17-5	1051	1058	-
66	786	0.82	(1-methylpropyl)-benzene	135-98-8	1066	1014	-
67	612	0.87	*m*-ethyltoluene	620-14-4	962	959	-
68	624	0.90	*p*-ethyltoluene	622-96-8	968	960	-
69	774	0.82	1-ethyl-2,3-dimethylbenzene	933-98-2	1058	1094	-
70	798	0.84	1-ethyl-2,4-dimethylbenzene	847-41-9	1078	1087	-
71	948	1.83	naphthalene	91-20-3	1183	1183	-
72	1356	0.65	(1-butylhexyl)-benzene	4537-11-5	1542	-	-
73	1416	0.70	(1-methylnonyl)-benzene	4537-13-7	1601	-	-
74	1482	0.67	(1-ethyloctyl)-benzene	4621-36-7	1669	-	-
75	1512	0.70	(1-methyldecyl)-benzene	4536-88-3	1701	-	-
76	1542	0.65	(1-butyloctyl)-benzene	2719-63-3	1734	-	-
77	1554	0.66	(1-propylnonyl)-benzene	2719-64-4	1747	-	-
78	1572	0.67	(1-ethyldecyl)-benzene	2400-00-2	1767	-	-
79	1608	0.70	(1-methylundecyl)-benzene	2719-61-1	1808	-	-
80	1632	0.66	(1-butylnonyl)-benzene	4534-50-3	1837	-	-
81	1644	0.66	(1-propyldecyl)-benzene	4534-51-4	1851	-	-
82	1698	0.70	(1-methyldecyl)-benzene	4536-88-3	1915	-	-
			Chlorinated compounds				
100	588	1.23	*o*-chlorotoluene	95-49-8	949	955	-
101	600	1.26	*p*-chlorotoluene	106-43-4	956	956	-
102	780	0.65	1-chlorooctane	111-85-3	1062	1064	-
103	924	0.64	1-chlorononane	2473-01-0	1164	1167	-
104	1056	0.64	1-chlorodecane	1002-69-3	1267	1256	-
			Esters				
105	108	0.52	ethyl acetate	141-78-6	607	608	609
107	450	1.39	1-methoxy-2-propyl acetate	-	879	-	-
108	876	0.76	methyl octanoate	111-11-5	1128	1126	-

Peak #	^1t_R [a] (s)	^2t_R [a] (s)	Compounds	CAS Number	RI_{calc} [b]	$RI_{lit.}$ [c] (GC)	$RI_{lit.}$ [d] (GC × GC)
109	972	0.70	ethyl octanoate	106-32-1	1201	1202	-
110	1122	0.74	methyl decanoate	110-42-9	1322	1326	-
111	1206	0.69	ethyl decanoate	110-38-3	1395	1397	-
112	1338	0.73	methyl dodecanoate	111-82-0	1524	1526	-
113	1344	1.66	ethyl-4-ethoxybenzoate	23676-09-7	1531	1522	-
114	1422	0.59	2-ethylhexyl 2-ethylhexanoate	7245-14-1	1607	-	-
115	1536	0.73	methyl tetradecanoate	124-10-7	1728	1727	-
116	1710	0.73	methyl hexadecanoate	112-39-0	1929	1928	-
			Furan compounds				
117	102	0.50	2-methylfuran	534-22-5	601	603	-
118	120	0.48	tetrahydrofuran	109-99-9	621	620	634
119	156	0.62	2,5-dimethylfuran	625-86-5	663	701	-
120	276	0.79	2-ethyl-5-methylfuran	1703-52-2	800	800	-
121	306	0.85	2,3,5-trimethylfuran	10504-04-8	814	817	-
122	432	2.28	2-(methylthio)-furan	13768-59-6	871	-	-
123	480	0.77	2,5-diethylfuran	10504-06-0	892	888	-
124	564	1.07	2-methyl-5-isopropenylfuran	-	936	-	-
125	672	0.76	2-pentylfuran		994	992	995
126	924	0.90	menthofuran	3777-69-3	1164	1164	-
127	990	1.42	4,7-dimethyl-1-benzofuran	28715-26-6	1215	1220	-
			Ketones				
128	150	0.92	2-pentanone	107-87-9	595	636	681
129	192	0.74	2-methyl-pentan-2-one	108-10-1	-	733	748
130	204	0.74	3-methylpentan-2-one	565-61-7	746	-	750
131	246	1.42	4-methyl-3-penten-2-one	123-54-6	790	798	-
132	252	0.86	2-hexanone	591-78-6	800	790	788
133	258	0.97	C_6 isomer (*m/z* 43, 58, 71)	-	808	-	-
134	258	1.46	1-hexen-3-one	1629-58-9	808	775	-
135	264	0.68	2,3-dimethyl-3-pentanone	565-80-0	810	806	-
136	354	0.89	4-methylhexan-2-one	17042-16-9	837	846	850
137	438	0.95	4-heptanone	123-19-3	873	895	884
138	636	0.80	3-octanone	106-68-3	991	989	989
139	660	1.10	6-methyl-5-hepten-2-one	110-93-0	991	989	985
140	828	0.84	2-nonanone	821-55-6	1093	1093	1089
141	894	1.02	1-nonen-3-one	24415-26-7	1142	1077	-
142	1086	0.82	2-undecanone	112-12-9	1296	1296	1293
143	1206	0.82	2-dodecanone	6175-49-1	1396	1396	-
144	1314	0.86	2-tridecanone	593-08-8	1501	1500	-
			Lactones				
146	828	3.53	δ-hexanolactone	823-22-3	1095	1084	1060
148	1632	0.97	Muskolactone	106-02-5	1837	1839	1841

Peak #	^1t_R [a] (s)	^2t_R [a] (s)	Compounds	CAS Number	RI_{calc} [b]	$RI_{lit.}$ [c] (GC)	$RI_{lit.}$ [d] (GC × GC)
			Norisoprenoids				
149	1068	0.75	γ-ionone	79-76-5	1277	1340	-
150	1092	0.70	dihydroedulan I	74006-61-4	1296	1298	-
151	1098	0.67	theaspirane A	66537-39-1	1301	1301	-
152	1110	0.80	edulan I	41678-29-9	1311	1313	-
153	1116	0.70	theaspirane B	36341-72-8	1316	1319	-
154	1158	1.00	1,2-dihydro-1,1,6-trimethylnaphthalene (TDN)	30364-38-6	1354	1354	-
155	1230	1.11	dihydro-β-ionone	20489-53-6	1419	1424	1450
156	1302	1.24	3,4-dihydro-β-ionone	17283-81-7	1490	1485	-
157	1470	1.47	methyl dihydrojasmonate	24851-98-7	1658	1649	-
			Sulphur Compounds				
158	192	0.96	dimethyl disulfide	624-92-0	753	730	747
159	228	1.03	2 methylthiophene	554-14-3	759	771	-
160	618	1.53	dimethyl trisulfide	3658-80-8	965	964	-
			Monotepenic compounds				
			Hydrocarbon type				
161	558	0.54	α-pinene	7785-70-8	933	933	940
162	582	0.58	camphene	79-92-5	945	947	959
163	594	0.66	verbenone	80-57-9	952	951	-
164	636	0.59	β-pinene	7785-70-8	975	975	987
166	684	0.63	α-phellandrene	99-83-2	1000	1000	1035
168	708	0.63	α-terpinene	99-86-5	1016	1016	1023
169	720	0.77	*o*-cymene	502-99-8	1024	1027	1042
170	726	0.64	limonene	5989-27-5	1027	1027	1037
171	732	0.65	1,8-cineole	470-82-6	1031	1031	1032
172	744	0.81	β-cimene	99-87-6	1039	1030	1038
173	774	0.66	γ-terpinene	99-85-4	1058	1059	1062
174	822	1.00	*p*-cymene	99-87-6	1089	1088	1039
175	852	0.83	p-mentha-1,5,8-triene	21195-59-5	1110	1110	-
176	888	0.84	*E,E*-2,6-dimethyl-1,3,5,7-octatetraene	460-01-5	1137	1134	-
			Ketone type				
177	828	1.11	6-camphenone	53803-33-1	1093	1093	1103
178	1026	1.38	carvone	2244-16-8	1244	1242	1242
			Alcohol type				
179	798	1.13	dihydromyrcenol	18479-58-8	1074	1076	1079
181	840	1.31	linalool	78-70-6	1101	1101	1098
182	900	1.70	verbenol	18881-04-4	1147	1147	-
186	942	1.17	4-terpineol	562-74-3	1178	1178	1181
187	948	2.64	p-cimeno-8-ol	1197-01-9	1184	1184	-
			Aldehyde type				

Peak #	^1t_R [a] (s)	^2t_R [a] (s)	Compounds	CAS Number	RI_{calc} [b]	$RI_{lit.}$ [c] (GC)	$RI_{lit.}$ [d] (GC × GC)
188	870	1.04	α-canfolenal	-	1124	1127	-
189	966	1.22	myrtenal	18486-69-6	1196	1195	-
			Ester type				
190	1080	0.82	bornyl acetate	76-49-3	1286	1285	-
			Sesquiterpenic compounds				
			Hydrocarbon type				
191	1224	0.63	α-cedrene	469-61-4	1412	1409	-
192	1296	0.66	aromadendrene	489-39-4	1483	1447	1496
193	1344	0.70	δ-cadinene	483-76-1	1530	1530	-
194	1362	0.91	α-calacorene	21391-99-1	1548	1546	-
			Alcohol type				
195	1344	0.79	spathulenol	6750-60-3	1530	1548	-
196	1380	1.06	nerolidol	7212-44-4	1566	1565	-
197	1440	1.16	γ-eudesmol	1209-71-8	1626	1629	-
198	1638	0.94	farnesol	4602-84-0	1844	1749	-
			Miscellaneous				
147	960	2.19	3-methyl-4-propyl-2,5-furandione	16493-20-2	1193	-	-
165	666	0.70	monoterpene m/z (91, 77, 119, 39, 134)	-	991	-	-
167	690	0.71	monoterpene m/z (91, 77, 119, 39, 134)	-	1004	-	-
183	906	1.58	monoterpene m/z (91, 77, 119, 39, 134)	-	1151	-	-
184	918	1.85	monoterpene m/z (91, 77, 119, 39, 134)	-	1161	-	-
185	930	1.85	monoterpene m/z (91, 77, 119, 39, 134)	-	1170	-	-

*- information not available
[a]- Retention times in seconds (s) for first (^1t_R) and second (^2t_R) dimensions.
[b]- RI: retention index obtained through the modulated chromatogram.
[c]- RI: retention index reported in the literature for one dimensional GC with a 5%-Phenyl-methylpolysiloxane GC column or equivalent.
[d]- RI: retention index reported in the literature for a comprehensive GC×GC system with Equity-5 for the first dimension.

The most reliable way to confirm the identification of each compound is based on authentic standard co-injection, which in several cases is economically prohibitive and often unachievable in the time available for analysis, or commercially unavailable standard[16]. With the use of NP/P columns chemically related compounds are grouped forming ordered patterns facilitating group-type analysis and conditional classification of unknown compounds[17].With this column combination, the chemical families are grouped based on the boiling point (^{1}D) and polarity (^{2}D) offering a clear visual separation between compound classes as shown in several reports4,[18]. The identification was also supported by experimentally determining the retention index (RI) values that were compared, when available, with values reported in literature for chromatographic columns similar to that used as the 1D column and whenever available compared to RI values obtained by GC×GC.

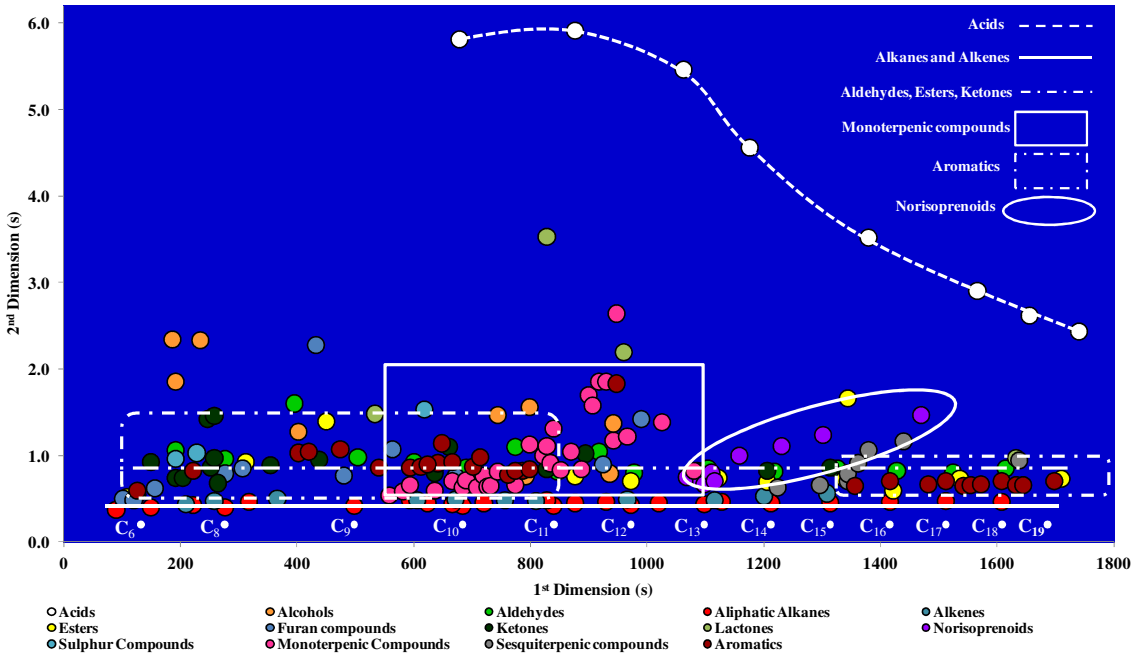


Figure 3.1 - Peak apex plot of the compounds identified in urine depicting all the chemical families present in Table 3.2.

In Figure 3.1, a peak apex plot can be visualized depicting all chemical families identified in urine. The increase in the number of carbons leads to an increase in the first retention time (^1t_R) due to the decrease in volatility. In the second retention time (^2t_R) the compounds are dispersed by polarity. The alkanes are the least polar compounds (^2t_R 0.370s) and the acids the most polar compounds (^2t_R 5.910s). It is also possible to clearly verify, for the acids family, that the increase of the carbon chain leads to a decrease in the ^2t_R.

The alkanes and alkenes behave in *quasi* linear fashion and the separation is based on volatility. The ramified alkanes and alkenes presented a slight higher ^2t_R when compared to the linear alkanes and alkenes (Table 3.2). The aromatics are depicted in Figure 3.1 in the rounded rectangle varying in ^1t_R between 126s to 948s and 1356s to 1698s and ^2t_R between 0.590s and 0.890s and. The exceptions were 2-phenylpropene and naphthalene that have a ^2t_R of 1.140s and 1.830s, respectively. This behavior is explained by the presence of the double bond in the substituted benzene for the former and the presence of another benzene ring for the latter. The introduction of a methyl group led to a significant increase in ^2t_R as it can be exemplified for benzene (0.590s) and p-xylene (1.040). However, when additional groups are present ^2t_R decrease is observed (0.820s for 1-ethyl-2,3-dimethylbenzene). It was also possible to identify clusters with monoterpenes ($C_{10}H_{16}$) found between ^1t_R 558 and 774s, monoterpene alcohols between ^1t_R 798 and 1026s and the sesquiterpenes from ^1t_R 1224 to 1638s (Table

3.2). As for the furan compounds, the presence of additional methyl groups led to a slight increase effect on ^2t_R (1.420s for 4,7-dimethyl-1-benzofuran) and a bigger increase is observed when the compound had sulphur in its composition (2.280s for 2-(methylthio)-furan). The norisoprenoids varied between 1068 and 1470s for ^1t_R and 0.670 and 1.470s for ^2t_R, as depicted in Figure 3.1. The sulphur compounds have increased polarity with the addition of a sulphur atom in its structure, 0.960s for dimethyl sulfide and 1.530s for dimethyl trisulfide. As for the chlorinated compounds, it can be observed from Table 3.2 that there is an increase in ^2t_R in the presence of a chlorinated toluene when compared with the substituted chloroalcane due to the increase in polarity of the former.

The general behavior of compounds with a carbonyl group can be observed in Figure 3.1 depicted by dashed line. Initially, for the ketones and aldehydes, there was an increase in polarity (consequently increase in ^2t_R) with the increase of carbons until C_8. After, there is a decrease and stabilization of ^2t_R due to the increase of hydrophobicity, and ^2t_R remains constant throughout the chromatogram. It was possible to verify the increase on ^2t_R when the compound has a double bond, as for example 4-methyl-3-penten-2-one and 1-hexen-3-one, with 1.460s and 1.020s respectively (Table 3.2). The presence of a benzene ring (ethyl-4-ethoxybenzoate) and even the presence of a metoxy group (1-methoxy-2-propyl acetate) promoted the increase on ^2t_R in these chemical families. In the linear alcohols, it was possible to confirm that the increase of the carbon chain led to a decrease in the ^2t_R. However, the ramified alcohols have a lower ^2t_R when compared to the correspondent linear alcohols. The same behavior is noted for the unsaturated alcohols, with the exception of 3-methyl-3-buten-1-ol mainly due to the presence of the double bond. The ^2t_R generally follows: alkanes < alkenes ≈ cyclic hydrocarbons <aromatic hydrocarbons ≈ esters ≈ terpenoids ≈ norisoprenoids < ketones ≈ aldehydes < sulphur compounds < alcohols < lactones < acids.

However, not all identified compounds are endogenous, i.e. there are compounds that are contaminants, not an internal metabolic product. For example, organochlorides or aromatic hydrocarbons from environmental cumulative exposure[19] or can have its origin from ingested foods as for example esters, furan compounds, lactones, norisoprenoids and terpenoids[20,21]. The metabolic pathways yielding sulphur compounds are regarded as detoxification routes[22]. Seventy eight compounds belonging to aldehydes, ketones, alkanes, alkenes and alcohols were selected. These metabolites are linked to the oxidative degradation of lipids (lipid peroxidation) due to increased oxidative stress that has been reported in asthma[30] and this study assessed the impact at the oxidative stress level on the fatty acids in individuals with asthma when compared to healthy individuals[23].

Targeting urinary asthma metabolomics toward oxidative stress impact

Figure 3.2 depicts the differences between the asthmatic and healthy individuals scaled chromatographic peak areas of the five selected families. It clearly shows differences in the selected dataset, with a highly altered chromatographic pattern for the asthmatic group as a whole.

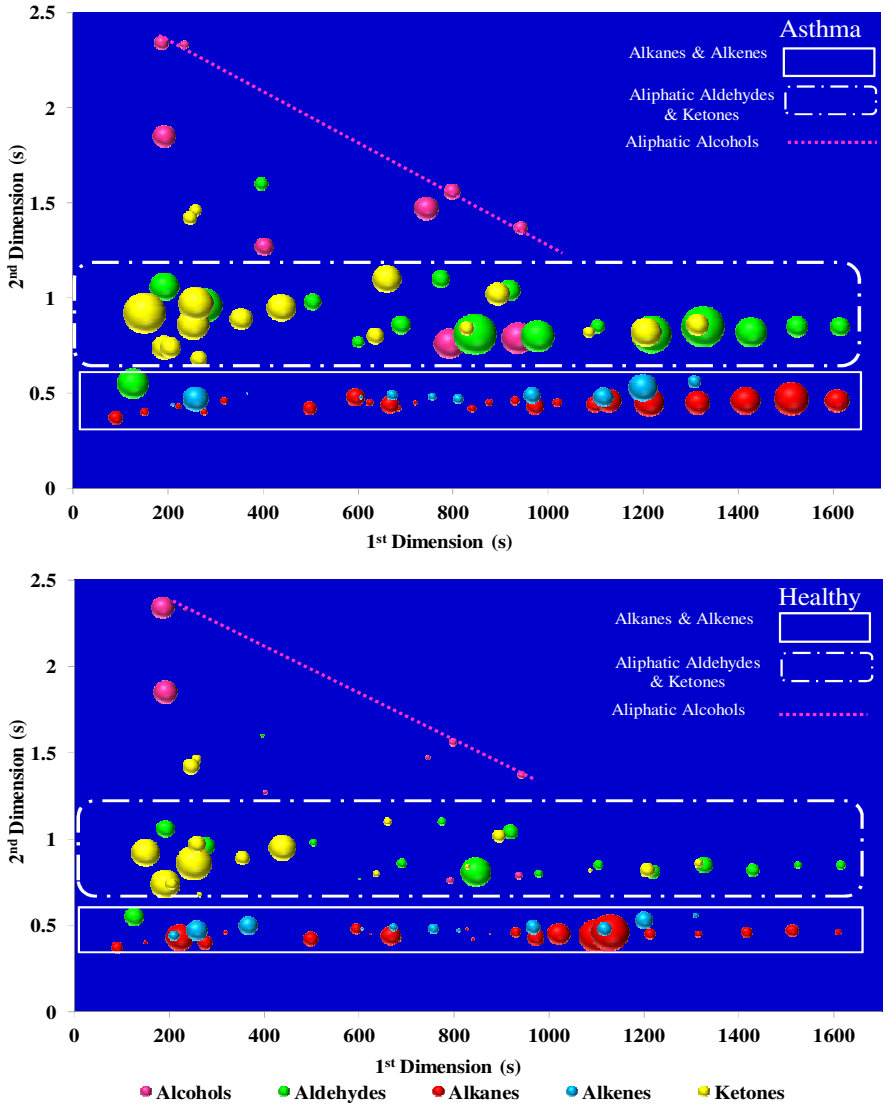


Figure 3.2 - Peak apex plots depicting five biochemically relevant selected families: alcohols, aldehydes, alkanes, alkenes and ketones. Comparison between the asthma and healthy groups scaled peak areas

However, the visualization of complex data through heat maps is a more suitable tool for a rapid inspection of the obtained data[24]. Mieth and co-authors[25] reported it in the analysis of exhaled breath and Rocha *et al.*[4] in urine. This approach was adapted to the scaled 78

compounds to quickly visualize the differences among asthma and healthy groups (Figure 3.3).

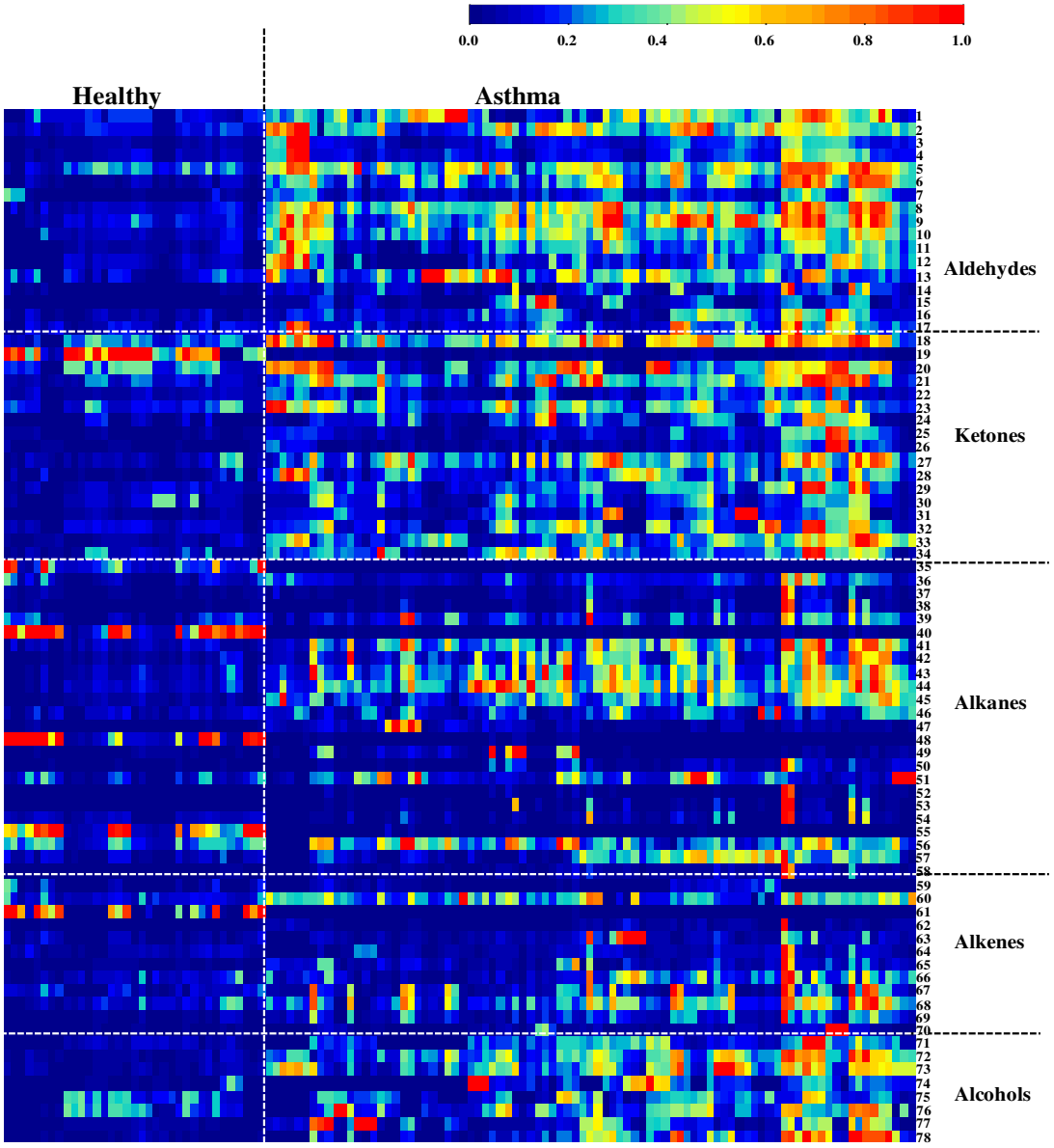


Figure 3.3 – Heat map representation of biochemically relevant selected families, aldehydes, ketones, alkanes, alkenes and alcohols. Each line represent a range- scaled and mean normalized volatile compound (organized by chemical families) and each column represents a subject (organized by healthy and asthma children). For peak assignment see Table 3.1.

At a first glance, it is confirmed that the urinary metabolomic composition is that the levels of urine volatile compounds of the asthmatics was higher in the majority (88%) of the selected compounds.

Methods of correction, i.e. normalization, are a crucial pre-processing step normally applied to urine samples to account for the different sample dilutions before applying multivariate tools to the dataset. Post sample analysis normalization allows investigating urine samples without full volume 24-h collection simplifying the overall process[26]. For this purpose, the mean normalization procedure was performed to the scaled chromatographic areas. After, PLS-DA was apllied to the selected dataset. Scores plot for the first two LV of PLS-DA model for volatile compounds are shown in Figure 3.4 **A**, while Figure 3.4 **B** (corresponding LV1 and LV2 loading weights plot) establishes the contribution of each volatile compound that promotes the observed distinction.

Figure 3.4 **A** shows that the healthy group is associated with LV1 and LV2 negative values whilst the asthma group is more dispersed by the remaining three quadrants. The healthy group is mainly characterized by octane, tridecane, 3-methylhexane, 2,2,4,6,6-pentamethylheptane, 6-methyldodecane, 3-ethyl-3-methylundecane, 3-methy-1-heptene and 2,4-dimethyl-1-heptene (Figure 3.4 **B**). The remaining compounds described the asthma group, however among these, the aldehydes, the heavier alkanes (from tetradecane to octadecane) and alcohols, have a bigger weight in discriminating the asthma group taking into account LV1 positive values. Comparing these results to the result in the heat map, differences are observed in the compounds that characterize the populations under study. From Figure 3.3, 4-methyl-3-penten-2-one had higher chromatographic areas in the healthy population (in the PLS-DA this compound characterized the asthma population) and from loading analysis (Figure 3.4 **B**) there were other compounds that characterized the healthy population such as 2,4,6,6-pentamethylheptane, 3-ethyl-3-methylundecane and 3-methy-1-heptene. These differences could be attributed to the normalization procedure adopted before applying MVA analysis due to the accounting for dilution factors in urine samples bringing all the compounds into proportion with one another.

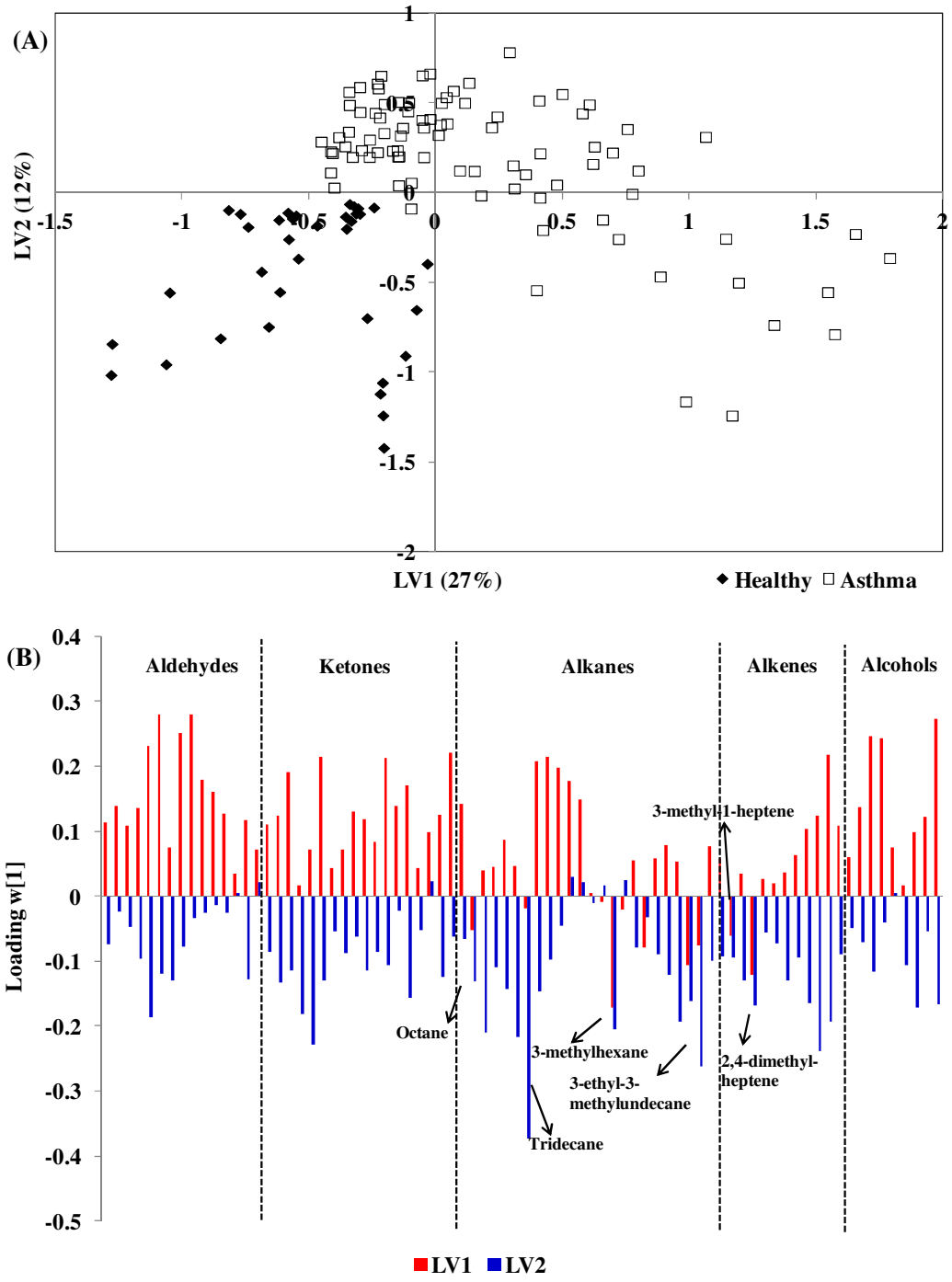


Figure 3.4 – **(A)** PLS-DA scores scatter plot showing a clear discrimination between allergic asthma and healthy children, **(B)** LV1 loading weights plot of the selected 78 metabolites with the identification of main compounds related to the healthy group.

As for validation purposes, an internal cross validation and MCCV described in the multivariate analysis section, were applied. In the performed internal cross validation, the R^2 was 0.907 whilst the validation results had a R^2 of 0.814. Therefore, to assess the predictive

ability of the classification model and evaluate the robustness of the developed models, MCCV was applied to add confidence to the obtained results.

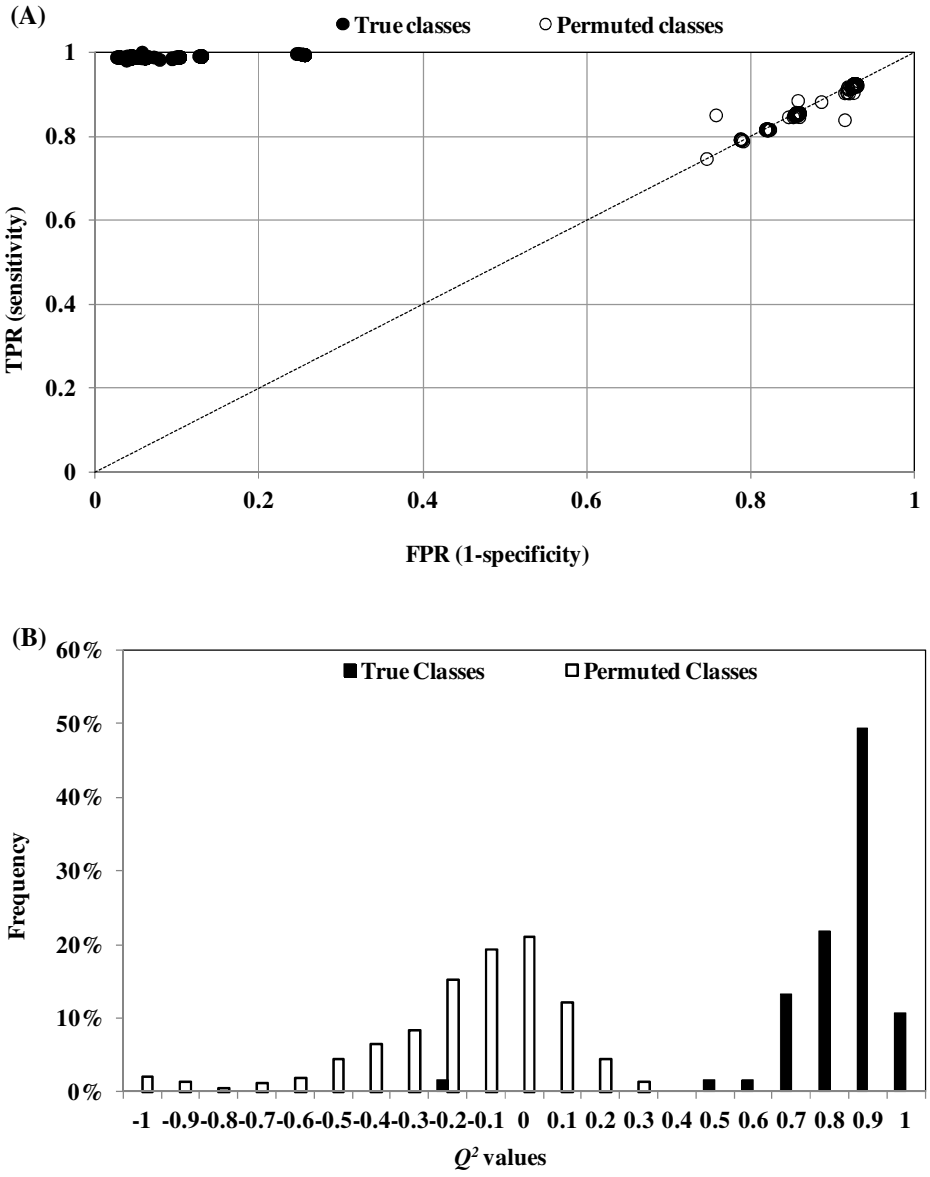


Figure 3.5 - ROC space **(A)** where each point represents a prediction result (sensitivity and 1-specificity) of the confusion matrices obtained from MCCV (500 iterations) of the PLS-DA model for the different data normalization techniques employed and Q^2 values distribution **(B)** of the original and permuted Monte-Carlo Cross Validation for PLS-DA of exhaled breath of full dataset.

The average Q^2 for the 500 runs was 0.9, with a large prevalence of values within the range 0.8-1.0. According to the resulting confusion matrix, the PLS-DA model showed 98.7% sensitivity, with approximately 1.3% of asthma patients being misclassified as controls, and 93.9% specificity, with 6.1% false positives. The classification rate (total number of samples correctly classified) was 97.3% .

The metabolic activity of children with asthma is different from those without asthma revealed by their urine volatile composition. Several metabolic pathways may be associated to asthma, nevertheless this study focused on the fatty acid oxidation that is summarized in Figure 3.6. Airway inflammation is closely linked to asthma[27,28] and studies have shown that ROS are mediators in the inflammatory process becoming clear that oxidative stress increases this process[29]. ROS alters key enzymatic and non-enzymatic antioxidants that lead to an oxidant-antioxidant imbalance in airways and this imbalance leads to known pathophysiological effects associated with asthma[30]. ROS are common initiators in living cells of the fatty acid oxidation process[30] and alcohols, aldehydes, alkanes, alkenes and ketones are commonly associated to lipid peroxidation that is linked to fatty acid oxidation5,6. Alcohols, aldehydes, alkanes and alkenes are linked to oxidative stress and inflammation[23]. The main mechanism that affects the release of these chemical families in the body is oxidative stress and is essentially formed via lipid peroxidation by ROS of polyunsaturated fatty acids (PUFA)[23]. PUFA, found in cellular and subcellular membranes in the body, undergo lipid peroxidation due to the existence of multiple double bonds, in particular the methylene groups that have especially reactive hydrogen, forming aldehydes, alkanes and alkenes. Heavier ketones, under metabolic conditions, can also be associated with the β-oxidation of branched fatty acids, a high oxidation rate of fatty acids[20,31]. Branched ketones may arise from branched aldehydes and can partially arise from bacterial action in the gut, probably by decarboxylation from the corresponding oxo-acids[20] Therefore, this study was focused on identification of these metabolites in urine of an asthmatic population and compared them to the healthy population. Taking into account the results where the majority of the selected compounds pertaining to the aldehydes, alkanes, and alkenes are increased (Figure 3.3) and characterized the asthma group (Figure 3.4 **B**) The alcohol levels are no exception to this behavior and are generally increased in the urine of the asthmatic patients characterizing the asthmatic population (Figure 3.3 **B**). Accordingly to Figure 3.4 **B**, ketones levels in urine are increased in the asthmatic population, . These results suggest the possibility of assessing lipid peroxidation through the measurement of these urinary volatile compounds linked to lipid peroxidation and consequently fatty acid oxidation.

3.2.5 Concluding remarks

In this study, GC×GC–ToFMS combined with SPME was applied, for the first time to the best of our knowledge, in the uncovering the urinary metabolome of a paediatric asthmatic population. The use of a powerful technique such as GC×GC–ToFMS allowed the identification of c.a. 200 compounds, common in all samples, belonging to 10 different chemical families. A targeted analysis was performed to assess the impact that asthma has in oxidative stress on fatty acids and for that purpose five biochemically relevant families linked to these processes were selected. A set of 78 compounds belonging to aldehydes, ketones, alkanes, alkenes and alcohols was used. A heat map, a quick visualization tool, was built and it was possible to verifyt that the urine of asthmatics was highly altered when compared to the urine of the healthy population. MVA tools were then applied and discrimination between the asthmatic and healthy populations was attained. Loading analysis enabled gathering metabolomic information through the identified compounds, therefore demonstrating that the levels of oxidative stress related to lipid peroxidation processes on asthma individuals was higher than in healthy individuals. Although the initial set of children is small (n=36), the achieved results led to the successful unravelling the asthma urinary volatile metabolome and led to the successful development of validated statistical models. The use of high throughput technology (GC×GC–ToFMS) in the analysis of urine from a pediatric asthmatic population yielded an easy and novel strategy for asthma assessment, which may be a valuable tool in assessing lipid peroxidation levels in a population affected by asthma.

3.3 Exploring the urinary metabolomic profile of children with asthma by ^{1}H Nuclear Magnetic Resonance

3.3.1 Abstract

Recently non-invasive sampling strategies have emerged as an attractive and useful approach combined to high throughput techniques such as ^{1}H NMR. Therefore, this study focused on the establishment of the urinary metabolomic pattern of asthmatic and healthy children using ^{1}H NMR in tandem with partial least squares discriminant analysis. Data pre-treatment is an important step in metabolomics previous to multivariate analysis. Thus, three methods were applied: normalization to creatinine signal, to total spectral area and probabilistic quotient normalization (PQN). PQN yielded the most positive impact when compared to the remaining studied methods. To further improve the obtain results variable selection was studied taking into account the compounds with variable importance in projection (VIP) higher than 1 and specific ^{1}H NMR spectral areas. The results showed that selecting VIP higher than 1 and for the spectral region between 0.5-3.0 and 6.0-9.5ppm was the best alternative. MCCV was also performed to assess both, the predictive power and classification models robustness of the developed models. The previously mentioned conditions yielded a classification rate of 83.9%, sensitivity of 90.3% and specificity of 72.1%.

Upon loading analysis, the compounds that characterized the healthy children were trimethylamine-N-oxide and trigonelline whilst a broader set of compounds characterized the asthma children β-aminoisobutyrate, lactate + threonine, histidine, phenylacetylglutamine, acetate, p-cresol, succinate, citrate, tyrosine and indoxyl sulphate. This metabolomic pattern allowed to obtain hypothesize on possible affected metabolic pathways by asthma such as TCA cycle, histidine metabolism, lactic acidosis, modification of free tyrosine residues after eosinophil stimulation, and DNA damage. The study led to a methodology able to monitor asthma patients allowing to ascertain urinary metabolomic changes providing the compounds involved in central metabolic pathways. The ^{1}H NMR urine analysis yielded a metabolomic pattern for asthma, and the identified compounds allowed a comprehensive study of asthma metabolic related pathways.

3.2.2 Framework

NMR is non-destructive technique enabling, highly reproducible, both qualitative and quantitative measurements while studying a number of metabolites in a biological fluid[32,33]. There has been a recent surge in the application of ^{1}H NMR in biofluids research, with metabolic profiles being used to characterize, diagnose, and predict pathological states[34,35].

The application of ^{1}H NMR spectroscopy to urine and plasma samples has been extensively used[36,37,38]. Saude *et al.*1, developed statistical models with 70 metabolites using urine of asthmatics analyzed by ^{1}H NMR proving the potential of this tecnique in asthma research. The study allowed the identification of biochemical pathways that are possibly influenced by asthma such as the TCA, stress on energy metabolism, the protein and amino acid metabolism and inflammation. These authors proposed other possible pathways that are related to the modification of free tyrosine residues by eosinophil activity and lactic acidosis.

Recently, Loureiro *et al.*[39] analyzed urine by ^{1}H NMR to evaluate the changes in urinary metabolites associated to asthma exarcebation and verified that lipid peroxidation associated to oxidative stress and TCA are the main altered metabolic pathways. Jung and co-authors[40] analyzed serum from patients with asthma using ^{1}H NMR and compared to healthy children. The metabolites responsible for the attained discrimination are reportedly involved in hypermethylation, response to hypoxia, immune reaction and lipid metabolism.

In this research study, the metabolomic urinary profile of an asthmatic childhood and healthy populations was explored using a ^{1}H NMR methodology in order to extract relevant information that can be used further on asthma management. Firstly, three normalization statistical techniques were tested to normalize the urine ^{1}H NMR spectra namely, total area normalization, normalization to creatinine reported in literature in studies related to asthma and PQN[41]. PLS-DA and Orthogonal Projections to Latent Structures (OPLS-DA) models were developed. MCCV was applied and loading analysis was performed to reveal the modifications on ^{1}H NMR urinary metabolome of childhood promoted by asthma condition.

3.2.3 Experimental

Subjects

Ninety-two patients with allergic asthma (from which 34 with also present allergic rhinitis) and 50 healthy subjects were enrolled in this study following informed consent and

approval of the study protocol by Hospital Infante D. Pedro E.P.E (Aveiro, Portugal) and the healthy population consisted of children of the faculty staff. The characteristics of the asthmatics and healthy population are presented in Table 3.3.

Table 3.3 - Characteristics of the studied population: allergic asthma patients and healthy children.

	Allergic asthma (n=92)	**Healthy Children**
Age (mean±SD)	9.5±3.2	8.8±3.5
Gender (male/female)	44/48	22/28
Pathology		
Allergic Asthma	58 (63%)	-
Allergic Asthma + Allergic Rhinitis	34 (37%)	-
Allergens[a]		
Dust mite	47 (51%)	-
Dust mite + gramineae	22 (23%)	-
Gramineae	10 (10%)	-
Dust mite + cat fur	5 (5%)	-
Dust mite + cat fur + dog fur	4 (4%)	-
Dust mite + gramineae + gat fur	2 (2%)	-
Gramineae + cat fur	2 (2%)	-
Gramineae + cat fur + dog fur	1 (1%)	-
Dust mite + dog fur	1 (1%)	-
Dust mite + olive tree	1 (1%)	-
Therapy		
Corticosteroid + bronchodilator	14 (15%)	-
Corticosteroid + leukotriene receptor antagonist + bronchodilator	12 (13%)	-
Corticosteroid + leukotriene receptor antagonist	9 (10%)	-
Nasal corticosteroid and bronchodilator	8 (9%)	-
Leukotriene receptor antagonist + bronchodilator	8 (9%)	-
Nasal corticosteroid and antihistamine	7 (8%)	-
Leukotriene receptor antagonist + bronchodilator + nasal	7 (8%)	-
Bronchodilator	5 (5%)	-
Antihistamine	5 (5%)	-
Leukotriene receptor antagonist + nasal corticosteroid	4 (4%)	-
Corticosteroid	4 (4%)	-
Corticosteroid + bronchodilator + antihistamine	2 (2%)	-
Corticosteroid + antihistamine	1 (1%)	-
No therapy	6 (7%)	-

[a] Results obtained by prick-tests

Asthma diagnosis was made based on characteristic clinical symptoms and exams (skin prick tests, lung function evaluation based on spirometry tests and IgE values). Appropriate therapy was prescribed by the patient's own physician and the allergic asthma population represented a controlled asthma status. No restrictions were applied regarding drugs or diet. The study was approved by the Hospital Ethics Committee. Each individual (either patient or healthy volunteer) provided a urine sample in a sterile cup. Then, aliquots of approximately 1 mL were transferred into vials, and stored at -80 °C (in average one month) until analysis being each sample thawed once only for analysis.

Sample Preparation and ^{1}H NMR analysis

For ^{1}H NMR analysis, urine samples were thawed and prepared as described by Carrola *et. al*.[42] Briefly, fine pH adjustment to 7.00 was performed by adding a few drops of 4 M solutions of KOD (98% atom D, Sigma-Aldrich, Madrid, Spain) or DCl (99% atom D, Sigma-Aldrich, Madrid, Spain). After, the samples were centrifuged (8000 rpm for 5 min) and transferred to a 5 mm NMR tube.

^{1}H NMR spectra were acquired on a Bruker Avance DRX-500 spectrometer operating at 500.13 MHz for ^{1}H observation, at 300 K based on the developed method by Carrola *et al*.[42] for urine analysis. For each sample, a standard 1D ^{1}H NMR spectrum was acquired, using a water suppression pulse sequence.

Data processing

All spectra were processed with a line broadening of 0.3 and a zero filling factor of 2 manually phased and baseline corrected. The chemical shifts were referenced internally to the sodium trimethlysilyl propionate-d4 (TSP) (98% atom D, Sigma-Aldrich, Madrid, Spain) signal at δ 0.00. The obtained data was matched to reference spectra in the BBIOREFCODE-2-0-0 database (Bruker Biospin, Rheinstetten, Germany), as well as other existing databases[43,44].

A total of 142 spectra corresponding to 92 allergic asthma patients and 50 healthy children have been considered for multivariate analysis. To build the data matrix, variable sized bucketing was performed in the δ 10.00-0.40 region of the standard 1D spectra, excluding the sub-region δ 4.55-6.05 to remove variability arising from water suppression and from possible cross-relaxation effect on the urea signal via solvent exchanging protons. The bucketing procedure, carried out in Amix version 3.9 (Bruker BioSpin, Rheinstetten, Germany), consisted of dividing the spectra into small regions (buckets) of 0.005 ppm[42]. These buckets were then integrated and normalized by: *i)* adjusting the total area to unity, *ii)* by creatinine signal and *iii)* through PQN. In the total area normalization, each observation is set to have total unit intensity, dividing each data point by the total spectral area[45]. Normalization by creatinine signal consists in the ratio of each data point to urinary creatinine signal at 4.06ppm[46] whilst PQN scales spectra, is estimated by analyzing the distribution of quotients of the amplitudes of the spectrum to be normalized with those of a reference spectrum[47]. PQN assumes that a majority of metabolites keep their concentrations unchanged across the samples. Therefore, normalization is carried out based on the maximum probability

of dilutions, which is estimated by the calculation of the most probable quotient between a target spectrum and a reference spectrum. The PQN method takes the median of many estimates rather than a single sum as the standard of normalization, and this makes it less susceptible to outliers[48]. Data normalization takes into account the inevitable variation in sample concentration and the efficiency of sample preparation procedure making data from all samples directly comparable with each other[49]. This step is necessary, especially with urine samples due to the variable nature of urinary concentration[50].

Then, after data normalizing, PLS-DA, and OPLS-DA were performed in SIMCA-P11.5 (Umetrics, Umeå, Sweden), employing a default 7-fold internal cross validation, from which Q^2 and R^2 values, representing, respectively, the predictive capability and the explained variance, were extracted. VIP is a summary of the importance of an X variable for both Y and X. It is weighted sum of squares of the PLS-weights calculated from the amount of Y-variance of each PLS component[51].

Model robustness was assessed by MCCV with 500 iterations. For each of the 500 randomly generated classification models, the number of LV, the Q^2 (expressing the cross validated explained variability), and the classification rate (percentage of samples correctly classified) were recovered. The selection of model complexity was based on the most frequent list of model properties that maximizes the classification rate (i.e., lower LV and higher Q^2). The sensitivity and the specificity of the model were then depicted from the confusion matrix resulting into a ROC map to further assess the results significance. Further validation was performed by permutation analysis, consisting of randomly permuting class membership and running 500 MCCV iterations. Sensitivity is calculated from the ratio between true positives (asthma samples correctly predicted) and the total number of modelled urine samples, whereas specificity is determined from the ratio between true negatives (control samples correctly predicted) and the total number of modelled control RMN data.

3.2.4 Results and discussion

Normalization of urinary compounds

Appropriate pre-treatment of metabolomic data is an essential step before the application of multivariate statistical methods as it takes into account the inevitable variation in sample concentration and the efficiency of sample preparation procedure[52]. In asthma related NMR studies, different normalization methods were applied. For example, Saude *et al.*1 referenced the identified metabolites in urine to creatinine levels whilst Jung *et al.*[53] normalized the identified serum metabolites to the total spectral area. However, PQN has been increasingly used in metabolomics as shown by Dieterle[47] and Kohl[52], among others. In this study, a comparison of the performance of the data pre-treatment, previously reported in asthma NMR studies, (creatinine signal and to total spectral area) and the increasingly used PQN was performed and the reliability of data pre-treatment was evaluated using PLS-DA models.

Scores plot for the first two latent variables of PLS-DA models for ^{1}H NMR data are shown in Figure 3 comparing different normalization procedures, total area normalization (Figure 3.6 **A**), creatinine normalization (Figure 3.6 **B**) and PQN (Figure 3.6 **C**). As observed, PQN transformation has a positive impact in the discrimination power and the models, consisting of two components, had R^2, which is the cumulative sum of squares of all the X's explained by all extracted components, for total area normalization of 0.03, creatinine normalization of 0.11 and using PQN 0.22. The application of PQN resulted in an improved R^2 when compared with the remaining two normalization procedures. Normalizing samples through creatinine has shown some disadvantages as this compound is not constant and may be related to muscle mass particularly in children[46]. PQN has shown that performs adequately in compensating for different urine dilutions and is more accurate even with low metabolic variations[47]. For further studies, the model that used PQN was chosen as it was demonstrated that the PQN normalization provided the best results in terms of group discrimination.

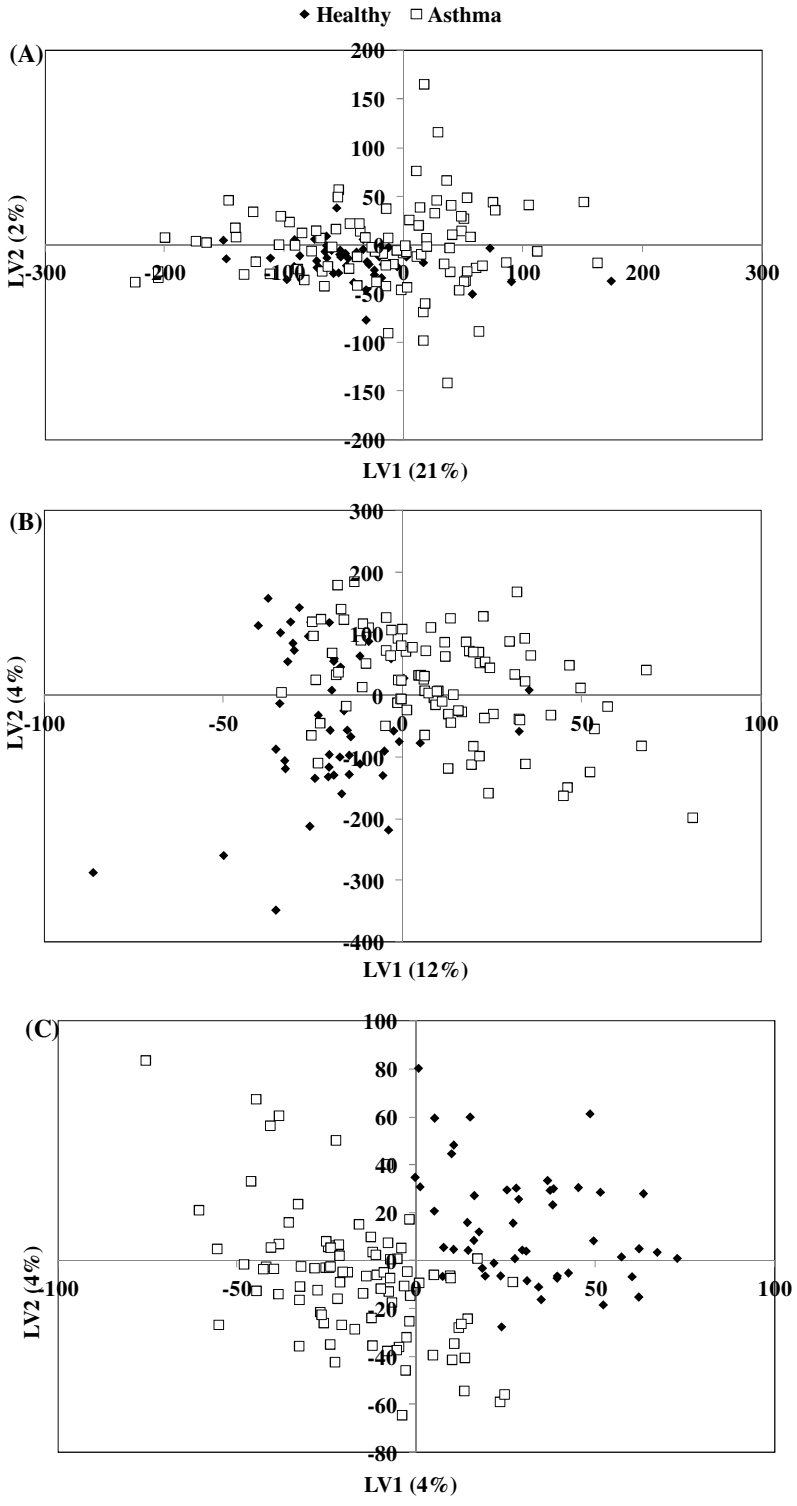


Figure 3.6 - Partial Least Squares Discriminant Analysis (PLS-DA) applied to ^{1}H NMR urine spectra (δ0.50 to 10.0, excluding 4.55-6.05) using different data normalization techniques: **(A)** normalized to total area, **(B)** normalized to creatinine signal and **(C)** by probabilistic quotient normalization (PQN). The R^2 for the total area, creatinine normalizations and PQN was 0.03, 0.11 and 0.22, respectively. The healthy subjects are in full symbols whilst the asthma patients are represented by open symbols.

PLS-DA is often used in the metabolomics field and this tool is known to overfit the data specially when a smaller number of samples is used whe compared to the number of varibales. Therefore, validating the obtained models is necessary and cross validation is often performed with the analysis of several parameters such as Q^2 value, true positives, true negatives, false positives, misclassifications and receiver operating characteristic curves[54]. MCCV statistics was applied to the PQN-PLS-DA model (Figure 3.6 **C**) to assess the predictive ability of the classification model.

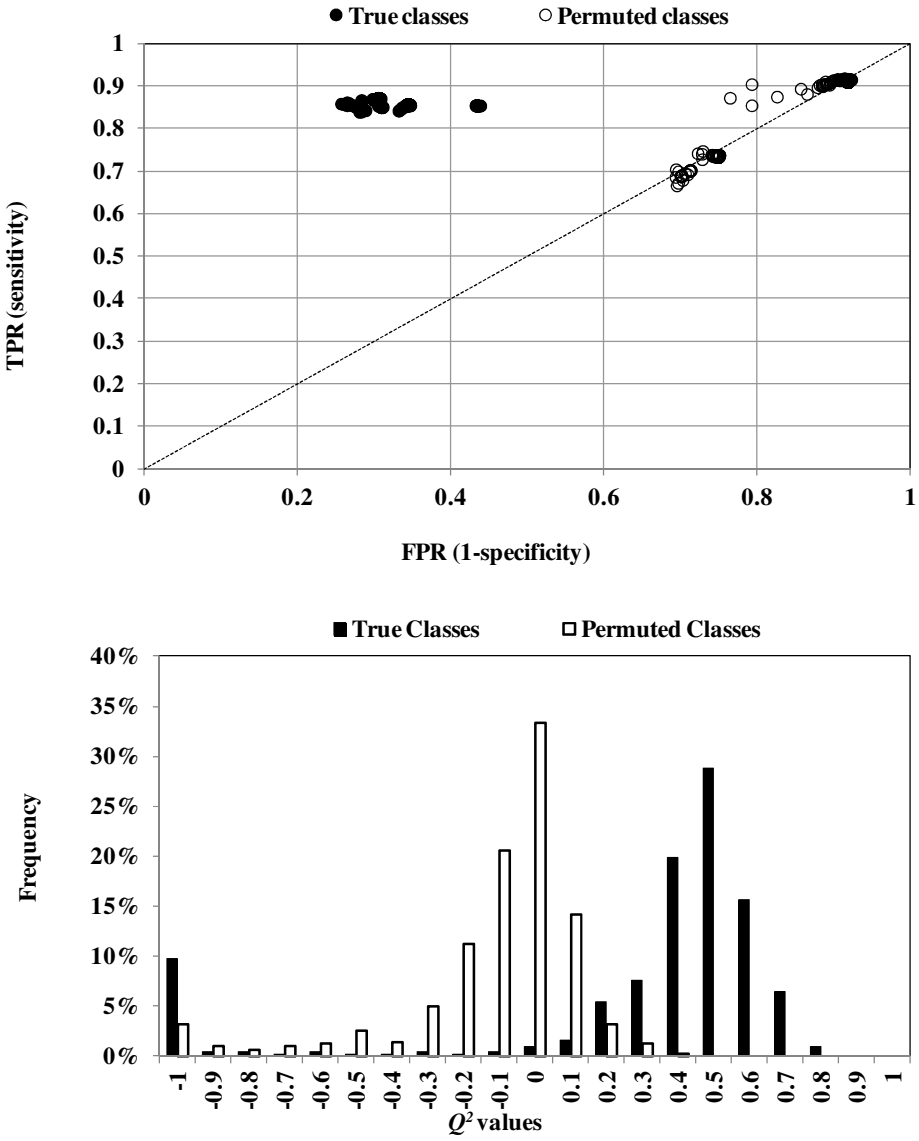


Figure 3.7 – ROC space, where each point represents a prediction result (sensitivity and 1-specificity) of the confusion matrices obtained from MCCV of the PLS-DA model with PQN; full symbols, prediction results in the original model (class membership, healthy or asthma, correctly assigned); open symbols, prediction results in the permuted model (class membership randomly permuted)

The average Q^2 for the 500 runs was 0.5, with a large prevalence of values in the range 0.4-0.6. According to the resulting confusion matrix, the PQN-PLS-DA model (Figure 3.7) showed approximately 85% sensitivity, with 15% of asthma patients being misclassified as healthy, and 72% specificity, with 18% false positives. The classification rate (total number of samples correctly classified) was 80%.

Improving metabolomic data through variable selection

The PLS-DA LV1 loadings were colored according to VIP and carefully inspected (Figure 3.8). The identification of compounds with VIP>1 was performed and 12 compounds were responsible for the attained discrimination.

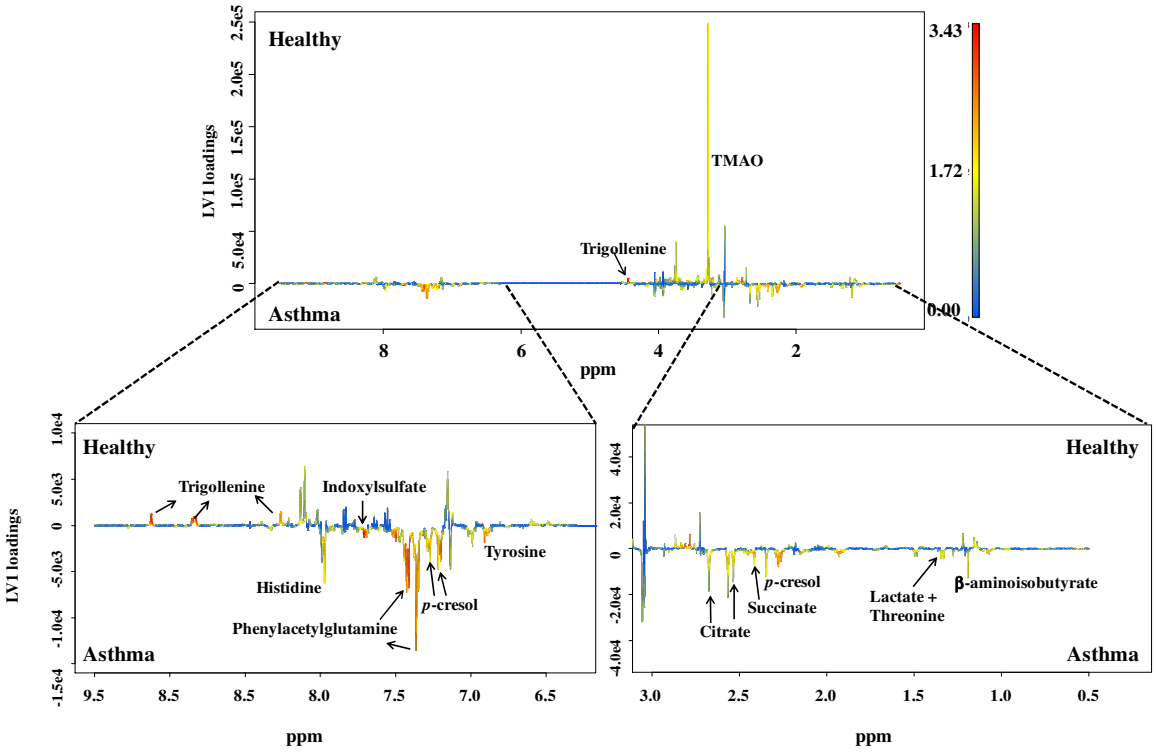


Figure 3.8 – PLS-DA LV1 loadings plot of all compounds colored as a function of VIP. Blue represents VIP starting from 0 and red represents the highest obtained VIP value

Positive signals with high VIP values (>1.0), corresponding to compounds with increased levels in healthy children, were found for trimethylamine-N-oxide and trigonelline. On the other hand, the signals showing negative intensity and high VIP values were from β-aminoisobutyrate, lactate + threonine, histidine, phenylacetylglutamine, acetate, p-cresol,

succinate, citrate, tyrosine and indoxyl sulphate meaning that these compounds were consistently elevated in the urine of asthma patients relatively to healthy children (Table 3.4).

Table 3.4 – Compounds (variables) contributing for the discrimination between healthy subjects and asthma patients (VIP>1; highest VIP and corresponding chemical shift highlighted) and comparison between the data available in the literature for each compound and the obtained results in this study.

Compounds	δ ^{1}H(ppm)	VIP	Possible biochemical origin(s)	↑	Comparison to literature results
Phenylacetylglutamine	1.93	2.70	Increased in syndromes of phospholipidosis impaired amino acid absorption; gut microflora	Asthma	-
	2.11	2.00			
	2.27	2.30			
	7.36	2.50			
	7.43	**2.97**			
Succinate	2.41	2.89	TCA cycle	Asthma	Saude *et al.*[1] **Asthma↑**
Acetate	1.93	2.70	TCA cycle	Asthma	Jung *et al.*[53] **Control↑**
Tyrosine	6.91	2.65	Modification of free tyrosine residues by eosinophil activity in the airways of humans with asthma; Amino acid oxidation	Asthma	Saude *et al.*[1] **Asthma↑**
Indoxyl sulphate	**7.51**	**2.42**	Oxidative stress	Asthma	-
	7.70	2.19			
p-Cresol	2.35	1.60	End-product of protein breakdown; compound of the amino acid tyrosine (gut microflora)	Asthma	-
	7.21	2.16			
	7.28	**2.18**			
Histidine	3.74	2.07	Histamine metabolism	Asthma	Saude *et al.*[1] **Control↑**
	3.75	2.08			
	7.97	**2.12**			
Lactate + threonine	1.33	1.95	Lactic acidosis	Asthma	Saude *et al.*[1] Asthma vs. **Emergency Department Asthma↑**
Citrate	**2.54**	**1.91**	TCA cycle	Asthma	-
	2.68	1.77			
β-Aminoisobutyrate	1.20	1.70	Thymine catabolism/metabolism	Asthma	-
Trigonelline	**4.44**	**3.28**	Diet; Impairment of nicotinate and nicotinamide metabolism	Healthy	Saude *et al.*[1] **Asthma↑**
	8.83	3.11			
	8.85	3.12			
	9.12	3.25			
Trimethylamine-N-oxide	3.27	1.89	Diet	Healthy	Saude *et al.*[1] **Control ↑**

Metabolomic studies benefit from variable selection methods, based on VIP or even from selecting specific chemical shifts creating new datasets improving the developed models[55]. The VIP assesses the significance of each variable in the projection, summarizing the contribution that a variable makes to the model and usually an appropriate threshold is 1.0[56,57]. The yielded R^2 values of PQN-PLS-DA model were low for studies in metabolomic field for complex sample analysis. With this in mind, VIP and spectrum region selection variable selection were performed. A new model was constructed using only the variables with VIP>1 and the results are shown in Figure 3.9 **A**. An improvement of Q^2 was observed from 0.221 to 0.558.

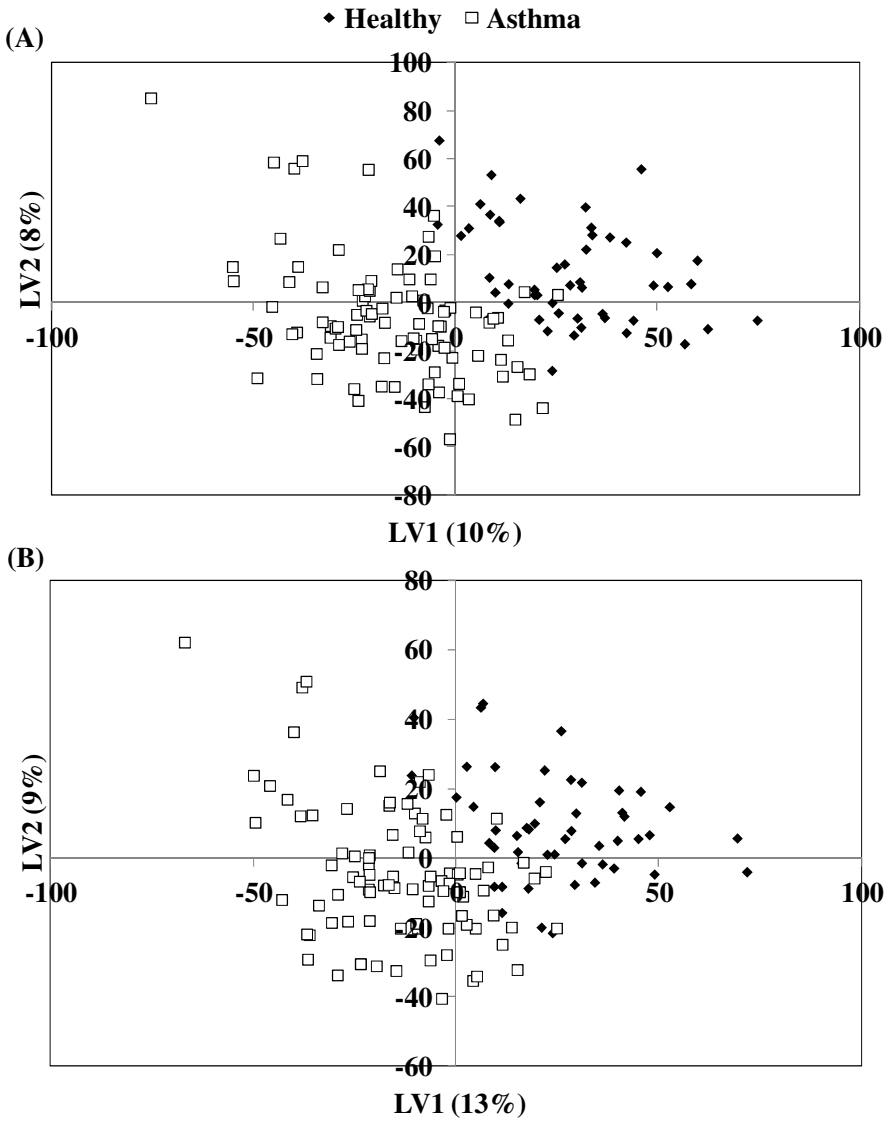


Figure 3.9 – Scores plot for the first two latent variables of PLS-DA models from ^{1}H NMR spectra of urine using PQN using two possible variable selection: **(A)** Variable Importance in Projection (VIP) higher than 1 and **(B)** by selecting regions between 0.5-3 ppm and 6-9.5ppm combined with VIP>1

Another variable selection tested was to select regions between 0.5-3 ppm and 6-9.5 ppm and choosing the signals with VIP>1 (Figure 3.9 **B**). With this selection, Q^2 improved but to a lesser extent of 0.539 and MCCV was also performed for the regions models and the results are shown in Table 3.5. Table 3.5 summarizes the results obtained in MCCV. These results show that variable selection (VIP>1 and δ 0.5-3+6-9.5 combined with VIP>1), in which subsets are created from the original dataset, improved predictive power and MCCV performance, which allowed to filter the information and focus on the significant compounds.

Table 3.5 – Prediction Results Obtained by MCCV (500 Iterations) of PLS-DA Models Built for the Classes: Healthy vs Asthma (n=142).Comparison between the PLS-DA using PQN, and a selection of variables with VIP higher than 1 (VIP>1) and by selecting regions between 0.5-3ppm and 6-9.5ppm combined with VIP>1 (regions)

Model	Q^2	Classification Rate (%)	Sensitivity (%)	Specificity (%)
PLS-DA PQN	0.221	80.3	85.2	71.8
PLS-DA PQN VIP>1	0.558	83.3	89.3	72.3
PLS-DA PQN regions	0.539	83.9	90.3	72.1

MCCV was performed to the two variable selections performed and as seen on Table 3.5 all parameters improved: classification rate of 83.3%, and method sensitivity of 89.3%, whilst the specificity remained at 72.3% (Figure 3.10). Another possibility to improve the developed statistical models and to reduce the number of variables is to choose specific ^{1}H NMR spectral regions. This was performed by choosing regions PQN δ0.5-3 and 6-9.5ppm selecting the compounds with VIP>1.

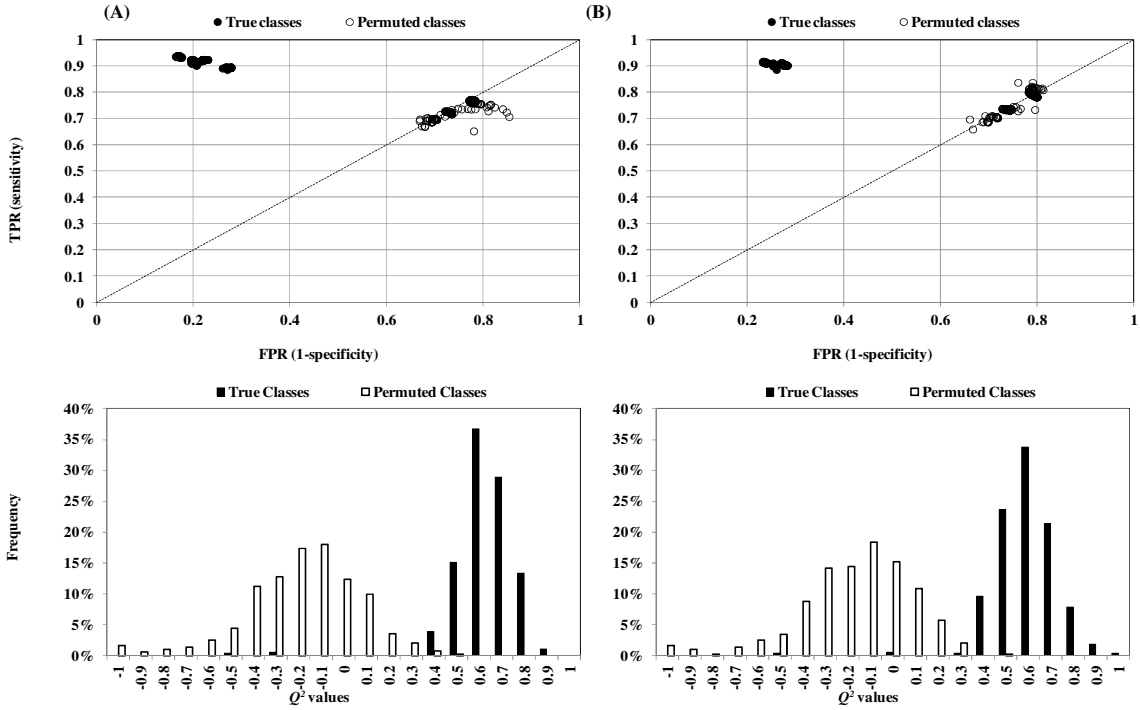


Figure 3.10 - ROC space, where each point represents a prediction result (sensitivity and 1-specificity) of the confusion matrices obtained from MCCV (500 iterations) of the **(A)** PLS-DA model with PQN and selecting variables VIP >1 and **(B)** model with PQN and selecting regions between 0.5-3ppm and 6-9.5ppm combined with VIP>1. Full symbols, prediction results in the original model (class membership, healthy or asthma, correctly assigned); open symbols, prediction results in the permuted model (class membership randomly permuted)

Asthma urinary asthma metabolomics based on ^{1}H NMR

From Table 3.4, it was possible to identify the compounds that discriminate asthma and healthy groups, where trimethylamine-N-oxide and trigonelline characterizes the healthy population and β-aminoisobutyrate, lactate + threonine, histidine, phenylacetylglutamine, acetate, p-cresol, succinate, citrate, tyrosine and indoxyl sulphate the asthmatics. The possible biochemical origins of the compounds are shown in Table 3.3 and a closer look into the metabolic pathways that might be affected by asthma will be made.

Succinate and citrate[58] are known compounds related to the TCA cycle, which have been reported to be increased in the asthma patients[1] show similar behavior in the results obtained in urina of the asthma population (Table 3.4), confirming that this biochemical pathway is affected by the asthma pathology. Citrate has been reported by Loureiro *et al.*[59] to be increased in asthmatic patients with a stable condition, relating this compound with the disturbance of the TCA cycle. Acetate, which is oxidized in TCA cycle, was found to be

elevated in urine of the asthma population (Table 3.4). Jung *et al.*[53] detected it in the sera of asthma patients, nevertheless exihibiting an opposite behavior from the one observed in our study. As urine is an excretion fluid, we may conjecture that the higher levels obtained in urine are due to kidneys activity. The decreased levels of these compounds may lead to conclude that the energy metabolism may be affected in asthmatics because hypoxia is known to affect all processes from the TCA cycle and it is a well known fact that asthmatics have decreased lung capacities and thus affecting the TCA cycle. Wang *et al.*[60] have found this compound to be elevated in COPD patients and associated it to an accelerated lipid catabolism to satisfy energy requirements. TCA cycle anomalies have been known to cause several diseases[61], as it is a crucial process for the cell basal metabolism.

Another biochemical pathway that might be affected by asthma pathology is lactic acidosis, which is a physiological condition characterized by low pH in body tissues accompanied by lactate build-up and which is indicative of hypoxia. This leads to metabolize glucose anaerobically and by this way lactate is formed[58]. Our results show that lactate + threonine are elevated in the asthma population under study (Table 3.4). Saude *et al.*[1], reported that the level of this compound was elevated when comparing stable asthma patients, emergency department asthma patients and healthy children. According to Loureiro *et al*[59]., its content seems to be higher in exarbeated condition when compared to patients with an asthma stable condition.

Histidine is a precursor for histamine biosynthesis and the compound histidine is increased in the asthmatic urine profile (Table 3.4). This behavior allowed to infer that the metabolic pathway that involves histidine may be affected by the asthma pathology as Jung *et al.*[53] demonstrated in their study. The hypothesis that can be formed by the elevated levels of histidine in asthmatics is that the higher bioavailability of this compound can lead to a higher rate in histidine decarboxylation to yield histamine. Histamine is a inflammatory mediator always present and associated to the asthma pathology as potent constrictor of airway smooth muscle[62].

Eosinophils promote tissue injury contributing to asthma pathogenesis and it has been verified that tyrosine is a part of the modification of free tyrosine residues after eosinophil stimulation and degranulation that occurs in the airways of humans with asthma[63]. This compound is also formed by amino acids oxidation induced directly by reactive oxygen species or indirectly by secondary by-products of oxidative stress reactions[64]. According to the results obtained (Table 3.4), this compound is also responsible for the discrimination

attained between the asthmatic and healthy population, being elevated in the former, which leads to confirm that this biochemical pathways may be affected by the asthma pathology.

The levels of phenylacetylglutamine are elevated in the asthma population, and observing the VIP of this compound in Table 3.4, we can verify that from all compounds it has the highest VIP (2.97) correspondent to the peak at 7.43ppm. This compound has shown to be increased in syndromes of phospholipidosis impaired amino acid absorption, states of increased gut absorption of phenylacetate[65], and the development of atopy and asthma may be influenced by gut permeability and gut microflora[66]. Asthma and gut permeability has been previously linked[67], as asthma is a T lymphocyte mediated inflammatory disease. It has been suggested that the whole mucosal system might be involved, with activated T lymphocytes migrating from one mucosal site to another[68]. The link between gut microflora and asthma is related to the "hygiene hypothesis". The "hygiene hypothesis" for allergies and asthma states that a lack of microbial stimulation results in aberrant immune responses to innocuous antigens later in life[69]. Evidence that sustains this hypothesis directs to the "microbiota hypothesis" where changes in the gastrointestional microbiota composition (specially in the Western geographical areas) change the mechanisms of mucosal immunologic tolerance owing to antibiotic use, dietary changes and other lifestyle variations. Epidemiologic and clinical data supporting this information includes, for example, a positive association between increasing risk for asthma/allergies and the increase use of antibiotics and the intake of oral probiotics and/or dietary changes in some individuals prevented/reduced allergies[70]. *p*-Cresol is also a compound generated from tyrosine by gut microflora[71]. As previously shown, the asthmatic gut microflora might be affected by asthma and elevated levels of p-cresol might show this effect.

β-Aminoisobutyrate is the product of the catabolism of the pyrimidine bases uracil and thymine in what constitutes the first step of pyrimidine degradation pathway[62]. This compound was found to be increased in urine of asthmatics (Table 3.4) and to the best of our knowledge this pathway has not been associated to asthma . Oxidative stress related to asthma is a studied phenomena[72]. This phenomenon damages several biomolecules as lipids, proteins and DNA. ROS react with DNA adding to the DNA double bond. By abstraction of an hydrogen atom from methyl group of thymine several pyrimidine radicals are formed[73]. Hasbal and co-authors[74] documented an increase in DNA strand breaks in children with asthma that can be formed by direct interaction with ROS and as β-aminoisobutyrate is related to the catabolism of pyrimidine bases this compound identified in our study may indicate the DNA damage that asthma causes.

Indoxyl sulphate is increased in urine of asthma patients (Table 3.4). Indoxyl sulphate, beside being a compound of the common amino acid tryptophan[75], induces oxidative stress in vascular endothelial cells[76] and strongly decreases the levels of glutathione, one of the most active antioxidant systems of the cells[77].

3.2.5 Concluding remarks

In this study, metabolomic urinary profile of an asthma pediatric population was explored by ^{1}H NMR. ^{1}H NMR technology in the analysis of urine combined with multivariate tools can be used to differentiate allergic asthma children from a healthy population.

The pre-treatment of metabolomic data is a ubitiquous step before the application of multivariate statistical tools. In this study three normalization methods were tested and PQN yielded the best results when compared to normalization to total area and creatinine signal. Variable (VIP >1) and spectrum region (0.5-3ppm and 6-9.5ppm) selection was also tested. The results showed that a combination between these selection gave the same results. MCCV was performed for all developed models. The improvement by the aforementioned selections was also observed by the validation results where all parameters from $Q^{2,}$ classification rate, sensitivity and specificity improved. Analysing the obtained loadings, the compounds responsible for the discrimination of the asthma and healthy groups were identified. These were trimethylamine-N-oxide and trigonelline for the healthy population and β-aminoisobutyrate, lactate + threonine, histidine, phenylacetylglutamine, acetate, p-cresol, succinate, citrate, tyrosine and indoxyl sulphate for the asthmatics.

A metabolic signature comprising an array of biochemical pathways altered in asthma patients was obtained. Overall, TCA cycle, lactic acidosis, histidine metabolism, modification of free tyrosine residues after eosinophil stimulation, alterations in intestinal microflora, DNA damage and oxidative stress were the possible identified biochemical pathways. It was also possible to obtain information on DNA damage produced by ROS probably derived from hypoxia state.

Urine analysis by ^{1}H NMR can be used as a diagnostic tool as it is easily applied in a clinical setting and can be incorporated into a laboratory, as other technology has been in the past. Moreover, urinary metabolomic profiles related to asthma can be made using ^{1}H NMR, bringing information on asthma-related changes in several biochemical pathways.

3.4 References

(1) Saude E.J., Skappak C.D., Regush S., Cook K., Ben-Zvi A., Becker A., Moqbel R., Skyes B.D., Howe B.H., Adamko D.J., Metabolomic profiling of asthma: diagnostic utility of urine nuclear magnetic resonance spectroscopy. J. Allergy Clin. Immunol. (2011)127: 757-764.

(2) Snowden S., Dahlén S.E., Wheelock C.E., Application of metabolomics approaches to the study of respiratory diseases. Bioanalysis (2012) 4: 2265-2960.

(3) Admako D.J., Skyes B.D., Rowe B.H., The metabolomics of asthma: novel diagnostic potential. Chest (2012) 141: 1295-1302.

(4) Rocha S.M., Caldeira M., Carrola J., Santos M., Cruz N., Duarte I.F., Exploring the human urine metabolomic potentialities by comprehensive two-dimensional gas chromatography coupled to time of flight mass spectrometry. J. Chromatogr. A (2012) 1252: 155-163.

(5) Kalluri U., Naiker M., Myers M.A., Cell culture metabolomics in the diagnosis of lung cancer—the influence of cell culture conditions., J. Breath Res. (2014) 8: 027109.

(6) Meager E.A., Fitzgerald G.A., Indices of lipid peroxidation in vivo: strengths and limitations. Free Radic. Biol. Med. (2000) 28: 1745-1750.

(7) van den Berg R.A., Hoefsloot H.C., Westerhuis J.A., Smilde A.K., van der Werf M.J., Centering, scaling, and transformations: improving the biological information content of metabolomics data. BMC Genomics (2006) 7: 142-157.

(8) Weber C.M., Cauchi M., Patel M., Bessant C., Turner C., Britton L.E., Willis C.M., Evaluation of a gas sensor array and pattern recognition for the identification of bladder cancer from urine headspace. Analyst (2011) 136: 359-364.

(9) R Development Core Team. R: A Language and Environment for Statistical Computing, 2.9.2 ; R Foundation for Statistical Computing: Vienna, Austria, 2010.

(10) Eriksson, L., Johansson, E., Kettaneh, N., Wold, N., Wold, S., Multi and Megavariate Data Analysis, Principles and Applications (2001) Umetrics AB Umea, Sweden.

(11) Picard, R.R., Cook, R.D., Cross-validation of regression-models. J. Am. Stat. Assoc. (1984) 79: 575-583.

(12) Xu Q.-S., Liang Y.-L., Du Y.-P., Monte Carlo cross-validation for selecting a model and estimating the prediction error in multivariate calibration. J. Chemometrics (2004)18: 112-120.

(13) Almstetter M.F., Oefner P.J., Dettmer K., Comprehensive two-dimensional gas chromatography in metabolomics. Anal. Bioanal. Chem. (2012) 402: 1993-2013.

(14) Zushi Y., Hashimoto S., Fushimi A., Takazawa Y., Tanabe K., Shibata Y., Rapid automatic identification and quantification of compounds in complex matrices using comprehensive two-dimensional gas chromatography coupled to high resolution time-of-flight mass spectrometry with a peak sentinel tool. Anal. Chim. Acta. (2013) 778:54-62.

(15) Silva B.J., Tranchida P.Q., Purcaro G., Queiroz M.E., Mondello L., Lanças F.M., Evaluation of comprehensive two-dimensional gas chromatography coupled to rapid scanning quadrupole mass spectrometry for quantitative analysis. J. Chromatogr. A. (2012) 1255: 177-183.

(16) von Mühlen C., Marriott P.J., Retention indices in comprehensive two-dimensional gas chromatography. Anal. Bioanal. Chem. (2011) 401: 2351-2360.

(17) Dallüge J., Beens J., Brinkman U.A., Comprehensive two-dimensional gas chromatography: a powerful and versatile analytical tool. J. Chromatogr. A. (2003) 1000: 69-108.

(18) Rocha S.M., Freitas R., Cardoso P., Santos M., Martins R., Figueira E., xploring the potentialities of comprehensive two-dimensional gas chromatography coupled to time of flight mass spectrometry to distinguish bivalve species: comparison of two clam species (Venerupis decussata and Venerupis philippinarum). J. Chromatogr. A, (2013) 1315: 152-161.

(19) Polkowska Z., Kozłowska K., Mazerska Z., Górecki T., Namieśnik J., Volatile organohalogen compounds in human urine: the effect of environmental exposure. Chemosphere (2006) 62: 626-640.

(20) Mills G.A., Walker V., Headspace solid-phase microextraction profiling of volatile compounds in urine: application to metabolic investigations. J. Chromatogr. B Biomed. Sci. Appl. (2001) 753: 259-268.

(21) Bouatra S., Aziat F., Mandal R., Guo A.C., Wilson M.R., Knox C., Bjorndahl T.C., Krishnamurthy R., Saleem F., Liu P., Dame Z.T., Poelzer J., Huynh J., Yallou F.S., Psychogios N., Dong E., Bogumil R., Roehring C., Wishart D.S., The human urine metabolome PLoS One (2013) 4: e73076.

(22) Tangerman A., Measurement and biological significance of the volatile sulfur compounds hydrogen sulfide, methanethiol and dimethyl sulfide in various biological matrices. J. Chromatogr. B (2009) 877: 3366-3377.

(23) Hakim M., Broza Y.Y., Barash O., Peled N., Phillips M., Amann A., Haick H., Volatile organic compounds of lung cancer and possible biochemical pathways. Chem. Rev. (2012):112 5949-5966.
(24) Auman J.T., Boorman G.A., Wilson R.E., Travlos G.S., Paules R.S., Heat map visualization of high-density clinical chemistry data. Physiol. Genomics 31 (2007) 352-356.
(25) Mieth M., Schubert J.K., Gröger T., Sabel B., Kischkel S., Fuchs P., Hein D., Zimmermann R., Miekisch W., Automated needle trap heart-cut GC/MS and needle trap comprehensive two-dimensional GC/TOF-MS for breath gas analysis in the clinical environment. Anal. Chem. (2010) 82: 2541-2551.
(26) Warrack B.M., Hnatyshyn S., Ott K.-H., Reily M.D., Sanders M., Zhang H., Drexler D.M., Normalization strategies for metabonomic analysis of urine samples. J. Chromatogr. B (2009) 877: 547-552.
(27) Nadeem A., Chhabra S.K., Masood A., Raj H.G., Increased oxidative stress and altered levels of antioxidants in asthma. J. Allergy Clin. Immunol. (2003) 111: 72-78.
(28) Celik M., Tuncer A., Soyer O.U., Saçkesen C., Tanju Besler H., Kalayci O., xidative stress in the airways of children with asthma and allergic rhinitis. Pediatr. Allergy Immunol. (2012) 23: 556-561.
(29) Henricks P.A.J., Nijkamp F.P., Reactive oxygen species as mediators in asthma. Pulm. Pharmacol. Ther. (2001) 14: 409-421.
(30) Nadeem A., Masood A., Siddiqui N., Oxidant--antioxidant imbalance in asthma: scientific evidence, epidemiological data and possible therapeutic options. Ther. Adv. Respir. Dis. (2008) 2: 215-235.
(31) Murray R., Granner D., Mayes P., Rodwell V., Harper's Illustrated Biochemistry, 27th ed.. (2006) McGraw-Hill Medical: New York, USA.
(32) Kolokolova T.N., Savel'ev O.Y., Sergeev N.M., Metabolic analysis of human biological fluids by 1H NMR spectroscopy. J. Anal. Chem. (2008) 63:104-120.
(33) Barding Jr G.A., Salditos R., Larive J.K., Quantitative NMR for bioanalysis and metabolomics. Anal. Bioanal. Chem. (2012) 404: 1165-1179.
(34) Duarte I.F., Gil A.M., Metabolic signatures of cancer unveiled by NMR spectroscopy of human biofluids. Prog. Nucl. Mag. Res. Sp. (2012) 62: 51-74.
(35) Malet-Martino M., Holzgrabe U., NMR techniques in biomedical and pharmaceutical analysis. J. Pharmaceut. Biomed. (2011) 55: 1-15.
(36) Keun H.C., Ebberls T.M., Antti H., Bollard M.E., Beckonert O., Schlotterbeck G., Senn H., Niederhauser U., Holmes E., Lindon J., Nicholson J., Analytical reproducibility in 1H NMR-based metabonomic urinalysis. Chem. Res. Toxicol. (2002) 15: 1380-1386.
(37) Dumas M.E., Malbaum E.C., Teague C., Ueshima H., Zhou B., Lindon J., Nicholson J., Stamler J., Elliot P., Chan Q., Holmes E., Assessment of analytical reproducibility of 1H NMR spectroscopy based metabonomics for large-scale epidemiological research: the INTERMAP study. Anal. Chem. (2006) 78: 2199-2208.
(38) Maher A.D., Zirah S., Holmes E., Nicholson J., Experimental and analytical variation in human urine in 1H NMR spectroscopy-based metabolic phenotyping studies. Anal. Chem. (2007) 79: 5204-5211.
(39) Loureiro C.C., Duarte I.F., Gomes J., Carrola J., Barros A.S., Gil A.M., Bousquet J., Bom A.T., Rocha S.M., Urinary metabolomic changes as a predictive biomarker of asthma exacerbation. J. Allergy Clin. Immunol. (2014) 133: 261-263.e5.
(40) Jung J., Kim S.H., Lee H.S., Choi G.S., Jung Y.S., Ryu D.H., Park H.S., Hwang G.S., Serum metabolomics reveals pathways and biomarkers associated with asthma pathogenesis. Clin. Exp. Allergy (2013) 43: 425-433.
(41) Kohl S., Klein M., Hochrein J., Oefner P., Spang R., Gronwald W., State-of-the art data normalization methods improve NMR-based metabolomic analysis. Metabolomics (2012) 8: 146-160.
(42) Carrola J., Rocha C.M., Barros A.S., Gil A.M., Goodfellow B.J., Carreira I.M., Bernardo J., Gomes A., Sousa V., Carvalho L., Duarte I.F., Metabolic Signatures of Lung Cancer in Biofluids: NMR-Based Metabonomics of Urine. J. Proteome Res. (2011) 10: 221-230.
(43) Wishart D.S., Tzur D., Knox C., Eisner R., Guo A.C., Young N., Cheng D., Jewell K., Arndt D., Sawhney S., Fung C., Nikolai L., Lewis M., Coutouly M.A., Forsythe I., Tang P., Shrivastava S., Jeroncic K., Stothard P., Amegbey G., Block D., Hau D.D., Wagner J., Miniaci J., Clements M., Gebremedhin M., Guo N., Zhang Y., Duggan G.E., Macinnis G.D., Weljie A.M., Dowlatabadi R., Bamforth F., Clive D., Greiner R., Li L., Marrie T., Sykes B.D., Vogel H.J., Querengesser L., HMDB: the human metabolome database. Nucleic Acids Res. (2007) 35: 521-526.
(44) Psychogios N N., Hau D.D., Peng J., Guo A.C., Mandal R., Bouatra S., Sinelnikov I., Krishnamurthy R., Eisner R., Gautam B., Young N., Xia J., Knox C., Dong E., Huang P., Hollander Z., Pedersen T.L., Smith S.R., Bamforth F., Greiner R., McManus B., Newman J.W., Goodfriend T., Wishart D.S., The human serum metabolome. Plos One (2011) 6: e16957.
(45) Craig A., Cloarec O., Holmes E., Nicholson J.K., Lindon J.C., Scaling and Normalization Effects in NMR Spectroscopic Metabonomic Data Sets. Anal. Chem. (2006) 78: 2262-2267.

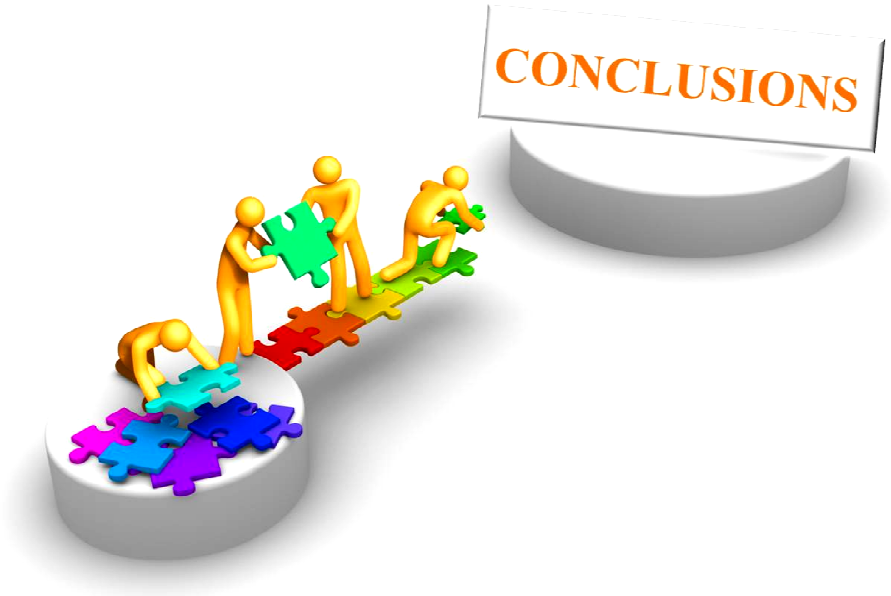
(46) Prevot A., Martini S., Guignard J.P., Glomerular filtration markers in paediatrics. Rev. Med. Suisse Romande (2002) 122: 625-630.
(47) Dieterle F., Ross A., Schlotterbeck G., Seen H., Probabilistic Quotient normalization as robust method to account for dilution of complex biological mixtures. Application in 1H NMR metabonomics. Anal. Chem. (2006) 78: 4281-4290.
(48) Torgrip R.J.O., Åberg K.M., Alm E., Schuppe-Koistinen I., Lindberg J., A note on normalization of biofluid 1D 1H-NMR data. Metabolomics (2008) 4: 114-121.
(49) Dong J., Cheng K.H., Xu J., Chen Z., Griffin J., Group aggregating normalization method for the preprocessing of NMR-based metabolomic data. Chem. Intell. Lab Syst. (2011) 108: 123-132.
(50) Wright C.A., Howles S., Trudgian D.C., Kessler B.M., Reynard J.M., Noble J.G., Hamdy F.C.,. Turney B.W, Label-free Quantitative Proteomics Reveals Differentially Regulated Proteins Influencing Urolithiasis. Mol. Cell. Proteomic (2011) 10: M110.005686.
(51) Wold S., Sjöström M., Eriksson L., PLS-regression: a basic tool of chemometrics. Chemometr. Intell. Lab. (2001) 58: 109-130.
(52) Kohl S., Klein M., Hochrein J., Oefner P., Spang R., Gronwald W., State-of-the art data normalization methods improve NMR-based metabolomic analysis. Metabolomics (2012) 8: 146-160.
(53) Jung J., Kim S.H., Lee H.S., Choi G.S., Jung Y.S., Ryu D.H., Park H.S., Hwang G.S., Serum metabolomics reveals pathways and biomarkers associated with asthma pathogenesis. Clin Exp Allergy (2013) 43:425-433.
(54) Westerhuis J.A., Hoefsloot H.C.J., Smit S., Vis D.J., Smilde A.K., van Velzen E.J.J., van Duijnhoven J.P.M., van Dorsten F.A., Assessment of PLSDA cross validation. Metabolomics (2008) 4: 81-89.
(55) Diaz S.O., Barros A.S, Goodfellow B.J., Duarte I.F., Galhano E., Pita C., Almeida M., Carreira I.S., Gil A.M., Second Trimester Maternal Urine for the Diagnosis of Trisomy 21 and Prediction of Poor Pregnancy Outcomes. J. Proteome Res. (2013) 12: 2946-2957.
(56) Bryan K. , Brennan L., Cunningham P., MetaFIND: A feature analysis tool for metabolomics data. BMC Bioinformatics (2008) 9: 470-483.
(57) Maraschin M., Somensi-Zeggio A., Oliveira S.K., Kuhnen S., Tomazzoli M.M., Zeri A.C.M., Carreira R., Rocha M., A Machine Learning and Chemometrics Assisted Interpretation of Spectroscopic Data – A NMR-Based Metabolomics Platform for the Assessment of Brazilian Propolis. Lect. Notes Comput. Sc. (2012) 7632: 129-140.
(58) Murray R.K., Granner D.K., Mayes P.A., Rodwell V.W., Harper's Illustrated Biochemistry. (2003) McGraw-Hill, New York, USA
(59) Loureiro C.C., Duarte I.F., Gomes J., Carrola J., Barros A.S., Gil A.M., Bousquet J., Bom A.T., Rocha S.M., Urinary metabolomic changes as a predictive biomarker of asthma exacerbation. J. Allergy Clin. Immunol. (2014) 133: 261-263.e5.
(60) Wang L., Tang Y., Liu S., Mao S., Ling Y., Liu D., He X., Wang X., Metabonomic Profiling of Serum and Urine by 1H NMR-Based Spectroscopy Discriminates Patients with Chronic Obstructive Pulmonary Disease and Healthy Individuals. Plos One (2013) 8: e65675.
(61) Brière J.J., Favier J., Gimenez-Roqueplo A.P., Rustin P., Tricarboxylic acid cycle dysfunction as a cause of human diseases and tumor formation. Am. J. Physiol. Cell Physiol. (2006) 291: 1114-1120.
(62) Dunford P.J., Holgate S.T., Histamine in Inflammation, The role of histamine in asthma (2010) Springer, New York, USA.
(63) Wu W., Samoszuk M.K., Comhair S.A., Thomassen M.J., Farver C.F., Dweik R.A., Kavuru M.S., Erzurum S.C., Hazen S.L., Eosinophils generate brominating oxidants in allergen-induced asthma. J. Clin. Invest. (2000) 105: 1455–1463.
(64) Dalle-Donne I., Giustarini D., Colombo R., Rossi R., Milzani A., Protein carbonylation in human diseases. Trend. Mol. Med. (2003) 9: 169-176.
(65) Delaney J., Neville W.A., Swain A., Miles A., Leonard M.S., Waterfield C.J., Phenylacetylglycine, a putative biomarker of phospholipidosis: its origins and relevance to phospholipid accumulation using amiodarone treated rats as a model. Biomarkers (2004) 9: 271–290.
(66) Hörmannsperger G., Clavel T., Haller D., Gut matters: Microbe-host interactions in allergic diseases. J. Allergy Clin. Immunol. (2012) 129: 1452-1459.
(67) Benard A., Desreumeaux P., Huglo D., Hoorelbeke A., Tonnel A.B., Wallaert B., Increased intestinal permeability in bronchial asthma. J Allergy Clin. Immunol. (1996) 97: 1173-1178.
(68) Hijazi Z., Molla A.M., Al-Habashi H., Muawad W.M., Molla A.M., Sharma P.N., Intestinal permeability is increased in bronchial asthma. Arch. Dis. Child. (2004) 89: 227-229.
(69) Wills-Karp M., Santeliz J., Karp C.L., The germless theory of allergic disease: revisiting the hygiene hypothesis. Nat. Rev. Immunol. (2001) 1: 69-75.
(70) Huffnagle G.B., The Microbiota and Allergies/Asthma. PLoS Pathog. (2010) 6: e1000549.

(71) Smith E.A., Macfarlane G.T., Studies on Amine Production in the Human Colon: Enumeration of Amine forming Bacteria and Physiological Effects of Carbohydrate and pH. J. Appl. Bacteriol. (1996) 81: 288-297.
(72) Araneda O. F., Tuesta M., Lung Oxidative Damage by Hypoxia. Oxid. Med. Cell. Longev. (2012) 2012: 1-18.
(73) Cooke M.S., Evans M.D., Dizdaroglu M., Lunec J., Oxidative DNA damage: mechanisms, mutation, and disease. FASEB J. (2003) 17: 1195-1214.
(74) Hasbal C., Aksu B.Y., Himmetoglu S., Dincer Y., Koc E.E., Hatipoglu S., Akcay T., DNA damage and glutathione level in children with asthma bronchiale: effect of antiasthmatic therapy. Pediatr. Allergy Immunol. 21 (2010) e674.
(75) Niwa T., Uremic toxicity of indoxyl sulphate. Nagoya J. Med. Sci. (2010) 72: 1-11.
(76) Tumur Z., Niwa T., Indoxyl sulfate inhibits nitric oxide production and cell viability by inducing oxidative stress in vascular endothelial cells. Am. J. Nephrol., (2009) 29: 551-557.
(77) Dou L., Jourde-Chiche N., Faure V., Cerini C., Berland Y., Dignat-George F., Brunet P., The uremic solute indoxyl sulfate induces oxidative stress in endothelial cells. J. Thromb. Haemost. (2007) 5: 1302-1308.

Chapter
4

CHAPTER 4

CONCLUSIONS AND FUTURE PERSPECTIVES



4.1 Conclusions

The challenges proposed for this PhD thesis were addressed and methodologies using high throughput techniques in the analysis of biological examples obtained in a non-invasive manner were developed. Nowadays, certain gold standards, such as spirometry, are routinely used to assess the asthma disease status, but do not provide sufficient information. The feasibility of sampling exhaled breath and urine in an effortlessly manner, namely in children, must also be a purpose to take into account. The use of exhaled breath and/or urine yields additional information that could change the current hallmark on diagnosis, prognosis, in following a prescribed therapy for a single patient or even verify the disease status.

The initial step in the analysis of exhaled breath volatile composition was the development of a HS-SPME method and then combine it with GC-MS. It was possible to verify the richness of the volatile composition of this matrix with the identification of several compounds belonging to different chemical families. The use of MVA tools was fundamental to verify that a pattern of compounds, belonging to the alkanes and aldehydes, was formed. It was observed that the asthmatic population was characterized by ramified alkanes and these compounds are connected to lipid peroxidation from the degradation of fatty acids that occurs due to the oxidant and antioxidant imbalance characteristic in asthma. Due to the advantages presented by the GC×GC-ToFMS, this analytical technique was used to obtain more information on exhaled breath. A more complete metabolomic profile was obtained and from the hundreds of identified compounds, an alkane/aldehyde pattern was obtained and this pattern characterized the asthmatic and healthy populations, respectively. This outcome, combined with the previous GC-MS results, links oxidative stress (measured in the lipid peroxidation products) to asthma. Complementarily, urine volatile analysis by HS-SPME/ GC×GC-ToFMS also revealed this information and it allowed to assess lipid peroxidation measuring compounds belonging to the alcohols, aldehydes, alkanes, alkenes and ketones. These compounds are associated to lipid peroxidation, and consequently to oxidative stress on fatty acids. Additionaly, the urine non-volatile metabolomic pattern was also obtained using ^{1}H NMR to supplement the information attained from the urine volatile analysis. It was possible to identify several other biochemical pathways affected by asthma namely TCA cycle, histidine metabolism, lactic acidosis, changes in gut

microflora, modification of free tyrosine residues by eosinophil activity in the airways of humans with asthma and oxidation of amino acids.

The use of the developed methodologies for clinical purposes was also tested, namely in exhaled breath. The first step was to reduce the GC×GC-ToFMS dataset (134 compounds) and still have a method with a high classification rate, specificity and sensitivity. This was obtained with 9 compounds within the alkane/aldehyde pattern. The applicability of using the exhaled breath metabolomic pattern was then tested through the follow-up: of a naive patient over 24 days, of a patient with severe asthma treated with Omalizumab® over 20 months, of a patient thoughout 3 years and of patients in exarcebation and stable conditions. A new cohort of asthmatic children was also tested in the verification of the developed metabolomic based models in diagnosis and monitoring the disease status through exhaled breath. The metabolite pattern obtained by GC×GC-ToFMS and the developed models were successful as there was a clinical positive evolution accompanied by exhaled breath change in composition towards the healthy group. A noteworthy result was obtained in the severe asthma case as this patient failed several treatments in the 20 month period and this reflected clinicaly as well as it could be observed in the exhaled breath volatile composition.

In conclusion, the analysis of two matrices obtained non-invasively using two high throughput techniques yielded a myriad of information, beside the successful development of statistical models with excellent results in terms of classification rate, sensitivity and sensibility. The information obtained from studying exhaled breath and urine, from a volatile point of view, was complementary and allowed to measure oxidation products that reflect lipid peroxidation in asthma. The non-volatile characterization of urine added information to that obtained in the volatile counterpart with several biochemical pathways being connected to asthma. The use of both matrices and both techniques was a paramount aspect in the work developed for this PhD thesis that allowed obtaining a more complete and complementary information on the metabolic changes in asthma.

The results presented demonstrate the potential of volatile and/or non-volatile exhaled breath and urine metabolomic profiling in asthma. This PhD thesis lays the foundations for future studies in asthma through the use of this matrices combined with the high throughput techniques.

4.2 Future Work

Exhaled breath and urine analysis are promising biofluids in monitoring asthma, being easily implemented in asthma management. These matrices produce an unique print and this can result in a new healthcare paradigm that intends more efficient drug uses, earlier/more accurate diagnosis resulting in a better medical outcome. Nevertheless there are still issues that can be studied from the results presented in this PhD thesis and that could be considered for the future endeavors:

- Enlarge the sample numbers to strengten the results and applying the developed methodologies to other locations in the country or even abroad;
- Use of other high throughput methodologies such as liquid chromatography coupled to mass spectrometry to attain more information of the studied matrices;
- Study the possiblity of characterizing different asthma phenotypes;
- Complementing the metabolomic information by performing proteomic and genomic studies;
- Deepening the biochemical origin of the found compounds analysing human bronchial cells;
- Clinical orientied studies for diagnosis, therapy follow-up and disease monitoring performing longitudinal studies and randomized controlled trials.
- Development of point-of-care platform to be implemented in medical practice as an aid to diagnosis, follow-up therapy and disease monitoring.

APPENDIX

QUESTIONÁRIO

Código do Voluntário: **Idade:** **Idade de início da doença:**

Peso (Kg): **Altura (cm):** **Crises (estimativa):**

Motivo da consulta: Asma Rinite Alérgenos:

Medicação:

A criança teve algum episódio de pieira ou chieira nos <u>últimos doze meses</u>? Sim Não

<u>Nos últimos doze meses</u> a criança fez tratamento imunoterapêutico ,ou seja, vacina para alergia? Sim Não

<u>Nos últimos doze meses</u> a criança teve crise asmática? Sim Não

<u>Nos últimos doze meses</u> a criança espirrou ou teve rinorreia sem que estivesse constipada? Sim Não

<u>Nos últimos doze meses</u> a criança quando fez exercício teve episódios de pieira ou chieira? Sim Não

HABITAÇÃO (Assinale as opções adequadas

Cidade	Apartamento	Nova	Ar condicionado	**Aquecimento:**
Campo	Vivenda	Velha	Desumidificador	Nenhum
	Outro:	Arejada		Óleo
		Poeirenta		Eléctrico
		Húmida		Lareira
		Zona Poluída		Braseira
				Central
				Outro:

QUARTO (Assinale as opções adequadas

Tipo de Pavimento: Alcatifa Tapete de lã Outro:

Colchão: Molas Espuma Palha Outro:

Almofada: Não Usa Espuma Fibra Sintética

Outros: Peluches Livros Expostos Material dos cortinados:

Cuidados Especiais com o colchão: Aspira de semana a semana Lava os lençóis a 60ºC

AMBIENTE (Assinale as opções adequadas

Tem animais domésticos: Canário Cão Gato Coelho Cavalo
Periquito Hamster Outros:

Há Fumadores em casa: Não Pai Mãe Outros:

PROBLEMAS (Assinale as opções adequadas

Época do ano em que passa pior: Primavera Outono Qualquer altura Verão Inverno
Meses Piores:

A criança tem problemas:

Tosse	Urticária
Expectoração	Inchaço
Farfalheira	Dorme de boca aberta
Pieira	Faz barulho a dormir
Falta de ar	Mau hálito de manhã
Comichão nos Olhos	Otites
Olhos a chorar	Amigdalites
Comichão no nariz	Adenodites
Perda do cheiro	Diarreia

Espirros frequentes Vómitos

Borbulhas na cara Outros:

Comichão na cara

Aparecimento dos sintomas: Dia Noite Madrugada Indiferente

Os sintomas são: Leves Moderados Graves Sempre Muitas Vezes Poucas vezes Raramente

Os sintomas interferem no dia-a-dia da criança? Não Pouco Moderamente Muito

Alguns factores podem ser a causa dos sintomas ou podem agravar:

Dentro de casa Zonas húmidas Cosméticos Leite e derivados

Fora de casa Poluição do ar Insecticidas Ovos

Tempo seco Poeiras Celeiros Frutas

Tempo húmido Ar condicionado Feno Chocolate

Dias quentes Fumo do cigarro Trigo Nozes, amêndoas

Dias Frios Papéis Cheiro de comida Amendoins

Dias ventosos Perfumes Peixe Legumes

Mudanças de tempo Medicamentos Carne Vegetais verdes:

Mais informações (familiares com doenças alérgicas e/ou outras doenças da criança):

Quem respondeu a este questionário:

Pai

Mãe

Outra pessoa

Data do Inquérito: / / 201__

Contactos (telemóvel/email):

[FICHA DO VOLUNTÁRIO]

Obrigado pela sua participação! Qualquer dúvida não hesite em contactar-nos!

Dados clínicos:

IgE:

Prick tests:

Espirometria (Fev_1; Fev_{25-75}; Peak Flow):

Em caso de dúvida não hesite em contactar-nos!

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UNIVERSIDADE da MADEIRA

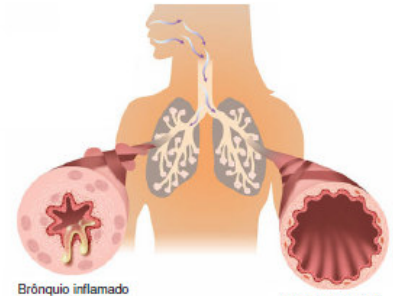
ALLERGY

Desenvolvimento de uma metodologia para a detecção de biomarcadores da asma

Contributos para a definição de um meio diagnóstico

ASMA

A asma é uma doença inflamatória crónica dos brônquios, que afecta ambos os sexos e todos os grupos etários, dos primeiros anos de vida ao indivíduo muito idoso.



Estima-se que mais de 10% das crianças portuguesas e 5 a 7% dos adultos tenham asma.

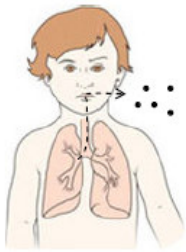
Estima-se que na próxima década estes valores sofram um incremento.

O diagnóstico precoce da asma e o adequado acompanhamento das terapias prescritas são fundamentais para o controlo da doença. Assim, o desenvolvimento de uma metodologia analítica simples, barata e não invasiva que permita a identificação de biomarcadores da asma, através da recolha de ar exalado, é crucial.

ANÁLISE DE AR EXALADO

A nível alveolar ocorrem trocas rápidas entre o sangue e o ar. Este conhecimento levou a que nos últimos anos se desenvolvessem métodos para avaliar marcadores de doenças no ar exalado.

A análise do ar exalado tem sido usada como matriz de estudo na área do desenvolvimento de meios de diagnósticos não invasivos e rápidos.



Com este projecto pretende-se recolher amostras de ar exalado de:

i) Crianças com asma acompanhadas no Hospital Infante D. Pedro, em Aveiro e no Hospital Dr. Nélio Mendonça, no Funchal.

ii) Crianças voluntárias que não tenham apresentado nunca episódios de asma ou outro tipo de alergia.

Saco de recolha do ar

Table S1 -Dataset of volatile compounds identified by GC×GC–ToFMS in exhaled breath used for projection purposes in Chapter 2.5

^1t_R [a] (s)	^2t_R [a] (s)	Compounds																											
		Hydrocarbons	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**	**S12**	**S13**	**S14**	**S15**	**S16**	**S17**	**S18**	**S19**	**S20**	**S21**	**S22**	**S23**	**S24**	**S25**	**S26**	**S27**	**S28**
		Alkanes																												
138	0.48	hexane	0.3	0.4	0.1	0.4	1.7	2.0	1.3	0.2	1.4	0.2	0.4	0.5	1.1	0.3	0.2	0.6	0.6	0.2	0.7	0.6	0.5	0.4	0.8	0.6	4.3	1.0	0.5	0.5
210	0.52	2,4-dimethylhexane	0.2	0.2	0.7	0.3	0.5	0.1	4.3	0.1	1.0	0.1	0.3	0.1	1.1	0.4	0.1	1.1	4.5	0.4	1.1	0.6	0.2	0.0	2.1	0.8	0.9	1.7	0.4	0.6
252	0.54	octane	1.4	0.7	4.0	1.0	0.9	1.2	2.1	0.5	1.7	0.3	0.6	0.6	1.9	0.8	0.4	0.5	1.9	0.7	2.0	1.1	0.6	0.3	0.4	1.5	2.0	2.8	1.1	1.4
276	0.55	2,2,4-trimethylhexane	0.1	0.1	0.2	0.6	2.3	1.7	3.7	1.7	2.9	0.5	0.9	1.0	3.6	1.1	0.0	4.2	3.9	1.3	1.5	1.6	0.1	0.1	2.7	3.1	2.4	3.6	0.2	0.3
288	0.55	2,4-dimethylheptane	1.0	1.0	0.7	0.6	0.3	0.8	0.2	0.3	1.5	0.0	0.2	0.4	0.5	0.0	1.0	0.2	0.8	0.2	0.6	0.6	0.9	0.7	0.1	0.4	1.2	1.6	3.1	2.6
318	0.56	4-ethyl-2-methylhexane	0.2	0.2	1.0	0.2	1.2	0.8	0.9	0.3	1.2	0.2	0.3	0.2	1.0	0.4	0.2	1.1	2.6	0.4	1.4	0.5	0.1	0.1	1.3	1.0	0.7	1.4	0.3	0.5
366	0.58	alkane isomer (*m/z* 43, 57, 85)	0.0	0.0	2.6	0.3	0.7	1.1	0.4	0.1	1.2	0.3	0.2	0.2	0.9	1.8	0.0	0.0	0.0	2.1	3.0	1.6	0.3	0.2	0.9	1.5	0.5	0.8	1.0	1.2
468	0.56	nonane	3.1	3.0	4.9	3.5	3.4	3.3	3.3	2.1	3.6	2.1	1.3	1.3	4.2	0.7	2.5	3.3	2.6	2.8	2.8	3.0	2.1	2.3	3.4	3.0	3.8	3.4	4.5	5.5
504	0.55	alkane isomer (*m/z* 43, 57, 85)	0.3	0.2	2.0	0.2	0.8	0.5	0.9	0.3	0.8	0.6	1.2	1.0	0.5	1.7	0.1	1.8	1.1	0.5	1.1	0.6	0.2	0.2	1.0	0.7	0.7	0.9	0.5	0.7
516	0.55	2,4-dimethyloctane	0.2	0.1	0.0	0.1	0.1	0.2	0.2	0.0	0.2	0.0	0.1	0.1	0.2	0.0	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.2	0.2	0.3	0.5
528	0.56	3-ethyl-3-methylheptane	0.2	0.1	2.2	0.2	0.3	0.3	0.2	0.1	0.3	0.1	0.2	0.1	0.2	0.1	0.2	0.2	0.5	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.3	0.7	0.3	0.5
540	0.55	2,6-dimethyloctane	0.0	0.9	7.1	1.0	0.7	0.7	1.0	0.4	1.6	0.5	0.3	0.2	1.4	0.7	0.5	0.7	0.8	0.6	0.7	0.6	0.4	0.6	0.7	0.6	0.8	0.9	1.4	1.7
552	0.55	3-ethyl-2-methylheptane	0.4	0.4	0.8	0.6	0.2	0.4	0.4	0.2	0.5	0.3	0.1	0.0	0.2	0.2	0.7	0.4	0.4	0.2	0.3	0.3	0.3	0.2	0.4	0.3	0.5	0.7	1.0	1.3
558	0.55	alkane isomer (*m/z* 57, 43, 71)	0.1	0.2	0.6	0.1	0.3	0.4	2.1	0.2	0.2	0.2	0.6	0.4	0.2	0.1	0.0	0.4	0.4	0.2	0.2	0.1	0.1	0.1	0.3	0.2	0.2	0.4	0.2	0.2
564	0.55	alkane isomer (*m/z* 43, 57, 71)	0.4	0.3	3.0	0.3	0.3	0.3	0.2	0.1	0.4	0.1	0.1	0.1	0.3	0.1	0.0	0.2	0.3	0.1	0.2	0.2	0.3	0.1	0.3	0.2	0.4	0.3	0.5	0.9
576	0.56	4-ethyloctane	0.1	0.1	1.5	0.2	0.2	0.2	0.2	0.1	0.8	0.2	0.2	0.2	0.2	0.3	0.0	0.4	0.3	0.2	0.2	0.2	0.1	0.1	0.3	0.2	0.3	0.2	0.2	0.3
582	0.55	4-methylnonane	0.2	0.2	1.1	0.4	0.4	0.4	0.5	0.2	0.6	0.4	0.2	0.2	0.3	0.2	0.1	0.3	0.6	0.2	0.2	0.5	0.1	0.2	0.4	0.4	0.5	1.2	0.2	0.4
588	0.54	alkane isomer (*m/z* 57, 43, 85)	0.6	0.2	3.5	0.4	0.2	0.3	0.4	0.3	0.5	0.1	0.1	0.1	0.2	0.0	0.4	0.1	0.1	0.3	0.1	0.2	0.3	0.2	0.4	0.2	0.5	0.4	0.6	1.1
594	0.55	alkane isomer (*m/z* 57, 43, 71)	1.1	0.7	3.7	1.3	0.8	0.8	0.6	1.7	1.1	0.5	0.4	0.4	0.8	0.6	5.8	0.9	1.1	0.5	1.2	0.6	0.8	0.5	0.7	0.5	1.0	1.0	1.4	2.5
600	0.55	2-methylnonane	0.5	0.4	3.4	1.0	0.8	0.7	0.6	0.4	0.8	0.4	0.1	0.1	0.6	0.5	0.8	0.6	0.7	0.5	0.6	0.6	0.5	0.4	0.7	0.6	0.8	0.9	0.9	1.3
606	0.55	alkane isomer (*m/z* 57, 43, 41)	0.7	0.2	4.3	0.6	0.3	0.4	0.2	0.2	0.5	0.1	0.5	0.4	0.4	0.5	2.4	0.2	0.3	0.5	0.2	0.6	0.4	0.2	0.3	0.6	0.5	0.4	0.7	1.5
612	0.55	3-methylnonane	0.5	0.4	2.9	0.8	0.7	0.5	0.7	0.4	0.7	0.4	0.1	0.1	0.4	0.6	0.5	0.7	0.6	0.7	0.6	0.0	0.4	0.4	0.7	1.0	0.7	1.1	0.7	1.0
636	0.54	2,2,4,6,6-pentamethylheptane	12.9	6.0	1.7	2.2	5.4	7.6	1.4	1.3	1.5	2.4	2.2	3.5	2.1	1.0	4.5	1.7	2.9	1.9	2.8	2.0	3.2	0.0	1.5	2.3	1.5	1.8	5.3	3.3
660	0.56	decane	6.3	4.0	6.2	11.0	3.9	3.8	3.8	3.4	5.9	4.3	3.0	3.1	4.9	1.1	4.6	4.2	3.4	3.4	4.2	5.2	3.7	4.1	4.3	3.5	3.9	3.5	4.8	5.2
672	0.54	alkane isomer (*m/z* 57, 41, 71)	1.1	1.2	1.1	0.0	0.4	0.6	0.1	0.1	0.2	0.2	1.2	2.7	0.3	0.3	4.6	0.1	0.1	0.2	0.0	0.1	0.2	0.1	0.5	0.1	0.4	2.4	0.2	0.8
720	0.54	3,9-dimethylnonane	0.8	0.2	2.3	0.4	1.5	1.3	0.1	0.4	0.8	0.4	6.8	5.1	1.2	2.7	0.9	0.5	5.5	0.2	4.6	0.8	0.6	0.8	0.6	0.2	1.0	0.1	0.5	0.6
756	0.54	2-methyldecane	6.5	4.0	2.7	3.2	2.5	5.4	1.4	3.3	3.1	3.8	0.7	2.6	2.3	1.5	10.1	3.2	1.4	1.7	1.5	1.5	10.0	3.1	1.9	2.3	2.7	3.6	12.3	10.7
768	0.54	alkane isomer (*m/z* 57, 43, 85)	0.7	0.3	1.6	0.3	0.7	0.8	0.5	0.3	1.0	0.4	0.4	0.3	1.1	0.5	6.4	0.4	0.5	0.4	0.7	0.5	0.6	0.4	0.5	0.5	0.5	0.9	0.6	0.6
774	0.55	3-methyldecane	1.2	0.0	3.3	1.2	0.5	0.5	0.4	0.3	0.5	0.3	0.3	0.3	0.5	0.6	1.2	0.2	0.6	1.0	0.7	0.8	1.0	0.3	0.4	0.9	0.5	0.5	1.1	1.2

Appendix

^1t_R [a] (s)	^2t_R [a] (s)	Compounds	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	S25	S26	S27	S28
864	0.56	2,3-dimethyldecane	0.5	0.5	0.8	0.0	0.5	0.6	0.1	0.0	0.2	0.1	0.9	0.3	0.2	0.9	0.7	0.9	0.1	1.1	0.7	0.1	0.4	0.5	0.3	0.4	1.1	0.2	0.9	0.5
876	0.56	alkane isomer (*m/z* 57, 43, 71)	0.3	0.3	0.3	0.3	1.9	1.8	1.1	1.3	1.4	1.2	1.7	1.3	1.7	1.1	0.2	1.5	1.3	1.7	1.2	1.2	0.2	2.0	2.3	1.6	2.0	1.2	0.3	0.1
894	0.55	5-methylundecane	0.0	0.6	1.3	0.8	0.7	0.9	0.3	0.1	0.3	0.1	0.3	0.4	0.6	0.5	1.1	0.2	0.3	1.0	0.3	0.0	0.7	0.8	0.9	0.7	0.6	0.9	0.7	0.8
906	0.57	alkane isomer (*m/z* 57, 43, 71)	0.7	0.8	1.3	1.0	0.5	1.0	0.4	1.0	2.6	0.4	0.6	0.3	0.4	0.7	0.5	0.4	0.5	0.5	0.5	0.5	0.7	0.7	0.6	0.6	0.5	1.2	0.7	0.6
912	0.57	3,9-dimethylundecane	0.6	0.6	1.1	0.9	3.8	0.7	0.7	1.9	0.8	1.0	2.2	1.4	0.8	1.0	0.4	0.1	1.1	0.9	0.4	0.9	0.5	0.5	1.0	0.9	1.0	0.5	0.5	0.5
948	0.57	dodecane	10.3	7.9	3.8	6.6	2.9	2.9	2.7	2.8	2.7	2.8	2.9	2.9	2.8	3.8	6.9	2.2	1.9	3.7	2.8	4.0	8.3	3.4	3.7	3.0	2.7	2.1	8.5	6.4
960	0.58	alkane isomer (*m/z* 57, 71, 43)	0.3	0.3	0.5	0.4	0.1	0.1	0.3	0.1	0.1	0.1	1.9	1.5	0.2	2.6	0.1	0.3	0.3	3.2	0.2	2.3	0.3	0.5	0.2	2.6	0.1	0.1	0.2	0.2
966	0.57	alkane isomer (*m/z* 57, 43, 71)	0.4	0.6	0.5	0.8	2.1	2.5	2.0	2.6	1.9	2.6	0.3	0.0	2.5	1.9	0.4	2.4	1.4	3.2	1.5	2.3	0.6	2.8	2.4	2.6	1.2	2.2	0.4	0.4
972	0.56	2,5,6-trimethyldecane	0.6	0.6	0.5	1.0	0.2	0.2	4.3	1.1	0.7	0.5	1.6	2.1	0.2	0.1	0.4	1.1	4.1	0.3	0.2	0.3	0.9	0.2	0.3	0.3	0.2	0.2	0.6	0.6
978	0.57	alkane isomer (*m/z* 57, 43, 71)	0.2	0.3	0.4	0.4	0.9	0.8	0.7	1.2	0.7	1.0	2.2	0.3	0.8	1.2	0.2	0.9	4.1	1.5	6.2	1.3	0.4	0.4	1.0	2.2	0.3	0.4	0.2	0.2
996	0.56	alkane isomer (*m/z* 57, 43, 71)	0.1	0.2	0.1	0.1	0.5	0.6	0.4	0.2	0.6	0.2	0.1	0.2	0.6	0.2	0.1	1.0	0.5	0.4	0.5	0.2	0.2	0.7	0.8	0.2	0.7	0.5	0.1	0.2
1002	0.57	alkane isomer (*m/z* 57, 43, 71)	0.2	0.3	0.1	0.2	0.8	1.3	0.8	0.9	0.6	0.7	1.4	1.2	0.6	0.9	0.1	0.4	0.7	0.5	0.5	0.6	0.2	1.4	1.6	0.5	0.7	0.5	0.1	0.1
1008	0.57	6-methyldodecane	0.3	0.4	0.1	0.3	2.0	2.0	1.5	1.8	2.2	1.8	1.9	1.5	1.7	1.9	0.2	1.7	1.4	2.2	1.4	0.7	0.4	2.0	2.3	1.8	2.0	2.1	0.2	0.2
1020	0.57	alkane isomer (*m/z* 43, 57, 71)	0.2	0.7	0.1	0.6	0.4	0.4	0.2	0.2	0.4	0.3	0.2	0.1	0.4	0.3	0.2	0.1	0.3	0.1	0.4	0.2	0.3	0.2	0.4	0.2	0.2	0.3	0.2	0.5
1044	0.55	alkane isomer (*m/z* 57, 43, 71)	0.7	1.3	0.3	0.9	1.8	1.7	1.4	2.1	0.7	2.1	0.8	0.3	0.7	0.6	0.7	1.8	0.6	0.7	0.6	0.7	1.7	0.7	2.1	0.6	1.7	0.5	1.0	1.0
1050	0.55	4-ethylundecane	3.3	3.9	1.4	2.7	3.0	2.3	2.3	3.7	2.0	3.3	1.3	1.7	2.5	1.9	3.6	2.0	1.5	2.1	1.9	2.1	6.0	2.3	0.5	3.8	2.6	1.4	3.8	3.8
1056	0.57	alkane isomer (*m/z* 57, 43, 71)	0.2	0.6	0.1	0.3	0.4	0.2	0.2	0.0	0.3	0.9	0.1	0.1	0.5	0.3	0.1	0.3	0.7	1.5	0.7	1.7	0.3	0.0	0.5	1.2	0.6	0.2	0.2	0.2
1062	0.55	alkane isomer (*m/z* 57, 71, 43)	0.9	1.2	0.3	1.1	0.3	0.3	1.1	1.1	0.3	0.4	1.3	0.6	0.4	1.0	1.4	0.3	0.4	0.3	0.5	0.5	1.2	0.6	0.8	0.5	0.3	0.4	0.9	1.0
1068	0.56	alkane isomer (*m/z* 43, 57, 71)	0.8	2.3	0.3	1.0	0.4	0.5	1.1	0.4	0.7	0.4	0.8	0.7	0.8	0.6	0.7	0.3	0.4	0.3	0.5	0.2	1.2	0.7	0.5	0.4	0.3	1.7	0.9	0.8
1074	0.57	tridecane	1.6	2.5	0.7	1.9	1.9	2.0	3.2	1.7	2.9	0.8	2.9	2.3	3.5	1.6	1.9	0.9	2.3	1.9	3.4	2.4	3.3	3.3	2.2	1.3	1.7	1.2	1.8	1.9
1092	0.57	2,2-dimethyldodecane	0.7	3.1	0.2	0.6	0.4	0.6	0.5	0.5	0.5	1.2	0.7	0.6	0.6	0.5	0.3	0.7	0.4	0.4	0.6	0.7	0.6	1.1	0.7	1.1	0.6	0.4	0.4	0.4
1104	0.57	alkane isomer (*m/z* 57, 43, 71)	3.9	4.8	1.5	3.4	3.3	3.3	3.4	4.5	4.3	6.5	1.5	2.8	4.8	2.8	6.6	3.5	3.2	3.8	2.5	3.9	6.5	4.1	2.6	3.9	3.0	2.9	4.3	4.0
1110	0.57	alkane isomer (*m/z* 57, 43, 71)	0.4	0.5	0.2	0.5	0.6	0.6	0.4	0.5	0.5	0.5	1.1	1.0	0.4	0.2	0.3	0.2	0.3	0.3	0.5	0.6	0.4	0.2	0.6	1.3	0.5	0.3	0.3	0.3
1116	0.57	alkane isomer (*m/z* 57, 71, 43)	1.8	2.5	0.8	1.7	1.6	1.3	2.1	2.4	2.0	1.4	1.1	1.1	2.4	2.1	1.5	1.1	1.9	2.3	2.0	2.5	3.2	2.6	1.5	2.3	1.6	0.8	3.2	3.7
1128	0.57	3-ethyl-3-methylundecane	1.0	1.5	0.4	1.2	2.1	1.9	3.2	3.0	2.3	4.2	2.0	1.9	3.7	2.0	1.6	2.6	2.3	2.5	6.4	0.5	1.6	3.7	2.1	0.8	1.6	2.7	1.1	1.2
1152	0.60	2-methyltridecane	0.4	0.0	0.2	0.6	1.3	1.3	1.0	1.5	1.4	1.8	1.7	1.6	1.0	1.1	0.3	1.8	0.7	2.3	1.1	2.5	0.6	1.2	1.6	1.5	1.0	1.5	0.3	0.4
1158	0.59	alkane isomer (*m/z* 57, 43, 71)	0.4	3.5	0.4	0.6	0.8	0.7	1.2	0.8	1.1	0.9	1.7	1.1	1.3	0.7	0.6	0.7	0.5	1.1	1.6	1.3	0.6	0.8	0.8	0.8	0.9	0.5	0.4	0.4
1170	0.61	alkane isomer (*m/z* 57, 43, 71)	0.2	0.4	0.1	1.2	2.0	1.0	0.4	1.6	0.4	1.2	2.7	1.1	0.5	1.1	0.1	0.7	0.2	1.5	0.5	1.7	0.3	0.4	1.4	1.1	1.4	1.4	0.2	0.2
1188	0.61	tetradecane	2.7	4.2	0.8	3.2	2.3	2.6	3.7	3.7	2.9	3.9	4.1	3.9	3.5	3.9	2.1	2.5	2.1	3.3	2.9	4.4	3.3	1.7	2.7	3.7	1.9	2.3	2.3	1.9
1284	0.58	alkane isomer (*m/z* 57, 43, 85)	0.2	0.4	0.1	0.3	0.2	0.3	0.4	0.3	0.3	0.4	0.3	0.5	0.3	0.5	0.3	0.4	0.2	0.5	0.3	0.6	0.5	0.5	0.5	0.4	0.4	0.2	0.4	0.3
1290	0.58	6,6-diethyldodecane	1.0	1.5	0.5	1.6	1.9	1.9	2.1	2.1	1.1	1.7	0.7	0.6	1.1	2.5	1.5	1.4	1.0	2.5	1.6	1.7	2.6	1.9	1.4	2.5	1.1	0.8	1.8	1.7

^1t_R [a] (s)	^2t_R [a] (s)	Compounds	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	S25	S26	S27	S28
		Cyclic																												
240	0.57	1,2,4-trimethylcyclopentane	0.3	0.4	0.2	0.3	0.1	0.3	0.2	0.0	0.3	0.1	0.1	0.1	0.2	0.1	0.0	0.2	0.6	0.1	0.5	0.2	0.3	0.1	0.3	0.1	0.7	0.5	0.2	0.2
534	0.60	propylcyclohexane	0.2	0.3	0.7	0.3	0.9	0.7	1.6	0.5	2.4	1.0	0.4	0.3	1.3	0.9	0.3	1.1	1.4	0.9	0.8	1.0	0.1	0.8	1.0	1.4	1.7	2.8	0.2	0.3
576	0.59	1,1,2,3-tetramethylcyclohexane	0.1	0.3	0.2	0.8	0.5	0.4	0.9	0.5	0.8	0.5	0.6	0.3	0.4	0.5	0.1	1.3	0.9	0.5	0.6	0.6	0.1	0.5	0.0	0.7	0.7	0.7	0.2	0.3
618	0.60	2-ethyl-1,3-dimethylcyclohexane	0.1	0.1	0.1	0.2	0.4	0.3	0.7	0.1	0.5	0.2	0.2	0.2	0.2	0.2	0.3	0.4	0.4	0.3	0.2	0.3	0.0	0.1	0.4	0.4	0.5	0.4	0.1	0.1
624	0.59	1-methyl-3-propylcyclohexane	0.5	0.3	3.0	0.9	0.5	0.5	0.3	0.2	0.7	0.2	0.4	0.4	0.4	0.2	0.5	0.3	0.5	0.2	0.3	0.2	0.2	0.3	0.4	0.2	0.7	0.6	0.5	1.1
630	0.58	1-methyl-3-(2-methylpropyl)cyclopentane	0.5	0.8	3.8	1.2	0.6	0.5	0.5	0.3	1.2	0.6	0.2	0.2	0.5	0.4	0.8	0.5	0.6	0.5	0.5	0.8	0.4	0.6	0.6	0.4	0.7	1.2	0.7	1.2
642	0.59	ethylpropylcyclopentane	0.2	0.1	1.4	0.3	0.2	0.3	0.2	0.1	0.2	0.1	0.1	0.0	0.3	0.2	0.1	0.3	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.2	0.2	0.2	0.3
654	0.59	1-methyl-2-propylcyclohexane	0.4	0.3	0.5	0.5	0.4	0.4	0.3	0.2	0.5	0.2	0.1	0.1	0.3	0.2	0.1	0.3	0.4	0.1	0.3	0.2	0.3	1.1	0.3	0.1	0.5	0.4	0.4	0.5
732	0.57	hexylcyclopentane	0.3	0.3	0.8	0.4	0.6	1.0	0.6	0.4	0.9	0.4	0.8	0.7	0.9	0.4	0.4	0.4	1.5	0.6	0.9	0.6	0.3	0.5	0.7	0.7	1.1	2.6	0.3	0.4
792	0.57	1,4-dimethylcyclooctane	0.5	0.7	1.8	0.5	0.9	0.8	0.8	0.6	0.9	0.6	0.8	0.9	1.0	0.7	0.7	0.7	3.7	0.7	1.4	0.5	0.7	0.7	0.9	0.7	0.9	0.9	0.8	1.0
804	0.59	1-ethyl-2-propycylohexane	0.3	1.1	0.4	0.3	1.0	1.4	0.9	0.8	1.6	0.8	0.3	0.3	2.6	1.4	0.2	1.1	1.1	1.0	1.4	0.9	0.3	2.2	1.5	1.9	1.1	1.0	0.4	0.3
996	0.62	hexylcyclohexane	0.1	0.1	0.1	0.2	0.6	0.5	0.2	0.9	0.4	0.9	0.3	0.8	0.4	0.3	0.1	0.2	0.6	0.9	0.4	1.2	0.2	0.5	1.0	0.9	0.6	0.3	0.1	0.2
1068	0.65	1-hexyl-3-methylcyclohexane	0.2	0.5	0.1	0.4	1.8	1.5	0.9	1.9	0.2	2.2	0.8	0.9	1.3	1.7	0.2	1.7	1.1	2.2	1.3	2.5	0.5	0.3	2.4	1.3	1.5	1.0	0.3	0.3
1080	0.60	1-butyl-2-propylcyclopentane	0.3	0.5	0.1	0.4	2.1	1.8	2.3	0.6	1.9	1.0	2.9	1.5	2.1	0.6	0.2	1.8	1.1	0.4	2.6	3.2	0.6	1.8	2.5	1.0	2.2	1.1	0.3	0.4
		Alkenes																												
252	0.60	3-octene	0.1	0.1	0.0	0.2	0.3	0.3	0.1	0.0	0.4	0.0	1.0	1.4	0.5	0.1	0.0	0.1	0.3	0.2	0.2	0.1	0.0	0.0	0.2	0.1	0.4	0.1	0.1	0.2
330	0.61	2,4-dimethyl-1-heptene	0.3	0.2	0.2	0.3	0.1	0.1	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.4	0.1	0.4	0.1	0.2	0.1	0.2	0.1	0.1	0.1	0.4	0.3
450	0.61	1-nonene	0.2	0.2	0.2	0.1	0.4	0.4	0.2	0.1	0.2	0.1	0.1	0.1	0.2	0.1	0.2	0.2	0.2	0.1	0.2	0.1	0.2	0.1	0.6	0.2	0.9	0.2	0.2	0.3
546	0.57	alkene isomer (*m/z* 55, 41, 69)	0.2	0.2	0.8	0.2	0.4	0.4	0.4	0.2	0.5	0.2	0.3	0.2	0.7	0.2	0.3	0.4	0.4	0.2	0.2	0.3	0.1	0.1	0.4	0.4	0.7	0.6	0.3	0.8
552	0.57	3-methyl-1-nonene	0.4	0.3	2.0	0.5	0.5	0.7	0.4	0.5	0.7	0.5	1.2	0.9	0.6	0.3	0.3	0.6	1.1	0.3	0.5	0.4	0.2	0.3	0.5	0.6	1.0	1.0	0.2	0.8
570	0.59	3,4-diethyl-2-hexene	0.1	0.1	0.4	0.1	0.5	0.4	0.7	0.3	0.6	0.4	0.6	0.5	0.6	0.6	0.5	0.8	0.5	0.6	0.4	0.6	0.1	0.2	0.7	0.6	0.5	0.7	0.1	0.1
582	0.59	alkene isomer (*m/z* 69,41,56)	0.2	0.1	0.8	0.4	0.2	0.2	0.3	0.1	0.2	0.1	0.0	0.0	0.4	0.1	0.0	0.2	0.1	0.1	0.1	0.1	0.1	0.3	0.2	0.1	0.2	0.4	0.1	0.3
606	0.59	alkene isomer (*m/z* 69,41,56)	0.5	0.6	1.7	0.6	0.5	0.5	0.4	0.3	0.3	0.3	0.3	0.2	0.4	0.1	0.4	0.4	0.5	0.1	0.4	0.2	0.2	0.2	0.5	0.3	0.7	0.6	0.5	0.3
612	0.59	7-methyl-1-nonene	0.1	0.2	0.4	0.3	0.4	0.3	0.3	0.2	0.4	0.3	0.2	0.1	0.3	0.2	0.1	0.3	0.4	0.3	0.2	0.6	0.1	0.1	0.3	0.3	0.4	0.5	0.1	0.1
648	0.59	4-decene	0.3	0.4	0.9	0.4	0.4	0.4	0.3	0.2	1.5	0.3	0.3	0.3	0.3	0.2	0.6	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.4	0.3	0.5	0.3	0.3	0.4
762	0.55	alkene isomer (*m/z* 55, 69, 41)	0.1	0.0	0.2	0.2	1.5	1.4	0.8	2.0	1.5	2.2	0.6	0.9	1.8	1.0	3.7	2.4	0.8	0.9	1.0	0.9	0.2	1.2	1.1	2.5	1.5	2.9	0.2	0.2
768	0.56	alkene isomer (*m/z* 55, 69, 41)	0.5	0.3	0.4	0.3	0.6	0.3	0.4	1.3	0.5	0.4	0.3	0.4	1.0	0.3	0.5	0.4	0.5	0.9	0.3	0.5	0.4	0.4	0.5	0.8	0.1	0.4	0.4	0.3
780	0.56	*(Z)*-2-decene	0.4	0.3	0.4	0.2	0.8	0.8	0.8	0.5	2.8	1.0	0.7	1.0	0.8	0.5	0.3	0.6	0.7	0.6	0.5	0.6	0.4	0.5	0.8	0.7	2.3	0.9	0.4	0.4
798	0.56	alkene isomer (*m/z* 55, 69, 41)	0.7	0.8	1.6	0.9	1.6	1.6	1.5	1.2	1.6	1.2	2.2	4.0	2.6	1.0	0.7	1.3	3.7	1.3	1.4	1.4	0.6	3.1	1.7	0.4	1.6	1.7	0.7	0.7

Appendix

^1t_R [a] (s)	^2t_R [a] (s)	Compounds	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	S25	S26	S27	S28
924	0.60	(*Z*)-5-methyl-5-undecene	0.2	0.3	0.2	0.4	0.2	0.2	0.8	0.8	0.8	0.6	0.7	0.5	0.9	0.7	0.2	0.7	0.8	0.8	0.7	0.8	0.4	0.8	0.2	0.7	1.0	0.9	0.2	0.2
942	0.60	1-dodecene	0.3	0.5	0.4	0.9	2.0	1.7	1.1	1.4	1.3	0.9	1.3	1.3	2.3	2.2	0.8	0.7	1.3	1.6	0.9	2.6	1.2	3.4	2.4	1.6	1.7	0.8	1.1	1.1
960	0.62	alkene isomer (*m/z* 55, 69, 41)	0.3	0.5	0.2	0.4	2.7	2.6	1.8	2.0	3.4	1.6	1.2	0.7	2.1	6.6	0.3	1.1	1.0	1.0	1.3	1.8	0.4	1.7	2.3	1.6	2.0	2.4	0.3	0.4
978	0.59	alkene isomer (*m/z* 55, 69, 43)	0.2	0.4	0.1	0.2	0.5	0.4	0.7	0.8	1.2	0.9	5.3	1.5	0.4	1.2	0.1	1.0	1.1	1.5	3.2	1.5	0.3	1.1	0.5	2.3	0.5	1.0	0.2	0.2
1062	0.61	1-tridecene	0.3	0.9	0.1	0.8	2.3	2.9	2.9	2.6	4.4	2.7	3.2	2.4	2.5	2.9	0.3	2.1	2.4	3.6	2.9	3.8	1.1	4.9	2.9	2.6	2.5	2.7	0.4	0.8
		Aldehydes																												
144	0.78	butanal	0.1	0.2	0.0	0.2	0.2	0.2	0.5	0.2	0.4	0.7	0.3	0.3	0.2	0.5	0.2	0.3	0.1	0.2	0.2	0.2	0.0	0.5	0.1	0.2	0.1	0.2	0.0	0.0
252	1.17	hexanal	1.2	0.3	0.3	2.2	0.7	0.6	0.8	1.6	0.4	1.2	0.1	0.0	0.4	3.6	0.9	1.3	0.6	1.4	0.3	1.1	0.9	1.7	1.2	1.3	0.8	0.3	0.6	2.5
474	1.22	heptanal	0.6	1.1	0.2	1.3	0.4	0.5	0.5	0.5	0.4	1.0	0.4	0.8	0.4	0.3	0.4	0.6	0.3	0.5	0.3	0.3	0.4	0.7	0.5	0.4	0.5	0.3	0.3	1.2
576	0.92	2-ethylhexanal	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.2
588	3.58	benzaldehyde	1.0	1.3	0.3	1.1	1.1	1.3	0.9	1.0	0.9	1.5	1.0	1.4	1.0	1.4	0.8	1.1	0.7	1.3	1.1	1.1	1.3	0.8	1.4	0.9	1.1	0.6	1.1	0.9
666	1.06	octanal	1.6	2.4	0.4	2.1	1.0	1.0	1.5	1.5	0.8	1.7	1.1	1.4	0.8	2.0	0.9	1.9	0.6	1.5	0.8	0.8	0.7	1.6	1.1	1.0	1.0	0.7	0.6	1.5
822	1.00	nonanal	12.5	7.8	1.7	8.7	2.3	2.6	3.7	6.5	2.7	5.5	4.9	6.3	2.0	8.7	3.4	5.9	1.8	4.8	1.6	3.3	2.8	4.8	2.7	3.9	2.4	1.7	2.2	5.3
900	1.28	*(E)*-2-nonenal	0.2	0.5	0.1	0.5	0.1	0.1	0.2	0.3	0.1	0.2	0.2	0.3	0.1	0.7	0.2	0.2	0.1	0.3	0.1	0.2	0.0	0.4	0.2	0.2	0.2	0.1	0.1	0.2
960	0.97	decanal	5.9	6.6	1.4	7.4	1.8	1.7	4.7	6.4	1.4	5.1	3.6	4.9	1.6	5.8	2.4	6.3	1.2	5.2	1.4	3.7	3.2	5.1	2.3	4.2	1.9	1.5	2.0	3.1
1038	1.03	aldehyde isomer (*m/z* 41, 55, 71)	0.2	0.3	0.1	0.2	0.5	0.4	0.5	1.1	0.4	1.1	0.6	1.0	0.4	0.8	0.2	0.8	0.3	0.9	0.4	0.9	0.0	0.9	0.3	0.9	0.4	0.3	0.0	0.0
1086	1.02	undecanal	0.2	0.2	0.0	0.3	0.1	0.1	0.3	0.5	0.1	0.3	0.3	0.4	0.1	0.3	0.1	0.5	0.0	0.3	0.0	0.3	0.3	2.2	0.1	0.3	0.1	0.3	0.1	0.1
1200	1.09	dodecanal	0.2	0.3	0.1	0.5	0.1	0.1	0.4	0.5	0.1	0.5	0.6	0.5	0.1	0.3	0.2	0.5	0.1	0.6	0.1	0.5	0.3	0.3	0.1	0.4	0.1	0.1	0.2	0.2
		Ketones																												
138	0.79	2-butanone	2.7	1.8	0.1	0.4	0.4	0.6	0.1	0.1	0.2	0.4	0.5	0.5	0.3	0.2	0.2	0.2	0.1	0.3	0.2	0.2	0.2	0.3	0.1	0.3	0.4	0.2	0.2	0.4
240	1.16	2-hexanone	0.1	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	1.0	0.0	0.1	0.0	0.0	0.1
438	1.19	3-heptanone	0.2	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.1	1.4	0.1	0.2	0.1	0.8	0.1	0.1	0.1	0.2	0.1	0.6	0.1	0.2	0.0	0.2	0.1	0.1	0.1	0.3
450	1.29	ketone isomer (*m/z* 43, 58, 71)	0.4	0.6	0.1	0.3	0.0	0.0	0.1	0.2	0.1	0.2	0.2	0.3	0.1	0.1	0.3	0.1	0.2	0.2	0.1	0.1	0.2	0.1	0.1	0.2	0.0	0.1	0.3	0.2
642	1.10	3-octanone	0.0	0.1	0.0	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.7	0.1	0.1	0.1	0.1	0.0	0.0	0.0
642	1.33	6-methyl-5-hepten-2-one	3.4	5.9	0.9	5.5	2.4	2.5	1.9	5.1	0.3	5.5	1.2	3.2	0.2	3.0	1.8	5.4	0.1	3.0	0.2	2.4	1.3	3.8	3.6	2.3	3.8	1.3	1.0	1.5
804	1.03	2-nonanone	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.1	0.0	0.1	0.1	0.1

^1t_R [a] (s)	^2t_R [a] (s)	Compounds	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	S25	S26	S27	S28
		Miscellaneous																												
630	2.13	1-octen-3-ol	0.2	0.4	0.1	0.4	0.1	0.2	0.2	0.3	0.1	0.1	0.0	0.0	0.1	0.3	0.2	0.2	0.1	0.3	0.1	0.3	0.9	0.3	0.1	0.4	0.2	0.0	0.2	0.1
720	1.78	2-ethyl-1-hexanol	0.4	0.5	1.5	0.5	5.2	5.1	0.3	7.4	0.3	5.6	4.0	6.8	0.4	1.0	0.4	5.3	5.3	0.4	4.9	0.3	0.4	0.4	4.4	0.3	4.8	3.6	0.4	0.3
774	0.99	2-nonen-1-ol	0.1	0.2	0.0	0.2	0.1	0.1	0.2	0.2	0.1	0.2	1.9	2.0	0.1	0.2	0.2	0.3	0.1	0.2	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1
780	1.86	1-octanol	0.2	0.2	0.0	0.2	0.1	0.1	0.1	0.2	0.1	0.2	0.0	0.1	0.1	0.2	0.2	0.2	0.1	1.0	0.1	0.2	0.6	0.2	0.1	0.2	0.1	0.1	0.1	0.1
912	0.97	2-decen-1-ol	0.3	0.3	0.1	0.3	0.3	0.1	0.4	0.5	0.3	0.5	0.5	0.6	0.3	0.4	0.1	0.6	0.3	0.5	0.2	0.1	0.2	0.5	0.4	0.1	0.1	0.3	0.1	0.2

Table S1 continued

^1t_R [a] (s)	^2t_R [a] (s)	Compounds	S29	S30	S31	S32	S33	S34	S35	S36	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	S47	S48	S49	S50	S51	S52	S53	S54	S55
		Hydrocarbons																											
		Alkanes																											
138	0.48	hexane	0.8	2.1	0.5	0.7	0.8	0.5	0.5	0.5	0.5	0.2	0.3	0.8	0.8	0.6	0.9	1.9	1.1	1.0	0.4	0.7	0.6	0.8	0.9	1.0	0.6	0.6	0.7
210	0.52	2,4-dimethylhexane	0.6	1.1	0.2	0.4	0.3	0.5	0.4	0.4	0.2	0.1	0.1	1.3	0.6	1.5	0.4	0.5	0.4	0.2	0.7	1.2	0.5	0.2	4.9	0.2	0.1	0.4	0.7
252	0.54	octane	1.0	1.4	0.6	1.1	1.1	1.4	1.4	0.6	0.7	0.4	0.5	0.6	0.8	2.3	1.6	1.1	1.1	1.3	0.6	1.4	1.3	1.5	2.6	1.8	0.3	0.4	0.9
276	0.55	2,2,4-trimethylhexane	0.2	0.3	0.1	0.1	0.8	0.3	0.2	0.1	0.1	0.0	0.1	3.5	2.4	3.9	3.3	0.7	1.4	1.9	3.1	1.4	1.9	1.7	4.1	3.3	0.1	1.8	1.7
288	0.55	2,4-dimethylheptane	1.2	2.7	0.4	0.9	0.8	3.0	1.5	0.5	0.9	0.7	1.1	0.1	0.9	0.6	1.7	0.7	1.0	1.1	0.2	0.5	1.0	1.1	1.6	0.8	0.3	0.5	0.1
318	0.56	4-ethyl-2-methylhexane	0.2	0.5	0.1	0.2	0.2	0.4	0.4	0.1	0.1	0.1	0.2	1.0	0.6	2.1	2.0	1.7	2.0	2.0	1.4	1.7	0.8	0.5	0.8	1.1	0.0	0.6	0.6
366	0.58	alkane isomer (*m/z* 43, 57, 85)	0.5	0.9	0.3	0.5	0.3	1.1	1.1	0.0	0.0	0.0	0.0	0.7	0.4	0.7	0.6	1.3	1.2	1.3	0.9	0.4	0.3	0.4	0.0	1.2	0.2	0.4	0.4
468	0.56	nonane	1.9	1.5	4.2	3.2	1.9	4.8	7.8	3.7	3.4	3.0	3.0	4.0	7.0	2.5	6.9	3.7	3.0	9.1	3.4	8.7	3.5	4.5	3.2	4.9	2.2	2.1	2.4
504	0.55	alkane isomer (*m/z* 43, 57, 85)	0.2	0.2	0.3	0.3	0.1	0.6	0.8	0.3	0.1	0.1	0.1	2.0	0.5	0.6	0.5	1.3	1.6	1.0	1.2	0.5	0.3	0.7	0.5	2.0	0.1	0.5	2.8
516	0.55	2,4-dimethyloctane	0.1	0.1	1.8	0.1	0.1	0.3	1.7	0.6	0.2	0.2	0.2	0.1	0.1	0.2	0.4	0.1	0.3	0.3	0.1	0.3	0.2	0.2	0.1	0.2	0.0	0.1	0.0
528	0.56	3-ethyl-3-methylheptane	0.2	0.1	0.4	0.2	0.1	0.2	0.8	0.3	0.3	0.3	0.3	0.2	0.2	0.3	0.2	0.6	0.7	0.6	0.2	0.4	0.4	0.4	0.3	0.3	0.1	0.1	0.1
540	0.55	2,6-dimethyloctane	0.6	0.3	1.0	0.9	0.4	1.5	2.0	0.3	0.5	0.5	3.1	0.9	0.8	0.7	0.7	1.3	1.3	0.6	0.9	0.6	0.5	1.6	1.0	1.1	0.6	0.8	0.7
552	0.55	3-ethyl-2-methylheptane	0.5	0.2	0.8	0.6	0.2	1.1	1.6	0.7	1.3	0.7	2.1	0.4	0.4	0.3	0.4	0.3	0.3	0.3	0.2	0.1	0.5	0.5	0.4	0.3	0.1	0.2	0.3
558	0.55	alkane isomer (*m/z* 57, 43, 71)	0.1	0.1	0.1	0.1	0.7	0.1	0.1	0.1	0.4	0.3	0.3	0.4	0.1	0.6	0.2	0.2	0.2	0.4	0.4	0.3	0.4	0.4	0.2	3.0	0.1	0.1	0.1
564	0.55	alkane isomer (*m/z* 43, 57, 71)	0.3	0.1	0.6	0.4	0.2	0.6	1.1	0.1	0.1	0.1	0.1	0.2	0.3	0.2	0.2	0.3	0.2	0.3	0.3	0.2	0.3	0.5	0.3	0.4	0.1	0.3	0.1

^1t_R [a] (s)	^2t_R [a] (s)	Compounds	S29	S30	S31	S32	S33	S34	S35	S36	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	S47	S48	S49	S50	S51	S52	S53	S54	S55
576	0.56	4-ethyloctane	0.2	0.1	0.3	0.2	0.1	0.3	0.4	0.2	0.0	0.0	0.0	0.3	0.2	0.2	0.2	0.3	0.4	0.3	0.3	0.2	0.2	0.3	0.2	0.4	0.1	0.3	0.3
582	0.55	4-methylnonane	0.2	0.1	0.3	0.3	0.2	0.3	0.3	0.2	0.1	0.0	0.1	0.6	0.3	0.3	1.2	0.7	0.8	0.6	0.5	0.4	0.4	1.5	0.6	0.9	0.2	0.3	0.2
588	0.54	alkane isomer (*m/z* 57, 43, 85)	0.4	0.2	1.2	0.2	0.1	0.8	1.8	1.1	0.5	0.4	0.4	0.4	0.1	0.7	0.1	0.5	0.3	0.5	0.5	0.7	0.1	0.4	0.4	0.4	0.1	0.1	0.1
594	0.55	alkane isomer (*m/z* 57, 43, 71)	0.9	0.4	2.5	1.1	0.6	1.7	3.5	2.2	8.9	8.2	4.8	1.0	1.4	0.7	1.3	3.3	0.3	0.7	0.9	0.6	1.1	1.2	0.8	1.1	0.4	0.4	0.5
600	0.55	2-methylnonane	0.6	0.3	1.1	0.8	0.4	1.0	1.5	0.9	1.1	0.9	0.9	0.9	2.4	0.7	0.6	2.9	1.0	1.5	0.9	0.6	0.6	1.0	0.6	0.9	0.4	1.5	0.5
606	0.55	alkane isomer (*m/z* 57, 43, 41)	0.5	0.2	2.3	0.5	0.3	0.9	2.2	2.2	3.7	4.1	3.5	0.2	0.2	0.2	0.2	1.2	0.4	0.3	0.3	0.2	0.4	0.6	0.4	0.0	0.1	0.2	0.5
612	0.55	3-methylnonane	0.5	0.2	0.2	0.7	0.3	0.8	0.8	0.6	0.7	2.4	0.6	1.1	0.9	0.6	0.7	1.2	1.3	1.0	0.9	0.7	0.5	0.9	0.8	0.8	0.4	0.5	0.7
636	0.54	2,2,4,6,6-pentamethylheptane	6.6	3.0	4.6	19.3	9.4	3.8	7.4	3.4	4.5	3.6	3.9	2.6	3.7	3.9	3.0	2.9	3.9	2.8	0.9	2.8	3.8	1.3	2.3	1.4	0.2	0.6	2.7
660	0.56	decane	4.6	3.3	6.8	7.2	3.9	5.0	8.6	6.9	5.5	3.6	5.4	3.4	5.2	3.3	3.2	7.6	6.1	6.9	4.0	3.4	4.1	4.5	3.4	4.4	3.5	2.9	3.4
672	0.54	alkane isomer (*m/z* 57, 41, 71)	0.5	0.1	1.4	0.3	1.7	0.2	0.4	6.9	5.5	3.6	5.4	0.1	0.2	0.1	0.1	0.2	0.4	0.2	0.4	1.7	1.7	1.5	0.7	0.1	0.1	0.1	0.3
720	0.54	3,9-dimethylnonane	0.5	0.1	1.3	1.2	0.3	0.5	0.7	1.2	0.6	0.5	0.5	7.0	6.5	6.3	9.7	0.5	0.7	1.1	0.5	0.1	0.1	0.1	0.1	0.1	0.7	7.9	0.1
756	0.54	2-methyldecane	9.0	7.9	2.8	3.2	5.5	11.7	4.0	5.1	8.6	8.4	10.3	1.6	1.6	1.8	2.2	3.1	2.7	2.4	2.1	2.1	3.4	4.1	4.2	2.2	4.4	5.4	1.7
768	0.54	alkane isomer (*m/z* 57, 43, 85)	0.5	0.4	3.0	0.9	0.6	0.6	0.7	5.1	5.4	5.4	6.3	1.5	0.4	0.5	0.5	1.1	1.3	1.5	0.6	0.3	1.0	0.5	0.3	0.4	0.3	0.9	0.4
774	0.55	3-methyldecane	0.9	0.5	3.0	1.8	0.8	1.1	2.5	1.9	1.5	1.4	1.3	0.7	0.8	0.7	0.8	0.5	0.5	0.4	0.4	0.4	0.7	0.5	0.4	0.3	0.3	0.5	0.6
786	0.55	alkane isomer (*m/z* 57, 43, 71)	0.9	0.2	1.4	1.0	1.0	0.9	1.0	0.5	0.5	0.4	0.6	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.1	0.2	0.1	0.2	0.2	0.1	0.1	0.1	0.2
798	0.55	alkane isomer (*m/z* 57, 43, 71)	0.9	0.4	0.7	1.1	0.6	1.0	1.1	0.6	0.9	0.6	0.5	1.0	0.2	0.2	0.2	1.1	0.2	0.1	0.8	0.2	0.2	0.2	0.3	0.4	0.3	0.1	0.2
804	0.55	alkane isomer (*m/z* 43, 71, 57)	0.8	0.4	0.5	1.0	0.4	0.9	0.9	0.5	0.4	0.5	0.4	0.1	0.1	0.2	0.5	0.4	0.2	0.2	0.1	0.1	0.2	0.2	0.2	0.1	0.2	0.1	0.1
822	0.55	undecane	13.8	20.9	5.9	7.6	3.4	19.0	6.2	1.9	2.6	7.7	4.0	3.0	2.6	3.5	4.5	5.7	5.2	5.4	3.7	6.1	4.7	3.7	4.4	5.4	7.6	6.5	4.4
864	0.56	2,3-dimethyldecane	0.4	0.3	0.7	0.7	0.4	0.4	0.4	1.0	0.4	0.4	0.0	0.2	0.6	0.2	0.0	0.4	0.2	0.1	1.6	0.2	0.2	0.2	0.2	0.3	0.6	0.4	0.4
876	0.56	alkane isomer (*m/z* 57, 43, 71)	0.3	0.4	0.3	0.4	0.4	0.2	0.2	0.8	0.4	0.2	0.4	1.6	1.3	1.3	1.3	1.7	1.6	1.3	2.2	1.4	1.5	1.4	1.3	1.6	1.3	1.2	1.5
894	0.55	5-methylundecane	0.7	0.5	0.7	1.1	0.0	0.7	0.6	1.2	1.3	1.0	1.4	1.0	0.8	0.4	0.4	0.4	0.4	0.9	0.7	0.5	0.4	0.2	0.7	0.3	0.5	0.6	0.7
906	0.57	alkane isomer (*m/z* 57, 43, 71)	0.7	0.5	0.9	0.8	0.8	0.7	0.7	0.8	0.6	0.5	0.6	0.7	5.0	0.6	1.3	0.0	0.7	1.5	0.4	0.9	1.7	0.6	1.6	0.4	0.4	0.5	1.2
912	0.57	3,9-dimethylundecane	0.5	0.3	0.7	0.6	0.5	0.6	0.5	0.6	0.5	0.5	0.5	0.5	0.6	0.7	0.5	0.7	0.8	0.7	1.0	0.8	0.6	0.8	0.4	0.9	0.8	1.0	0.9
948	0.57	dodecane	10.6	9.2	3.7	6.3	6.6	8.7	7.7	3.2	5.2	3.6	7.6	2.4	2.6	2.5	2.5	2.7	3.0	2.7	2.9	2.3	2.8	2.5	2.4	2.8	2.9	2.8	3.1
960	0.58	alkane isomer (*m/z* 57, 71, 43)	0.2	9.2	0.1	0.4	0.3	0.2	0.2	0.1	0.2	0.1	0.2	0.2	0.6	0.2	0.2	1.1	0.1	0.1	0.2	0.1	0.2	0.1	0.1	0.2	0.1	0.3	2.6
966	0.57	alkane isomer (*m/z* 57, 43, 71)	0.4	0.3	0.4	0.4	0.5	0.4	0.3	0.3	0.3	0.3	0.3	2.2	1.9	1.5	1.5	2.4	0.9	1.1	1.2	1.6	2.2	1.9	2.0	2.4	1.8	2.1	2.6
972	0.56	2,5,6-trimethyldecane	0.5	0.3	0.6	0.7	0.8	0.6	0.4	0.5	0.5	0.4	0.5	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.3	2.1	5.9	5.7	4.5	1.9	0.2	0.2	0.7
978	0.57	alkane isomer (*m/z* 57, 43, 71)	0.3	0.1	0.2	0.1	0.4	0.2	0.2	0.6	0.2	0.5	0.2	1.0	0.4	0.7	0.7	0.4	2.9	0.4	3.3	0.4	0.4	0.7	0.3	0.7	0.0	0.7	0.2
996	0.56	alkane isomer (*m/z* 57, 43, 71)	0.2	0.2	0.1	0.2	0.3	0.1	0.2	0.1	0.3	0.1	0.3	0.5	0.7	0.6	0.6	0.5	0.7	0.5	0.8	0.5	0.9	0.5	0.3	0.2	0.8	0.4	0.5
1002	0.57	alkane isomer (*m/z* 57, 43, 71)	0.2	0.1	0.1	0.2	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.6	0.5	0.6	0.5	0.6	0.9	0.9	1.5	1.0	0.7	0.9	0.5	1.0	1.1	0.5	1.2
1008	0.57	6-methyldodecane	0.2	0.2	0.2	0.2	0.4	0.2	0.0	0.1	0.2	0.1	0.2	2.6	0.7	2.6	0.5	1.5	2.4	1.5	2.2	1.4	0.6	1.8	1.4	1.5	1.6	1.5	1.9

^1t_R [a] (s)	^2t_R [a] (s)	Compounds	S29	S30	S31	S32	S33	S34	S35	S36	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	S47	S48	S49	S50	S51	S52	S53	S54	S55
1056	0.57	alkane isomer (*m/z* 57, 43, 71)	0.2	0.1	0.1	0.2	0.5	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.9	0.8	0.8	0.3	0.6	0.5	0.4	0.4	0.6	0.5	0.4	0.3	0.8	0.6	0.3
1062	0.55	alkane isomer (*m/z* 57, 71, 43)	1.0	0.8	0.4	0.6	1.1	1.0	0.8	0.4	1.0	1.2	0.7	0.3	0.2	0.3	0.4	0.5	0.3	0.5	0.4	0.5	0.5	0.4	0.3	0.4	0.6	0.6	0.3
1068	0.56	alkane isomer (*m/z* 43, 57, 71)	0.9	0.6	0.3	0.7	1.4	0.9	0.7	0.3	1.0	0.5	0.4	0.4	0.5	0.4	0.3	0.8	0.4	0.5	0.1	0.5	0.4	0.5	0.8	0.4	0.5	0.8	0.3
1074	0.57	tridecane	2.5	1.5	1.7	1.3	2.5	2.1	2.1	1.5	1.3	1.3	2.2	2.0	1.4	2.5	1.6	1.4	3.5	3.0	2.0	1.5	2.9	1.4	2.8	2.7	1.5	1.2	1.5
1092	0.57	2,2-dimethyldodecane	0.3	0.3	0.8	0.3	1.0	0.3	0.3	0.9	0.8	0.3	0.3	0.4	0.3	0.8	0.5	0.5	0.6	0.5	1.1	0.4	0.4	0.4	0.4	0.4	0.9	0.8	0.5
1104	0.57	alkane isomer (*m/z* 57, 43, 71)	4.9	5.0	1.4	3.6	5.0	4.3	3.4	1.8	3.8	6.4	6.0	1.9	2.3	1.6	1.7	7.3	4.0	4.6	2.8	3.9	2.4	2.9	3.7	4.0	5.3	4.7	4.7
1110	0.57	alkane isomer (*m/z* 57, 43, 71)	0.4	0.5	0.2	0.4	0.9	0.3	0.3	0.2	0.2	0.2	0.3	0.4	0.4	0.5	0.4	0.5	0.4	0.4	0.4	0.5	0.4	0.4	0.4	0.3	0.4	0.4	0.2
1116	0.57	alkane isomer (*m/z* 57, 71, 43)	3.6	2.2	1.1	3.6	4.9	3.3	1.8	0.9	1.8	2.0	1.4	1.8	3.4	1.9	1.0	2.1	2.0	2.3	1.4	2.0	1.8	1.6	1.2	2.2	4.9	5.6	2.4
1128	0.57	3-ethyl-3-methylundecane	1.2	1.4	0.6	0.0	0.0	1.1	0.0	0.5	0.6	0.7	0.7	2.6	1.0	4.7	4.8	2.2	1.3	2.7	1.8	3.2	2.8	1.1	0.7	2.6	1.8	2.6	2.0
1152	0.60	2-methyltridecane	0.5	0.3	0.5	0.6	0.0	0.4	0.4	0.5	0.4	0.3	0.3	0.8	0.7	1.0	0.9	1.4	2.3	1.5	1.6	0.8	0.9	0.9	0.6	0.8	1.2	1.1	1.1
1158	0.59	alkane isomer (*m/z* 57, 43, 71)	0.5	0.3	0.6	0.8	0.6	0.4	0.7	0.5	0.4	0.4	0.3	1.1	0.6	0.7	0.7	1.0	1.1	1.1	0.8	1.0	1.2	0.6	1.0	0.6	1.2	0.8	0.8
1170	0.61	alkane isomer (*m/z* 57, 43, 71)	0.2	0.2	0.3	0.2	0.5	0.2	0.2	0.2	0.2	0.2	0.1	0.8	0.5	1.8	0.6	0.6	1.2	0.5	0.8	0.3	0.5	0.2	1.4	1.0	1.0	0.3	1.0
1188	0.61	tetradecane	3.5	3.2	3.0	2.6	4.3	2.2	3.2	2.6	2.3	1.6	1.9	3.9	1.8	2.5	2.6	2.2	3.4	3.1	2.4	1.9	3.1	2.6	3.4	2.6	3.3	3.8	5.5
1284	0.58	alkane isomer (*m/z* 57, 43, 85)	0.4	0.3	0.3	0.3	0.3	0.4	0.4	0.3	0.3	0.2	0.2	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.5	0.3	0.3	0.3	0.3	0.3	0.6	0.4	0.5
1290	0.58	6,6-diethyldodecane	1.7	1.8	1.2	1.8	1.6	1.8	1.5	1.1	1.3	1.3	1.1	1.2	1.6	0.9	1.1	1.2	1.1	1.2	0.6	1.2	1.5	1.2	1.1	1.8	2.4	0.4	1.6
1296	0.64	pentadecane	0.7	0.6	1.0	0.9	1.0	0.6	0.7	0.9	0.7	0.4	0.5	0.2	0.4	0.3	1.0	0.3	0.3	0.3	0.5	1.1	1.1	0.3	0.6	0.4	2.9	1.9	0.5
1302	0.59	5-ethyl-5-methyltridecane	0.4	2.5	0.3	0.2	0.5	0.2	0.2	0.3	0.2	0.1	0.2	0.9	0.9	0.7	0.7	0.5	0.2	0.3	0.4	0.7	0.7	0.3	0.3	0.8	0.3	1.7	0.6
1338	0.61	3-ethyl-3-methyltridecane	2.6	2.3	2.0	2.5	2.0	3.0	2.4	1.9	1.5	1.5	1.7	1.2	0.3	1.5	0.5	0.3	0.4	0.4	0.5	0.9	0.9	1.1	1.0	0.5	0.8	0.8	1.8
		Cyclic																											
240	0.57	1,2,4-trimethylcyclopentane	0.5	0.5	0.4	0.3	0.4	0.2	0.2	0.2	0.1	0.0	0.0	0.2	0.2	0.5	0.6	0.4	0.5	0.4	0.7	0.3	0.3	0.5	0.5	0.3	0.1	0.2	0.1
534	0.60	propylcyclohexane	0.1	0.3	0.3	0.2	0.3	0.2	0.4	0.1	0.3	0.3	0.2	1.6	1.2	0.7	1.7	1.9	2.7	1.7	1.3	1.0	1.0	2.2	1.6	2.5	0.5	0.8	0.7
576	0.59	1,1,2,3-tetramethylcyclohexane	0.3	0.1	0.5	0.5	0.3	0.4	0.3	0.2	0.1	0.1	0.1	1.1	0.7	0.7	0.3	0.2	0.4	0.6	1.3	0.7	0.8	0.5	0.5	1.9	0.2	0.4	0.6
618	0.60	2-ethyl-1,3-dimethylcyclohexane	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.4	0.1	0.3	0.4	0.3	0.3	0.3	0.5	0.7	0.4	0.5	0.3	0.3	0.5	0.3	0.5	0.1	0.2	0.2
624	0.59	1-methyl-3-propylcyclohexane	0.4	0.2	0.3	0.7	0.3	0.6	1.2	1.0	0.7	0.8	0.6	0.5	0.4	0.4	0.4	0.5	0.8	0.6	0.6	0.4	0.5	1.6	0.5	0.5	0.3	0.2	0.2
630	0.58	1-methyl-3-(2-methylpropyl)cyclopentane	0.6	0.5	1.1	1.0	0.7	0.5	1.3	1.4	1.1	1.2	0.9	1.7	0.7	0.9	0.6	0.7	1.0	0.8	0.8	0.5	1.1	2.6	0.5	0.7	0.4	0.6	0.4
642	0.59	ethylpropylcyclopentane	0.1	0.1	0.4	0.2	0.1	0.2	0.4	0.3	0.2	0.3	0.2	0.3	0.4	0.2	0.4	0.2	0.6	0.8	0.5	0.2	0.2	0.5	0.2	0.3	0.1	0.1	0.1
654	0.59	1-methyl-2-propylcyclohexane	0.4	0.2	0.6	0.6	0.4	0.4	0.7	0.3	0.2	0.3	0.2	0.4	0.4	0.3	0.3	0.3	0.5	0.4	0.4	0.3	0.3	0.8	0.3	0.3	1.4	0.3	0.2
732	0.57	hexylcyclopentane	0.4	0.2	0.6	0.5	0.4	0.3	0.5	1.0	0.8	0.4	0.6	1.3	2.5	2.4	1.2	1.1	0.6	0.4	0.9	0.6	0.7	1.0	1.8	0.6	0.4	0.4	0.5
792	0.57	1,4-dimethylcyclooctane	0.6	0.5	2.9	1.3	0.6	0.5	0.9	3.5	2.2	2.6	0.8	1.6	2.4	2.9	3.0	0.7	0.7	0.7	1.0	1.4	0.9	0.6	0.7	0.9	0.7	0.7	0.8
804	0.59	1-ethyl-2-propycylohexane	0.2	0.4	0.9	0.8	0.7	0.3	0.2	0.9	0.7	0.2	0.3	1.1	2.4	1.3	1.2	1.4	1.0	0.7	0.9	1.4	1.2	0.9	0.5	2.3	2.2	1.6	1.4
996	0.62	hexylcyclohexane	0.2	0.1	0.2	0.2	0.3	0.2	0.1	0.2	0.1	0.1	0.1	0.6	0.6	0.7	0.7	0.4	0.5	0.3	0.7	0.6	0.6	0.3	0.2	0.9	0.4	0.3	0.8

Appendix

^1t_R [a] (s)	^2t_R [a] (s)	Compounds	S29	S30	S31	S32	S33	S34	S35	S36	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	S47	S48	S49	S50	S51	S52	S53	S54	S55
1068	0.65	1-hexyl-3-methylcyclohexane	0.4	0.2	0.3	0.2	1.3	0.3	0.2	0.3	0.2	0.2	0.2	1.3	1.2	1.3	1.3	1.0	1.8	1.2	1.8	1.2	1.1	1.2	0.9	1.1	0.2	0.3	1.8
1080	0.60	1-butyl-2-propylcyclopentane	0.4	0.2	0.3	0.2	0.5	0.3	0.2	0.2	0.2	0.2	0.3	1.7	1.2	2.5	1.5	1.3	1.6	1.5	3.1	1.7	1.3	2.1	1.5	1.4	1.6	0.5	0.5
		Alkenes																											
252	0.60	3-octene	0.2	0.4	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.0	0.2	0.5	0.2	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1
330	0.61	2,4-dimethyl-1-heptene	0.3	0.5	0.3	0.3	0.3	0.4	0.2	0.2	0.2	0.1	0.2	0.2	0.2	1.1	0.7	0.3	0.1	0.2	0.1	0.1	0.2	0.1	0.2	0.2	0.2	0.1	0.2
450	0.61	1-nonene	0.2	0.2	0.2	0.1	0.3	0.2	0.1	0.2	0.2	0.1	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.5	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1
546	0.57	alkene isomer (*m/z* 55, 41, 69)	0.1	0.1	0.4	0.3	0.1	0.4	0.8	0.4	0.2	0.1	0.5	0.4	0.3	0.3	0.3	0.5	0.7	0.8	0.5	0.0	0.3	0.7	0.4	0.6	0.1	0.2	0.2
552	0.57	3-methyl-1-nonene	0.3	0.1	0.5	0.4	0.3	0.1	0.8	0.4	1.3	0.5	0.6	1.5	0.7	0.7	0.7	0.8	0.7	0.6	0.6	0.5	0.7	1.1	0.9	0.3	0.2	0.3	0.3
570	0.59	3,4-diethyl-2-hexene	0.1	0.1	0.1	0.2	0.1	0.2	0.2	0.1	0.5	1.6	0.6	0.7	0.4	0.4	0.4	0.3	0.4	0.4	0.8	0.4	0.3	0.4	0.4	0.9	0.1	0.4	0.6
582	0.59	alkene isomer (*m/z* 69,41,56)	1.4	0.1	0.2	0.3	0.2	0.2	0.3	0.3	0.0	0.2	0.2	0.3	0.1	0.1	0.2	0.2	0.4	0.2	0.3	0.2	0.2	0.4	0.3	0.3	1.2	0.1	0.1
606	0.59	alkene isomer (*m/z* 69,41,56)	0.4	0.1	0.4	0.7	0.4	0.4	0.7	0.6	0.5	0.4	0.4	0.5	0.5	1.4	0.4	0.1	0.1	0.1	0.7	0.4	0.5	1.1	0.8	0.7	0.1	0.1	0.1
612	0.59	7-methyl-1-nonene	0.2	0.2	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.4	0.3	0.3	0.3	0.4	0.7	0.5	0.5	0.3	0.2	0.4	0.3	0.5	0.2	0.2	0.3
648	0.59	4-decene	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.4	0.3	0.3	0.6	0.4	0.7	0.3	0.4	0.5	0.7	0.8	0.5	0.3	0.3	0.5	0.2	0.5	0.2	0.3	0.2
762	0.55	alkene isomer (*m/z* 55, 69, 41)	0.0	0.0	0.2	0.3	0.1	0.2	0.2	2.9	3.2	3.0	4.0	0.9	1.1	1.1	0.9	1.4	1.8	1.8	1.3	1.1	1.6	2.4	1.6	1.3	1.5	1.5	0.9
768	0.56	alkene isomer (*m/z* 55, 69, 41)	0.5	0.2	0.3	0.5	0.0	0.6	0.6	0.2	0.2	0.1	0.6	0.5	0.4	0.4	0.4	0.4	0.4	0.4	0.6	0.2	0.3	0.5	0.3	0.1	0.3	0.5	0.4
780	0.56	*(Z)*-2-decene	0.5	0.2	0.6	0.5	0.3	0.4	0.4	0.8	1.4	0.4	0.4	2.4	1.0	0.6	0.8	0.7	0.6	0.6	1.0	0.6	1.0	0.6	0.6	2.6	0.4	0.5	0.6
798	0.56	alkene isomer (*m/z* 55, 69, 41)	1.0	0.3	2.9	1.6	0.6	0.7	0.8	1.2	0.8	0.8	0.8	1.6	2.4	2.9	3.0	1.3	1.5	1.2	2.2	1.3	1.3	1.4	1.8	1.6	4.7	0.9	3.2
810	0.55	alkene isomer (*m/z* 69, 55, 41)	0.7	0.3	0.8	0.6	0.3	0.6	0.4	0.7	0.5	0.6	0.5	0.8	0.6	0.8	0.7	0.8	0.7	0.8	1.2	1.6	3.0	2.3	1.9	0.8	0.6	0.7	0.7
828	0.57	alkene isomer (*m/z* 55, 69, 41)	0.6	0.2	0.6	1.0	0.4	0.4	0.4	2.4	0.9	0.6	0.3	3.6	2.9	2.9	0.2	1.1	2.9	1.9	2.0	3.2	1.3	1.4	1.4	1.2	1.9	1.9	3.8
870	0.59	2-methyl-1-undecene	0.2	0.2	0.5	0.7	0.3	0.4	0.3	0.2	2.3	0.6	1.5	1.4	1.2	1.4	1.7	2.3	1.3	1.2	1.9	1.1	2.2	1.2	1.7	1.3	1.3	1.3	1.3
918	0.60	(*E*)-5-methyl-4-undecene	0.5	0.3	0.5	0.2	0.2	0.1	0.1	0.2	0.2	0.1	0.2	0.9	0.7	0.8	0.8	0.8	0.7	0.6	1.1	0.6	0.7	0.8	0.6	0.8	0.7	0.6	0.4
924	0.60	(*Z*)-5-methyl-5-undecene	0.3	0.1	0.5	0.3	0.2	0.2	0.2	0.4	0.2	0.2	0.2	0.9	0.8	0.7	0.7	0.8	0.7	0.7	1.1	0.8	0.8	0.9	0.7	0.8	0.6	0.5	0.7
942	0.60	1-dodecene	1.1	1.1	0.7	0.4	0.4	1.0	0.8	0.6	1.0	1.0	1.1	1.6	1.2	0.8	1.0	2.0	2.5	1.3	2.7	1.3	1.3	3.2	0.9	1.3	2.9	1.4	1.9
960	0.62	alkene isomer (*m/z* 55, 69, 41)	0.4	0.4	0.3	0.4	0.4	0.4	0.3	0.3	0.3	0.2	0.3	1.6	0.7	1.5	1.4	2.3	3.0	2.9	2.3	4.7	1.9	1.6	2.0	1.9	1.8	0.3	2.3
978	0.59	alkene isomer (*m/z* 55, 69, 43)	0.2	0.1	0.2	0.1	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.4	1.2	0.4	0.4	0.9	0.8	2.1	0.5	0.7	1.2	1.8	0.8	0.3	0.8	0.3	1.3
1062	0.61	1-tridecene	0.4	0.4	0.3	0.3	0.7	0.7	0.3	0.5	0.6	0.5	0.8	2.8	2.5	3.1	2.8	2.4	3.9	3.3	3.2	3.1	2.6	2.1	2.4	2.0	4.3	3.5	2.6
		Aldehydes																											
144	0.78	butanal	0.2	0.3	0.1	0.1	0.3	0.0	0.0	0.1	0.3	0.1	0.3	0.2	0.2	0.8	0.5	0.9	0.2	0.2	0.1	0.4	0.2	0.2	0.4	0.2	0.3	0.3	0.2
252	1.17	hexanal	1.3	1.8	5.6	1.2	1.6	0.6	1.0	5.1	1.0	3.3	1.0	0.6	0.7	0.4	0.5	0.7	0.4	0.6	0.8	0.8	0.7	0.5	0.4	0.5	1.5	1.8	2.1

^1t_R [a] (s)	^2t_R [a] (s)	Compounds	S29	S30	S31	S32	S33	S34	S35	S36	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	S47	S48	S49	S50	S51	S52	S53	S54	S55
474	1.22	heptanal	0.6	0.8	2.1	1.1	1.0	0.4	0.5	1.8	0.5	1.3	0.5	0.3	0.4	0.3	0.4	0.3	0.3	0.4	0.4	0.5	0.4	0.3	0.4	0.4	0.7	0.5	0.4
576	0.92	2-ethylhexanal	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0
588	3.58	benzaldehyde	1.3	1.8	1.1	1.4	1.5	1.0	0.8	0.9	0.8	0.7	0.9	1.1	1.1	1.0	1.2	1.1	0.9	1.1	1.1	1.0	0.9	0.7	0.9	0.8	1.1	1.2	1.1
666	1.06	octanal	1.0	1.1	3.0	1.1	2.0	0.6	1.1	2.7	1.1	2.0	1.0	0.8	0.6	0.6	0.7	0.6	0.7	0.9	0.6	0.8	0.8	0.7	0.7	1.0	1.6	1.7	1.4
822	1.00	nonanal	3.7	2.7	8.6	3.5	7.7	1.9	3.2	7.4	3.7	5.9	3.5	2.1	2.0	1.5	2.3	2.1	1.8	2.0	2.5	2.5	2.3	1.8	2.2	2.4	4.8	4.3	5.1
900	1.28	*(E)*-2-nonenal	0.5	0.1	0.3	0.3	0.3	0.1	0.1	0.5	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.2
960	0.97	decanal	3.2	3.4	5.3	3.8	7.4	1.8	3.1	4.6	2.9	3.4	2.5	1.7	1.6	1.1	2.3	1.7	1.5	1.8	2.0	2.4	1.9	1.8	1.9	2.9	5.9	5.0	4.9
1038	1.03	aldehyde isomer (*m/z* 41, 55, 71)	0.2	0.1	0.1	0.2	0.3	0.0	0.2	0.1	0.2	0.1	0.1	0.3	0.3	0.4	0.4	0.3	0.3	0.5	0.4	0.3	0.3	0.3	0.5	0.7	1.0	1.1	0.7
1086	1.02	undecanal	0.2	0.2	0.2	0.1	0.2	0.1	0.1	0.3	0.1	0.1	0.1	0.1	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.7	0.1	0.2	0.3	0.3	0.2
1200	1.09	dodecanal	0.3	0.2	0.4	0.4	0.3	0.1	0.2	1.1	0.3	0.2	0.2	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.3	0.4	0.5	0.2
		Ketones																											
138	0.79	2-butanone	0.5	0.7	0.2	0.8	1.3	0.2	0.5	0.8	0.3	0.3	0.3	0.2	0.1	0.1	0.2	0.2	0.1	0.4	0.4	0.5	0.3	0.2	0.4	0.1	0.3	0.2	0.2
240	1.16	2-hexanone	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
438	1.19	3-heptanone	0.1	0.1	0.4	0.0	0.0	0.1	0.2	0.3	0.1	0.3	0.1	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.2	0.0	2.3
450	1.29	ketone isomer (*m/z* 43, 58, 71)	0.5	0.4	0.4	0.1	0.5	0.2	0.3	0.3	0.6	0.2	0.3	0.1	0.2	0.2	0.0	0.2	0.2	0.2	0.2	0.4	0.3	0.1	0.2	0.1	0.2	0.2	0.1
450	2.07	ciclohexanone	1.6	1.1	0.1	0.0	0.3	0.2	0.1	1.3	0.9	1.0	0.9	0.7	0.3	0.2	0.2	0.4	0.3	0.2	0.5	1.1	1.3	0.7	1.2	0.7	0.2	0.2	0.4
642	1.10	3-octanone	0.0	0.2	0.0	0.2	0.1	0.0	0.0	0.0	0.1	0.0	0.1	0.1	0.1	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1
642	1.33	6-methyl-5-hepten-2-one	2.3	2.9	2.4	2.7	7.1	1.0	2.1	2.1	1.8	2.0	2.8	0.2	0.2	0.2	0.2	0.2	0.1	0.2	2.7	1.5	1.8	1.5	2.3	2.0	5.2	5.5	3.0
804	1.03	2-nonanone	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.1
810	1.02	ketone isomer (*m/z* 43, 58, 71)	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	1.7	1.7	1.8	1.6	0.1	0.1	0.1	0.1
		Miscellaneous																											
630	2.13	1-octen-3-ol	0.2	1.1	0.1	0.2	0.6	0.2	0.2	0.1	0.3	0.1	0.2	0.3	0.1	0.1	0.2	0.1	0.1	0.2	0.1	0.1	0.2	0.2	0.2	0.2	0.3	0.4	0.2
720	1.78	2-ethyl-1-hexanol	0.6	0.1	0.9	0.3	0.5	0.4	0.3	0.4	0.6	0.4	0.4	3.9	6.3	5.4	5.2	0.3	0.3	0.4	3.5	4.9	4.5	3.1	4.1	0.3	0.4	0.4	0.5
774	0.99	2-nonen-1-ol	0.1	0.1	0.1	0.1	0.2	0.0	0.1	0.3	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.2
780	1.86	1-octanol	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.3	0.2	0.2
912	0.97	2-decen-1-ol	0.2	0.1	0.3	0.3	0.3	0.1	0.1	0.2	0.1	0.2	0.1	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.4	0.5	0.5	0.2

Table S1 continued

^1t_R [a] (s)	^2t_R [a] (s)	Compounds	S56	S57	S58	S59	S60	S61	S62
		Hydrocarbons							
		Alkanes							
138	0.48	hexane	0.7	0.7	1.2	0.4	0.3	0.4	0.6
210	0.52	2,4-dimethylhexane	0.2	0.8	0.5	0.1	0.1	0.1	1.0
252	0.54	octane	0.2	0.2	0.7	0.2	0.4	0.9	1.4
276	0.55	2,2,4-trimethylhexane	2.4	2.7	1.1	0.7	0.2	1.2	2.7
288	0.55	2,4-dimethylheptane	0.0	0.1	0.1	0.0	0.1	0.0	0.3
318	0.56	4-ethyl-2-methylhexane	0.7	0.9	0.5	0.3	0.2	0.4	0.8
366	0.58	alkane isomer (*m/z* 43, 57, 85)	1.8	1.6	2.3	1.7	1.3	1.5	0.8
468	0.56	nonane	2.9	3.5	2.1	4.4	2.7	2.9	2.7
504	0.55	alkane isomer (*m/z* 43, 57, 85)	3.6	4.2	0.5	0.8	0.6	1.1	0.9
516	0.55	2,4-dimethyloctane	0.1	0.1	0.0	0.0	0.0	0.0	0.0
528	0.56	3-ethyl-3-methylheptane	0.7	0.1	0.6	0.0	0.0	0.0	0.1
540	0.55	2,6-dimethyloctane	0.6	0.7	0.5	0.4	0.5	0.5	0.5
552	0.55	3-ethyl-2-methylheptane	0.2	0.4	0.2	0.2	0.2	0.2	0.3
558	0.55	alkane isomer (*m/z* 57, 43, 71)	0.3	0.3	0.1	0.3	0.0	0.3	0.3
564	0.55	alkane isomer (*m/z* 43, 57, 71)	0.1	0.1	0.1	0.0	0.1	0.0	0.1
576	0.56	4-ethyloctane	0.3	0.4	0.1	0.2	0.2	0.2	0.2
582	0.55	4-methylnonane	0.2	0.0	0.2	0.2	0.3	0.4	0.3
588	0.54	alkane isomer (*m/z* 57, 43, 85)	0.2	0.1	0.2	0.0	0.0	0.0	0.1
594	0.55	alkane isomer (*m/z* 57, 43, 71)	0.5	0.7	0.4	1.4	1.3	1.4	0.5
600	0.55	2-methylnonane	0.5	0.6	0.4	0.4	0.4	0.5	0.5
606	0.55	alkane isomer (*m/z* 57, 43, 41)	0.5	0.6	0.4	0.1	0.1	0.1	0.1
612	0.55	3-methylnonane	1.7	0.7	2.0	0.4	0.4	0.5	0.5
636	0.54	2,2,4,6,6-pentamethylheptane	5.6	1.1	0.6	1.0	0.6	2.1	1.0
660	0.56	decane	2.7	5.1	4.6	4.5	5.1	4.1	4.7
672	0.54	alkane isomer (*m/z* 57, 41, 71)	0.2	0.2	0.3	0.1	0.2	0.1	0.2
720	0.54	3,9-dimethylnonane	0.0	0.1	0.1	0.3	0.2	0.2	0.4
756	0.54	2-methyldecane	2.1	2.3	1.3	1.8	2.7	1.6	2.2

^1t_R [a] (s)	^2t_R [a] (s)	Compounds	S56	S57	S58	S59	S60	S61	S62
768	0.54	alkane isomer (*m/z* 57, 43, 85)	0.4	0.8	0.4	0.4	0.5	0.5	0.4
774	0.55	3-methyldecane	0.8	0.8	0.5	0.4	0.3	0.4	0.4
786	0.55	alkane isomer (*m/z* 57, 43, 71)	0.2	0.2	0.1	0.1	0.1	0.1	0.1
798	0.55	alkane isomer (*m/z* 57, 43, 71)	1.1	0.1	0.3	0.7	0.4	0.4	0.1
804	0.55	alkane isomer (*m/z* 43, 71, 57)	0.2	0.1	0.1	0.2	0.1	0.1	0.1
822	0.55	undecane	4.8	6.1	3.6	5.6	5.9	6.5	5.1
864	0.56	2,3-dimethyldecane	0.1	0.2	0.2	0.1	0.1	0.4	0.1
876	0.56	alkane isomer (*m/z* 57, 43, 71)	1.3	1.2	1.6	0.6	0.4	0.6	1.2
894	0.55	5-methylundecane	0.3	0.4	0.7	0.3	0.5	0.4	0.2
906	0.57	alkane isomer (*m/z* 57, 43, 71)	0.5	0.3	0.0	1.3	0.4	1.2	0.6
912	0.57	3,9-dimethylundecane	0.6	0.5	0.7	0.1	0.1	0.2	1.0
948	0.57	dodecane	3.1	2.7	3.7	2.8	3.4	3.4	2.9
960	0.58	alkane isomer (*m/z* 57, 71, 43)	2.7	2.4	3.2	0.1	0.1	0.2	0.2
966	0.57	alkane isomer (*m/z* 57, 43, 71)	2.1	2.1	3.2	2.2	2.5	1.0	2.4
972	0.56	2,5,6-trimethyldecane	0.0	0.2	0.8	1.5	1.1	0.8	1.1
978	0.57	alkane isomer (*m/z* 57, 43, 71)	0.0	0.0	1.6	1.2	1.2	1.4	1.3
996	0.56	alkane isomer (*m/z* 57, 43, 71)	0.3	0.2	0.4	0.2	0.2	1.5	0.2
1002	0.57	alkane isomer (*m/z* 57, 43, 71)	0.7	0.4	0.7	0.0	0.9	0.2	0.7
1008	0.57	6-methyldodecane	1.6	1.6	2.4	1.9	2.1	0.9	1.9
1020	0.57	alkane isomer (*m/z* 43, 57, 71)	0.2	0.3	0.5	0.4	0.1	0.2	0.3
1044	0.55	alkane isomer (*m/z* 57, 43, 71)	0.5	0.6	0.6	1.7	2.1	2.3	1.9
1050	0.55	4-ethylundecane	3.0	2.4	2.2	4.6	2.5	4.2	1.7
1056	0.57	alkane isomer (*m/z* 57, 43, 71)	0.5	0.3	0.2	1.3	1.0	1.3	1.4
1062	0.55	alkane isomer (*m/z* 57, 71, 43)	0.5	0.7	0.1	0.5	0.7	0.7	0.3
1068	0.56	alkane isomer (*m/z* 43, 57, 71)	0.7	0.6	0.4	0.5	0.7	0.3	0.4
1074	0.57	tridecane	1.4	1.2	1.8	1.2	1.1	1.5	1.1
1092	0.57	2,2-dimethyldodecane	0.4	0.4	0.7	1.1	0.8	0.9	0.1
1104	0.57	alkane isomer (*m/z* 57, 43, 71)	5.1	4.8	3.3	8.0	4.3	8.3	3.9
1110	0.57	alkane isomer (*m/z* 57, 43, 71)	0.1	0.3	0.0	1.1	0.9	1.0	0.3
1116	0.57	alkane isomer (*m/z* 57, 71, 43)	2.6	2.4	2.9	1.3	1.7	1.4	2.2
1128	0.57	3-ethyl-3-methylundecane	1.7	1.5	2.6	1.1	1.2	1.3	1.4

$^{1}t_{R}$ [a] (s)	$^{2}t_{R}$ [a] (s)	Compounds	S56	S57	S58	S59	S60	S61	S62
1152	0.60	2-methyltridecane	2.1	0.7	1.6	0.8	0.8	1.0	2.7
1158	0.59	alkane isomer (*m/z* 57, 43, 71)	0.6	0.6	1.0	1.3	1.0	1.3	0.9
1170	0.61	alkane isomer (*m/z* 57, 43, 71)	0.4	0.7	0.8	0.5	1.5	0.7	1.0
1188	0.61	tetradecane	3.0	2.6	4.3	3.7	3.2	3.4	2.9
1284	0.58	alkane isomer (*m/z* 57, 43, 85)	0.4	0.3	0.5	0.5	0.4	0.4	0.4
1290	0.58	6,6-diethyldodecane	1.8	1.2	2.9	1.6	2.1	1.9	1.4
1296	0.64	pentadecane	0.6	1.1	1.4	0.8	0.7	1.3	1.8
1302	0.59	5-ethyl-5-methyltridecane	0.5	0.3	0.5	0.7	0.5	0.6	0.4
1338	0.61	3-ethyl-3-methyltridecane	1.9	1.2	2.4	2.3	4.7	2.7	1.0
		Cyclic							
240	0.57	1,2,4-trimethylcyclopentane	0.1	0.2	0.1	0.1	0.1	0.1	0.1
534	0.60	propylcyclohexane	0.9	1.5	0.8	0.5	0.5	0.3	0.8
576	0.59	1,1,2,3-tetramethylcyclohexane	0.7	0.7	0.4	0.7	0.6	0.7	0.7
618	0.60	2-ethyl-1,3-dimethylcyclohexane	0.3	0.3	0.2	0.2	0.2	0.2	0.2
624	0.59	1-methyl-3-propylcyclohexane	0.2	0.3	0.2	0.2	0.2	0.2	0.2
630	0.58	1-methyl-3-(2-methylpropyl)cyclopentane	0.4	0.5	0.4	0.3	0.4	0.4	0.5
642	0.59	ethylpropylcyclopentane	0.1	0.3	0.1	0.2	0.3	0.2	0.2
654	0.59	1-methyl-2-propylcyclohexane	0.2	0.3	0.2	0.2	0.2	0.3	0.2
732	0.57	hexylcyclopentane	0.4	0.4	0.4	0.1	0.3	0.0	0.1
792	0.57	1,4-dimethylcycloctane	0.7	1.0	0.8	0.7	0.7	0.7	0.8
804	0.59	1-ethyl-2-propycylohexane	1.5	1.8	1.6	0.9	0.8	0.8	0.4
996	0.62	hexylcyclohexane	0.8	0.6	1.1	0.8	1.0	0.7	0.9
1068	0.65	1-hexyl-3-methylcyclohexane	1.5	1.5	2.4	2.2	2.4	3.1	2.2
1080	0.60	1-butyl-2-propylcyclopentane	0.4	0.3	0.3	0.8	0.5	0.2	0.5
		Alkenes							
252	0.60	3-octene	0.0	0.1	0.0	0.1	0.0	0.0	0.1
330	0.61	2,4-dimethyl-1-heptene	0.6	0.0	0.0	0.0	0.3	0.1	0.1
450	0.61	1-nonene	0.1	0.2	0.1	0.1	0.2	0.1	0.2
546	0.57	alkene isomer (*m/z* 55, 41, 69)	0.4	0.3	0.2	0.2	0.2	0.2	0.2

^1t_R [a] (s)	^2t_R [a] (s)	Compounds	S56	S57	S58	S59	S60	S61	S62
552	0.57	3-methyl-1-nonene	0.4	0.4	0.3	0.2	0.3	0.2	0.6
570	0.59	3,4-diethyl-2-hexene	0.6	0.9	0.5	0.4	0.3	0.5	0.4
582	0.59	alkene isomer (*m/z* 69,41,56)	0.1	0.2	0.4	0.1	0.1	0.1	0.1
606	0.59	alkene isomer (*m/z* 69,41,56)	0.1	0.2	0.1	0.2	0.3	0.3	0.3
612	0.59	7-methyl-1-nonene	0.2	0.4	0.1	0.2	0.3	0.3	0.3
648	0.59	4-decene	0.2	0.3	0.2	0.2	0.3	0.4	0.3
762	0.55	alkene isomer (*m/z* 55, 69, 41)	1.0	2.1	1.6	0.6	0.8	0.6	2.1
768	0.56	alkene isomer (*m/z* 55, 69, 41)	0.4	0.8	0.9	0.6	0.6	0.7	0.4
780	0.56	*(Z)*-2-decene	0.6	0.6	0.5	0.4	0.4	0.4	0.6
798	0.56	alkene isomer (*m/z* 55, 69, 41)	1.2	3.6	1.7	0.8	2.8	1.1	1.3
810	0.55	alkene isomer (*m/z* 69, 55, 41)	0.6	0.7	0.6	1.0	0.9	1.5	0.6
828	0.57	alkene isomer (*m/z* 55, 69, 41)	0.9	1.4	1.2	1.0	1.0	1.0	1.2
870	0.59	2-methyl-1-undecene	1.3	1.4	1.6	1.2	1.3	1.2	1.3
918	0.60	(*E*)-5-methyl-4-undecene	0.5	0.9	0.9	0.8	0.9	1.0	0.9
924	0.60	(*Z*)-5-methyl-5-undecene	0.6	0.7	0.8	0.5	0.6	0.6	0.6
942	0.60	1-dodecene	1.8	1.8	2.8	1.2	1.2	1.7	0.7
960	0.62	alkene isomer (*m/z* 55, 69, 41)	3.1	5.2	4.2	1.4	1.6	1.6	1.6
978	0.59	alkene isomer (*m/z* 55, 69, 43)	1.0	1.0	0.5	0.9	0.8	1.1	0.2
1062	0.61	1-tridecene	2.7	2.2	4.0	1.5	1.6	2.2	2.5
		Aldehydes							
144	0.78	butanal	0.3	0.3	0.5	0.2	0.3	0.3	0.2
252	1.17	hexanal	1.3	1.6	0.8	1.9	1.5	1.0	1.7
474	1.22	heptanal	0.4	0.4	0.3	0.5	0.6	0.2	0.5
576	0.92	2-ethylhexanal	0.0	0.0	0.0	0.1	0.1	0.1	0.1
588	3.58	benzaldehyde	1.1	1.0	1.7	3.8	1.4	1.7	1.3
666	1.06	octanal	1.6	0.5	0.5	1.6	1.6	1.9	1.9
822	1.00	nonanal	4.9	5.2	4.9	5.9	6.5	4.7	6.4
900	1.28	*(E)*-2-nonenal	0.1	0.2	0.2	0.3	0.3	0.3	0.3
960	0.97	decanal	6.2	4.3	4.3	5.3	6.6	4.5	6.0
1038	1.03	aldehyde isomer (*m/z* 41, 55, 71)	0.8	1.0	0.9	0.9	1.1	1.3	0.8

^1t_R [a] (s)	^2t_R [a] (s)	Compounds	S56	S57	S58	S59	S60	S61	S62
1086	1.02	undecanal	0.3	0.2	0.2	0.5	0.6	0.4	0.5
1200	1.09	dodecanal	0.3	0.3	0.3	0.7	0.7	0.6	0.7
		Ketones							
138	0.79	2-butanone	0.3	0.0	0.3	0.4	0.2	0.2	0.2
240	1.16	2-hexanone	0.0	0.0	0.0	0.0	0.0	0.0	0.0
438	1.19	3-heptanone	1.8	1.2	0.2	0.1	0.1	0.1	0.1
450	1.29	ketone isomer (*m/z* 43, 58, 71)	0.2	0.1	0.2	0.2	0.1	0.1	0.2
450	2.07	ciclohexanone	0.7	0.7	0.4	0.6	0.6	0.9	0.5
642	1.10	3-octanone	0.2	0.1	0.1	0.1	0.1	0.1	0.1
642	1.33	6-methyl-5-hepten-2-one	3.1	3.5	4.0	5.2	5.0	3.6	6.0
804	1.03	2-nonanone	0.1	0.1	0.1	0.2	0.1	0.1	0.0
810	1.02	ketone isomer (*m/z* 43, 58, 71)	0.1	0.1	0.1	0.0	0.0	0.0	0.1
		Miscellaneous							
630	2.13	1-octen-3-ol	0.6	0.2	0.2	0.6	0.3	0.2	0.2
720	1.78	2-ethyl-1-hexanol	0.5	0.5	0.4	4.9	5.9	4.4	6.3
774	0.99	2-nonen-1-ol	0.2	0.2	0.1	0.2	0.3	0.2	0.3
780	1.86	1-octanol	0.1	0.1	0.3	0.1	0.1	0.1	0.2
912	0.97	2-decen-1-ol	0.2	0.5	0.4	0.2	0.2	0.2	0.5