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**Electronic nose for analysis of volatile organic  
compounds in exhaled breath**

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**MASTER IN ENVIRONMENTAL ENGINEERING**

**by**

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*Confucius*



## Abstract

Human exhaled breath volatile organic compounds (VOCs) are of growing interest to scientists and have shown promising results when discriminating individuals with adverse respiratory effects from healthy controls. Being a convenient, low-cost and non-invasive method, it may represent a useful tool for early diagnosis and screening of subjects with risky respiratory occupational exposures, which require regular health check-ups. However, most of the current research are in the early stages; so the current knowledge is far from comprehensive. This work aimed to investigate the potential of the analysis of exhaled breath VOCs as a research methodology in indoor exposure science hoping to build a steppingstone for future researches. The specific objectives of this work were to characterise VOCs patterns in exhaled breath in an adult population by electronic nose (eNose), and to identify the relevant sociodemographic and behavioural subject characteristics for VOC profiles.

Exhaled breath air samples from 94 adult subjects were collected and analysed using an eNose composed by 32 sensors (Cyranose 320<sup>®</sup>). Relevant physiological and sociodemographic characteristics, data on lifestyle, behaviour, living conditions, professional and environmental exposures, information on diet and health status were collected through a self-administered questionnaire. Routine and personal habits (i.e., physical exercise, time and type of meals and drinks consumed, brushing teeth, smoking, information on health and number of hours at the workplace up to the time of sampling) of the participants on the day of breath collection was collected via a face-to-face interview. A multivariate cluster analysis model, using resistance data from the 32 sensors, was built to discriminate the VOC patterns between individuals separating the study population in 2 clusters. Logistic regression models were used to estimate the associations between relevant parameters and the clusters.

The study population ( $37.5 \pm 12.1$  years old) was predominantly composed of males (57.1%) with significantly higher BMI ( $25.0 \pm 3.5$  kg/m<sup>2</sup> vs.  $23.5 \pm 3.9$  kg/m<sup>2</sup> for female subjects), thus possibly indicating a starting obesity for the respective study subgroup. Current smokers represented 23.1% of the population, 11.0% of the participants were former smokers; a significant ( $p < 0.05$ ) positive correlation was observed between smoking and coffee consumption. Water intake prior to the breath air collection sample had a notable effect on the majority of eNose sensors. Further, the study also showed that water intake shall be limited to at least 2.5 hours prior to the exhaled breath samples since study participants exhibited differences in the VOC patterns of exhale.

While in future occupational studies, the analysis of the VOC profiles may provide a fast and non-invasive complementary early diagnostic and screening for individuals, the respective sampling protocol shall be adapted in terms of liquids intake. This study indicated that the use of the eNose may represent a suitable tool to be used in the context of indoor air studies, although further research and a larger population sample size are needed to confirm results and unravel more pronounced associations.

**KEYWORDS:** Volatile organic compounds, electronic nose, occupational exposure, exhaled breath, public risks



## Resumo

Os compostos orgânicos voláteis (COV) no ar exalado têm assumido um interesse crescente na comunidade científica dado os resultados promissores na discriminação, por exemplo, de indivíduos com problemas respiratórios e controlos saudáveis.

Como mostram as evidências científicas, por ser um método conveniente, de baixo custo e não invasivo, a análise do perfil de COV no ar exalado usando a tecnologia do nariz eletrónico (eNose) pode representar uma ferramenta útil, nomeadamente, no diagnóstico precoce e triagem de indivíduos com exposições ocupacionais de risco. Estas evidências também apontam para a necessidade de mais estudos de forma a colmatar lacunas no conhecimento contribuindo para a promoção da saúde.

Esta tese teve como objetivo geral investigar o potencial de aplicação, em estudos epidemiológicos, da análise de COV no ar exalado. Os objetivos específicos foram caracterizar, através da recolha e análise por um eNose composto por 32 sensores (Cyrano<sup>®</sup> 320), amostras de ar exalado de 94 adultos, e identificar as características sociodemográficas e a comportamentais relevantes para os perfis de COV.

Informação individual foi recolhida através de um questionário preenchido por cada participante. No presente estudo foram analisadas em particular características fisiológicas, sociodemográficas e de saúde, bem como variáveis relacionadas com o estilo de vida. Outros indicadores sobre hábitos e rotinas (por exemplo, exercício físico, tempo e tipo de refeições e bebidas consumidas, higiene oral, tabagismo, saúde e número de horas no local de trabalho) relativos ao dia e hora da colheita de ar exalado dos participantes foram obtidos durante uma entrevista.

Um modelo de análise multivariada de clusters foi construído para discriminar os padrões de COV entre os participantes; e as associações entre as variáveis individuais e os clusters foram analisadas por modelos de regressão logística. Os resultados mostram que os participantes, com idade média de  $37.5 \pm 12.1$  anos, eram predominantemente do género masculino (57.1%) apresentando um índice de massa corporal significativamente maior ( $25.0 \pm 3.5$  kg/m<sup>2</sup> vs.  $23.5 \pm 3.9$  kg/m<sup>2</sup>) indicando, possivelmente uma fase de obesidade inicial para este subgrupo de acordo com a classificação da Organização Mundial de Saúde.

A avaliação da distribuição das participantes de acordo com os seus hábitos tabágico mostra que 23.1% dos participantes eram fumadores e 11.0% ex-fumadores. Observou-se ainda uma correlação positiva significativa ( $p < 0.05$ ) entre o tabagismo e o consumo de café.

Entre os resultados relevantes deste trabalho, destaca-se a influência da ingestão de água antes da colheita de ar exalado por parte dos participantes na resposta da maioria dos sensores do eNose. Em particular, os resultados apontaram que a ingestão de água deve ser limitada a pelo menos 2.5 horas antes da colheita de ar exalado tendo em consideração as diferenças observadas nos padrões de COV.

Embora em futuros estudos, a análise dos perfis de COV por eNose possa ser usada como uma ferramenta promissora de diagnóstico precoce e rastreio complementar rápido e não invasivo na população em geral e em populações ocupacionalmente expostas a COV, o respetivo protocolo de amostragem deve ser adaptado tendo em consideração a ingestão de líquidos por parte dos participantes. Contudo, mais estudos científicos confirmatórios deverão ser realizados, nomeadamente numa amostra mais alargada de participantes.

**PALAVRAS-CHAVE:** Compostos orgânicos voláteis, nariz eletrónico, exposição ocupacional, ar exalado, riscos públicos



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## Nomenclature

### Abbreviations

**BMI** *Body Mass Index*

**CO<sub>2</sub>** *Carbon dioxide*

**CO** *Carbon monoxide*

**CSV** *Comma-separated values*

**EFFIS** *European Forest Fire Information System*

**eNose** *Electronic Nose*

**ETS** *Environmental Tobacco Smoke*

**EU** *European Union*

**GHG** *Greenhouse Gas*

**IARC** *International Agency in Cancer Research*

**INSA, IP** *National Institute of Health Doctor Ricardo Jorge, IP*

**IPCC** *Intergovernmental Panel on Climate Change*

**NO<sub>2</sub>** *Nitrogen dioxide*

**NOAA** *National Oceanic and Atmospheric Administration*

**O<sub>3</sub>** *Ozone*

**PM<sub>2.5</sub>** *Particulate matter which passes through a size-selective inlet with a 50% efficiency cut-off at 2.5  $\mu\text{m}$  aerodynamic diameter*

**PM<sub>10</sub>** *Particulate matter which passes through a size-selective inlet with a 50% efficiency cut-off at 10  $\mu\text{m}$  aerodynamic diameter*

**R<sub>0</sub>** *Baseline Resistance*

**R<sub>max</sub>** *Maximum Resistance*

**SR** *Sensor Response*

**VOCs** *Volatile Organic Compounds*

**WHO** *World Health Organization*

# 1. Preface

## 1.1 Relevance and motivation

The consequences of climate changes have been alarming for nowadays society. Due to its geographical characteristics, Portugal is one of the European countries most vulnerable to climate change (Schleussner et al., 2019). The existing predictions show that in the foreseeable future Mediterranean countries will experience the consequences of the rising temperature in forms of extreme events (heat waves, droughts, or wildfires) even at a larger scale than up to now (IPCC, 2019). Increasing temperatures and reduced precipitation also leads to a significant decrease in the amount of soil moisture (Parente et al., 2018), causing a secondary fire risk (Turco et al., 2019). Future warming may therefore increase the number, duration and amplitude of heat waves and thus wildfires (especially in the north-east region as Bragança - Annex 1). Due to a general improved effectiveness of firefighting mechanisms and a reduction in the total number of incidents, the total area affected by forest fires has decreased in recent years in other Mediterranean countries (Hernández, 2019). Unfortunately, in Portugal the opposite trend is true with forest fires consequences on rise (Hernández, 2019).

Firefighting is of the riskiest professions (IARC, 2010). During the fire suppression, firemen are exposed to a wide range of toxic and or health-risk relevant gaseous pollutants and particulates. This is especially relevant for wildfire or forest fire firefighters who do not use self-contained breathing apparatus and at the same time execute more physically demanding work tasks. Therefore, regular screening and health controls of the workers are necessary in the

occupationally exposed subject in order to prevent the possible negative consequences on their health.

Exhaled breath volatile organic compounds (VOCs) have shown promising results when discriminating individuals with adverse respiratory effects (Ratiu et al., 2021). Being non-invasive and with various methodological advantages, it may also represent a useful tool for screening of subjects with risky respiratory occupational exposures, who require medical controls on a frequent basis.

## 1.2 Objectives

This study sought to investigate the potential of the analysis of exhaled volatile organic compounds (VOCs) as a research methodology in air exposure science in a general group of individuals. The experiments designed for this thesis are intended as a pathfinder study to complement other ongoing research in occupational exposure scenarios, such as forest firefighters', to further the understanding of this under-explored scientific area, since we hypothesise that VOCs patterns will differ between persons occupationally exposed to VOCs and those unexposed controls.

To that end, the specific objectives of this work were:

- i) To characterise VOCs patterns of exhaled breath by means of the electronic nose (eNose) on adult subjects; and
- ii) To identify the relevant socio demographic and behavioural subject' characteristics for exhaled breath profiles.

## 1.3 Thesis outline

The thesis is divided into 5 chapters. The content of each chapter is given below.

**Chapter 1** (present chapter) is the preface of the thesis. It explains the relevance and motivation of the work as well as its objectives.

**Chapter 2** is a short overview into the problematic of forest fires. It clarifies the increasingly relevant impacts of climate change and explains the relevance of VOCs. The significance of control population is also emphasised. The technology of electronic nose (eNose) is described.

**Chapter 3** provides the materials and methods used for this work. It describes the applicability of the electronic nose and provides details of the methodology, which was used for exhaled breath analysis. It also describes the studied control population, as well the process of the data collection in the study and respective statistical analysis.

**Chapter 4** considers the results and discussions of the work. The first part of the chapter concerns the key steps related with the method optimization, while the second part of the chapter is dedicated to the characterization of the studied population. Finally, it contains the major results of the exhaled breath samples analysis.

The conclusions of the thesis and the suggestions for future work are addressed in **Chapter 5**.

## **2. State of the art**

### **2.1 Air pollution**

Climate change is a phenomenon closely related to air pollution. Climate change occurs gradually over time but the increase in human activity has been accelerating this transition, with serious consequences, one of them being forest fires. Forest fires are a significant emissions source of greenhouse gases (GHGs) and health hazardous air pollutants in general (Vilén & Fernandes, 2011). Their duration and frequency have been increasing in the last decades, with longer fire seasons and more powerful incidents. Fires and their frequency also impact on ecosystems by disrupting life cycles of the individual's. In addition, forest fires may impact the economy of a landscape, farming lands, rainwater or cause wind erosion (Flannigan, 2000). Forest fires undoubtedly represent a significant risk not only to the general public but also to those occupationally exposed.

In correlation, Global warming a term used to describe the rise in the annual average global surface temperature on Earth. According to the results of a variety of climate model simulations, our planet's average temperature in 2100 might be 1.1 to 5.4°C higher than it is currently (IPCC, 2007). The main cause of this temperature rise is carbon dioxide (CO<sub>2</sub>) production and other heat-trapping "greenhouse" gases (GHG) caused by anthropogenic activities. The greatest source of additional CO<sub>2</sub> comes from anthropogenic activities, such as burning coal and other fossil fuels (IPCC, 2007). According to National Oceanic and Atmospheric Administration

(NOAA), 2019 was the second warmest year on record ( $1.15^{\circ}\text{C}$  above pre-industrial average of (1880–1900)), (Ahmed, 2020). Worldwide annual average land and ocean surface temperature has risen at a pace of  $0.07^{\circ}\text{C}$  each decade since 1880 (Ahmed, 2020). This increase in global annual average temperature has led to changes in the climate and weather such as temperature, humidity, precipitation and wind speed as well as on the levels of air pollution (Sun et al., 2019).

As a consequence, the human health and the ecosystem are altered; such fact is not only associated to the emissions of the anthropogenic air pollutants but also due to the production of secondary pollutants released into the atmosphere through a photochemical process in which VOCs are oxidised by nitrogen oxides.

For instance, a number of scientific papers and reports have been published in the Mediterranean region (*San-Miguel-Ayanz et al., 2020*) about the high incidence of forest fires in the region, increasing the risk of serious health consequences enhanced by climate change (*Figure 2-1*).

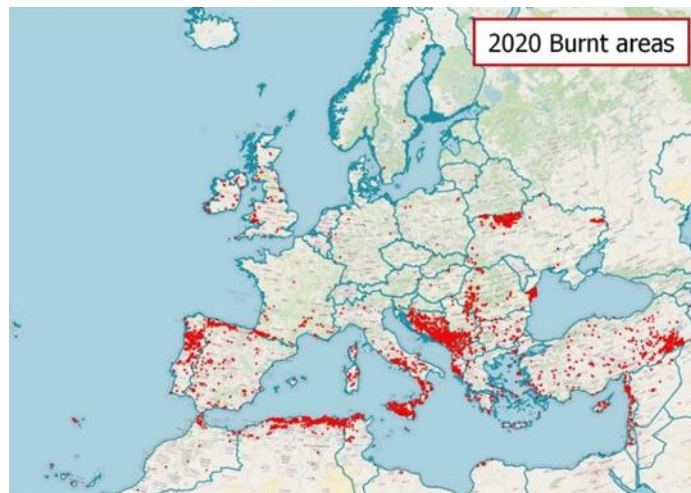


*Figure 2-1- Europe burnt area in 2020; adapted from (San-Miguel-Ayanz et al., 2020)*

Between 2000 and 2017, wildfires were responsible for 8.5 million hectares (ha) burnt (approximately 480 800 ha/year) in Europe (Vallejo Calzada et al., 2018) and resulted in a loss of 611 firefighters and citizens. These economic damages corresponded to more than 54 billion € on European territory (Vallejo Calzada et al., 2018). More specifically, between 2010 to 2016, more than 40 000 fires per year were reported in the five Mediterranean countries (i.e., Greece, Spain, France, Italy and Portugal), which burnt areas accounted for around 85% of total burns in Europe (Vallejo Calzada et al., 2018).

2020 was again a year, in which wildfires destroyed large areas of European wildland, despite the increased level of preparations in European Union (EU) countries. Around 340,000 ha were burned, an area 30% larger than Luxembourg. The 2021 fire season was even more severe. Up to the release of this report 0.5 million ha were burned, out of which 25% were within "Natura 2000" (European Commission, 2021) – i.e., a European network of protected nature areas where certain species of animals and their natural habitats are protected in order to preserve biodiversity.

This increase in the number of fires means that firefighters will be even more exposed to VOCs released from burning forests with increased frequency and in higher quantities, resulting in an increased risk of developing diseases and even death in serious cases due to their occupational exposure, making the study of these VOCs so crucial.

Table 2-1- Occurrence of forest fires in the European Union (EU) since 1980; adapted from (San-Miguel-Ayaz et al., 2020)

| Year | Belgium | Switzerland | Cyprus | Czech Republic | Denmark | Estonia | Spain | Finland | France | Greece | Croatia | Hungary | Italy | Lithuania | Latvia | Netherlands | Poland | Portugal | Romania | Sweden | Slovenia | Slovakia | Turkey |
|------|---------|-------------|--------|----------------|---------|---------|-------|---------|--------|--------|---------|---------|-------|-----------|--------|-------------|--------|----------|---------|--------|----------|----------|--------|
| 1980 |         | 87          |        |                |         |         |       |         |        |        |         |         |       |           |        |             | 2349   |          |         |        |          |          |        |
| 1981 |         | 153         |        |                |         |         |       |         |        |        |         |         |       |           |        |             | 6730   |          |         |        |          |          |        |
| 1982 |         | 86          |        |                |         |         |       |         |        |        |         |         |       |           |        |             | 3626   |          |         |        |          |          |        |
| 1983 |         | 120         |        |                |         |         |       |         | 945    |        |         |         |       |           |        |             | 4542   |          |         |        |          |          |        |
| 1984 |         | 183         |        |                |         |         |       |         | 1184   |        |         |         |       |           |        |             | 7356   |          |         |        |          |          |        |
| 1985 |         | 114         |        |                |         | 12235   | 3732  | 1417    |        |        |         | 12931   |       |           |        |             | 8441   |          |         |        |          |          |        |
| 1986 |         | 87          |        |                |         | 7514    | 2657  | 1088    |        |        |         | 6115    |       |           |        |             | 5036   |          |         |        |          |          |        |
| 1987 |         | 121         |        |                |         | 8816    | 2116  | 1234    |        |        |         | 8506    |       |           |        |             | 7705   |          |         |        |          |          |        |
| 1988 |         | 79          |        |                |         | 9440    | 2240  | 1798    |        |        |         | 9785    |       |           |        |             | 6131   |          |         |        |          |          |        |
| 1989 |         | 189         |        |                |         | 20250   | 3321  | 1203    |        |        |         | 8328    |       |           |        |             | 21896  |          |         |        |          |          |        |
| 1990 |         | 257         |        |                |         | 12914   | 3297  | 1283    |        |        |         | 11560   |       |           |        |             | 10745  |          |         |        |          |          |        |
| 1991 |         | 152         |        |                |         | 13529   | 2372  | 1036    |        |        |         | 7580    |       |           |        |             | 14327  |          |         |        |          |          |        |
| 1992 |         | 86          |        |                |         | 15956   | 2708  | 2008    |        |        |         | 10044   |       |           |        |             | 14954  |          |         |        |          |          |        |
| 1993 |         | 83          |        |                |         | 14253   | 4766  | 2707    |        |        |         | 14317   |       |           |        |             | 16101  |          |         |        |          |          |        |
| 1994 |         | 86          |        |                | 706     | 19249   | 4728  | 1955    |        |        |         | 7153    |       |           |        | 24361       | 19983  |          |         |        |          |          |        |
| 1995 |         | 96          |        |                | 525     | 25557   | 6539  | 1494    |        |        |         | 5505    |       |           |        | 23816       | 34116  |          |         | 44     |          |          |        |
| 1996 |         | 130         |        |                | 822     | 16586   | 6401  | 1527    | 2363   |        |         | 6064    |       |           |        | 23582       | 28626  |          | 4854    | 47     |          |          |        |
| 1997 |         | 179         |        |                | 276     | 22320   | 8001  | 2271    | 2648   |        |         | 11608   |       |           |        | 25068       | 23497  |          | 7057    | 55     |          |          |        |
| 1998 |         | 121         |        |                | 592     | 22003   | 6289  | 605     | 4096   |        |         | 9565    |       |           |        | 21342       | 34676  |          | 2503    | 143    |          |          |        |
| 1999 |         | 50          |        |                | 794     | 17943   | 4881  | 513     | 2592   |        |         | 6956    |       |           |        | 32646       | 25477  |          | 4707    | 55     |          |          |        |
| 2000 |         | 70          | 285    |                | 930     | 23574   | 4343  | 1469    | 5477   |        |         | 8609    |       |           |        | 31809       | 34109  |          | 4708    | 100    |          |          |        |
| 2001 |         | 67          | 299    |                | 373     | 19099   | 4259  | 1313    | 2505   |        |         | 7227    |       |           |        | 24511       | 28915  |          | 4831    | 60     |          |          |        |
| 2002 |         | 117         | 243    |                | 278     | 19929   | 4097  | 572     | 3428   | 429    |         | 4607    |       |           |        | 38154       | 28993  |          | 6490    | 64     |          |          |        |
| 2003 |         | 304         | 427    |                | 1238    | 18616   | 7023  | 622     | 4904   | 373    |         | 9716    |       |           |        | 79013       | 28087  |          | 8282    | 227    |          |          |        |
| 2004 |         | 94          | 221    | 957            | 300     | 21396   | 3767  | 739     | 1704   | 104    | 6341    | 430     | 647   |           |        | 36315       | 27829  | 34       | 4955    | 50     | 153      |          |        |
| 2005 | 251     | 110         | 185    | 653            | 299     | 65      | 25492 | 2631    | 4698   | 718    | 2180    | 150     | 7918  | 267       | 365    |             | 46542  | 41689    | 64      | 4573   | 74       | 287      | 1530   |
| 2006 | 393     | 110         | 172    | 697            | 717     | 248     | 16334 | 6314    | 4608   | 764    | 2210    | 97      | 5651  | 1444      | 1929   |             | 35630  | 24243    | 105     | 4618   | 106      | 238      | 2227   |
| 2007 | 1479    | 120         | 111    | 809            | 435     | 64      | 10932 | 2813    | 3382   | 1226   | 3759    | 603     | 10736 | 245       | 426    |             | 31303  | 25132    | 478     | 3787   | 129      | 463      | 2706   |
| 2008 | 582     | 63          | 114    | 470            | 560     | 71      | 11656 | 3161    | 2781   | 1071   | 228     | 502     | 6648  | 272       | 716    |             | 35786  | 18958    | 91      | 5420   | 68       | 182      | 2135   |
| 2009 | 314     | 103         | 91     | 520            | 575     | 47      | 15642 | 2746    | 4808   | 354    | 181     | 608     | 5423  | 471       | 890    |             | 30912  | 29783    | 190     | 4180   | 122      | 347      | -      |
| 2010 | 222     | 88          | 133    | 731            | 525     | 30      | 11722 | 3100    | 3828   | 540    | 131     | 109     | 4884  | 106       | 319    |             | 24443  | 26113    | 70      | 3120   | 33       | 123      | 1861   |
| 2011 | 635     | 114         | 85     | 1341           | 515     | 24      | 16417 | 2871    | 4283   | 953    | 279     | 2021    | 8181  | 137       | 373    |             | 39011  | 29782    | 340     | 3534   | 114      | 303      | -      |
| 2012 | 876     | 75          | 78     | 1555           | 451     | 5       | 15978 | 1050    | 3713   | -      | 570     | 2657    | 10345 | 81        | 162    |             | 53907  | 25352    | 911     | 2213   | 168      | 517      | 2449   |
| 2013 | 408     | 58          | 135    | 671            | 355     | 15      | 10797 | 2864    | 2061   | -      | 137     | 761     | 2077  | 119       | 420    |             | 25652  | 23129    | 118     | 4907   | 75       | 233      | 3755   |
| 2014 | 151     | 60          | 68     | 870            | 251     | 91      | 9806  | 3637    | 1729   | -      | 43      | 1042    | 1821  | 155       | 695    |             | 38115  | 9388     | 83      | 4374   | 35       | 153      | -      |
| 2015 | 439     | 166         | 87     | 1738           | 594     | 67      | 11810 | 1644    | 2891   | -      | 176     | 1069    | 5424  | 247       | 704    |             | 60176  | 19643    | 250     | 2700   | 93       | 242      | -      |
| 2016 | 584     | 82          | 119    | 899            | 407     | 84      | -     | 2101    | 2761   | -      | 176     | 452     | -     | 98        | 641    |             | 25791  | 16104    | 174     | 5454   | 90       | 136      | -      |
| 2017 | 513     | 110         | -      | 988            | 176     | 61      | -     | 2263    | 3201   | -      | 328     | 1454    | -     | 80        | 423    | 321         | 25193  | 21006    | 447     | 5276   | 108      | 162      | -      |
| 2018 | 222     | 153         | -      | 2033           | 1216    | 230     | -     | 4401    | 1616   | -      | 57      | 530     | -     | 211       | 972    | 949         | 33227  | 12273    | 158     | 8181   | 32       | 262      | -      |
| 2019 | 888     | -           | -      | 59910          | 845     | -       | -     | 3046    | 2886   | -      | 123     | 2088    | -     | 280       | 1107   | 547         | -      | 10832    | 425     | 5483   | 84       | -        | -      |

Portugal is one of the EU countries that has always been one of the most severely affected by wildfires (Table 2-1). Moreover, as demonstrated, forest fires have been increasing in more recent years, both in numbers of occurrences and in burnt areas across the whole EU region (Figure 2-2).

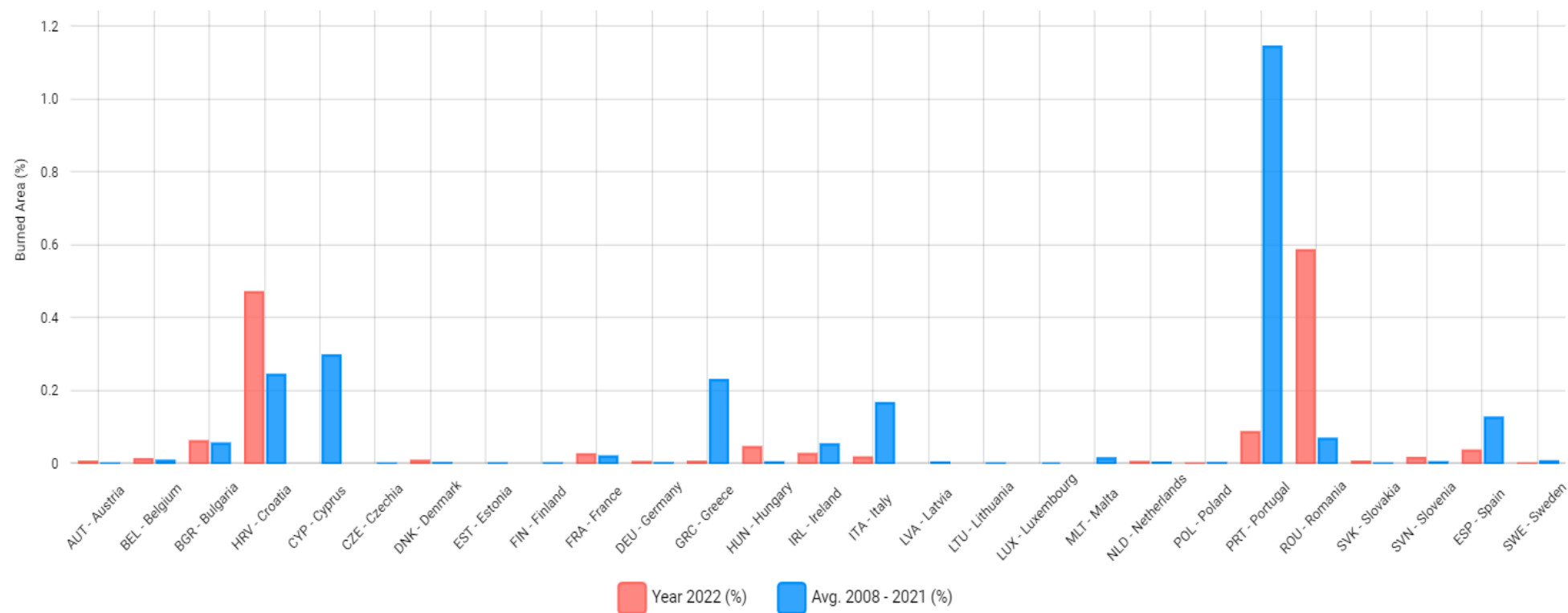


Figure 2-2- Burnt area (% by country area) due to forest fires across European Union Member States; adapted from (European Forest Fire Information System ,2022)

## 2.2 Volatile organic compounds

Volatile organic compounds (VOCs) are defined according to their chemical and physical properties. Indeed, it is an organic chemical, excluding methane, which consists of at least one element of carbon and one or more other elements such as hydrogen, oxygen, nitrogen, sulphur, phosphorus, silicon, and halogens. These compounds, as their name indicates, are highly volatile under normal conditions of pressure and ambient temperature, with boiling points ranging from 0°C to 400 (Hodgson et al., 1994). VOCs are very numerous, and they are a part of large chemical families such as linear hydrocarbons, saturated or not, as heptane, hexane, pentane, mineral spirits, etc.; monocyclic aromatic hydrocarbons or polycyclic aromatic hydrocarbons such as benzene, toluene, and xylene; oxygenated VOCs as aldehydes, alcohol, ketones, or esters; chlorinated VOCs (tetrachloroethylene, trichloroethylene, dichloromethane); chlorofluorocarbons; plasticizers (dioctyl phthalate, etc.); nitrogen compounds (amines, nitriles, etc.); and sulphur compounds (thiols, dimethyl sulphide, etc.) ((Bensouilah et al., 2020).

### 2.2.1 Non-occupational exposure

A variety of VOCs have been detected indoors due to numerous sources (Hodgson et al., 1994). Many indoor goods, including paints, varnishes, cleaning products, cosmetics, insecticides, building materials, office equipment, adhesives, etc. are potential major sources of indoor VOCs. Other significant sources of VOCs, primarily in ambient air, include the combustion products of wood, gasoline, and fuels; automotive emissions are the most significant source from the outdoors, while tobacco smoke is the most significant source indoors (Barro et al., 2009).

Typically, VOCs are characterised by their indoor/outdoor concentration ratios (Hodgson et al., 1994). The concentration of VOCs varies depending on the environment; it is 2–5 times higher in indoor conditions than outdoors (Barro et al., 2009). In case of occupational exposure the respective levels can be 1000 times higher than outdoor background levels (Barro et al., 2009). VOCs measured indoors are generally regarded as "non-reactive" as single substances, except for acrolein and formaldehyde (Hodgson et al., 1994). BTEX (i.e., refers to benzene, toluene, ethylbenzene and xylene) chemicals traditional indicators for human VOCs exposure, however

other compounds are nowadays being monitored to provide a more accurate assessment of human health risks (Barro et al., 2009).

The ability of VOCs to cause health effects varies greatly from those that are highly toxic, to those with no known health effect; some organics can cause cancer in animals, others are suspected or known to cause cancer in humans (Puzyn, 2012). Eye, nose and throat irritation, headaches, loss of coordination and nausea, and damage to liver, kidney and central nervous system are among the key health effects (Puzyn, 2012).

So, characterising and understanding of the hazardous of VOC or VOCs profiles is a key step in identifying the actions necessary to avoid and reduce the adverse impact of these pollutants -commonly found in air inside homes, offices, schools, or other private and public buildings where people spend a large part of their life – on people's health and well-being.

### **2.2.2 Occupational exposure**

Exposure to VOCs also occurs in a wide variety of occupational settings since they are used in solvents, fixing agents, paints and glues (IARC, 2010). For example, occupational exposure of painters has been classified Group 1 by IARC in 2010; it causes cancers of the lung and of the urinary bladder. In addition, most of the chemical compounds used in the shipyard painting activity are VOCs, such as benzene, toluene, xylene, known compounds with carcinogenic properties. Past exposure assessments studies of wildland firefighters have also measured acrolein, benzene, formaldehyde, polycyclic aromatic hydrocarbons, and other VOCs from occupational exposure to wildland fire smoke. The IARC classified occupational exposure of fires as potentially carcinogenic to humans as many of the carcinogenic combustion products identified are VOCs and can be found in most burned materials (IARC, 2010).

The exposure to VOCs via inhalation and/or by dermal absorption by a fireman performing hard labour without proper individual protection is typically more than that of a worker with a regular work (Reh et al., 1994). Studies have shown that firefighting uniforms worn in firefighting conditions may contribute to indirect exposure as well increase dust concentrations at the locations where this gear is stored (Banks et al., 2021). Estimating the exposure of firefighters to gases, including VOCs, released in fires is complex due to a wide range of factors such as working hours, wide variations between the time spent firefighting, type of exposure (mixture of gases, vapours and particulate matter) unknown impact of the heat, the density of

the smoke and the struggle to sample unexpected locations in a perilous and quickly changing environment (IARC, 2010). The current knowledge on the occupational health effects is far from comprehensive (IARC, 2010). A better understanding of the occupational impacts of fire emissions on workers exposure and health is required. This is especially relevant in countries such as Portugal, that is severely affected by the occurrence of forest fires. In addition, raising awareness and preventing these occurrences are key tools in reducing the number of forest fires outbreaks (San-Miguel-Ayanz et al., 2020).

## **2.3. Exhaled breath**

In exhaled breath, VOCs include many molecular families such as hydrocarbons, compounds containing nitrogen, oxygen or sulphur. Under normal conditions, the exhaled breath contains many VOCs that are both endogenous, produced during the body's metabolic processes, and exogenous, absorbed by the external environment. Exogenous compounds enter into the body through the skin, inhalation or ingestion and then are metabolised, modified by physiological processes such as the absorption in the epithelial lining fluid of airways, and by biochemical alteration such as oxidation processes and exhaled (Cazzola et al., 2015). A recent review indicates some 1840 VOCs in exhaled breath of healthy subjects (Cazzola et al., 2015).

The major advantage for breath analysis is the non-invasive collection procedure. In this thesis we will focus on eNose technology.

### **2.3.1 Electronic nose**

Scientist Alexander Graham Bell, in 1914 popularised the idea that measuring a smell was difficult. In the decades after Bell's observation no such science of odour was achieved until the 1950s. A common issue with odour recognition technology is that it does not sense energy but rather physical particles (Wise et al., 2000).

eNose is a versatile instrument typically portable, based on arrays of partially selective gas sensors system (Cosio et al., 2010). It can typically detect a large range of VOCs to provide discrimination among samples classified according to their chemical composition (Stitzel et al., 2011). Among a manifold of applications, some eNose have been used to study biological olfactory functions identifying complex volatiles by reproducing the structure and characteristics of the olfactory sense. The significance of olfactory uses in the food industry, environmental detection, and medical care is vast (Fitzgerald et al., 2017). It is frequently

utilised in odour detection, raw material inspection, quality signing, and the control of sprinkling processes. It is one of the most important instruments for quality assurance and control. The eNose is employed in fruit and vegetable quality evaluation, maturity detection, and species identification (Fitzgerald et al., 2017) but can be used for identifying odours such as wine, cigarettes, and others.

More recently, the medical industry has been investing in this technology due its benefits. It allows for quick diagnostics, with minimum risks for the individual and at acceptable cost. Thus, it may be a viable tool in detection and diagnosis of diseases such as lung cancer, asthma and other major respiratory diseases (Fitzgerald et al., 2017). The current study is based on some of these applications, namely in what concerns health risks, such as the diagnosis of asthma through volatile organic compounds as biomarkers that can be done quickly and non-invasively (Farraia et al., 2020) and (Cavaleiro Rufo et al., 2019).

eNose provides information on the respiratory profile characterised by a set of several VOCs but does not provide specific information about individual molecules. To identify the compounds in the exhaled breath is necessary to use other techniques to separate the different compounds such as Gas chromatography–mass spectrometry (GC-MS) that is the most widely used instrument for that purpose.

### **3. Materials and methods**

#### **3.1 Study participants**

The cross-sectional study was conducted in the framework of the BioFirEx project (PCIF/SSO/0017/2018) which was approved by the Ethical Committee of the University of Porto (Approval report 92/CEUP/2020) and was conducted in accordance with the Helsinki convention and its amendments. Participants provided a written informed consent.

Adult subjects attending a public University in Porto city were invited on a random and voluntary basis to participate in the study. Those who agreed to participate were clearly informed about the objectives and purpose of the study. Ninety-four individuals were included in the study.

Relevant physiological characteristics (sex, age, body height and body weight), sociodemographic characteristics, professional status and the respective exposure, information on life style behaviour (including smoking habits and exposure to environmental tobacco smoke (ETS) - at work, home and leisure spaces), data on living conditions, environmental exposure, information on diet and health status were collected through a self-administered questionnaire, which was adapted from other epidemiological studies (Csobod et al., 2014). Additional information to assess the routine and personal habits of the study participants on the day of breath air collection was also gathered (i.e., physical exercise, time and type of meals and drinks

consumed, brushing teeth, smoking, information on health and number of hours at the workplace up to the time of sampling) via a face-to-face interview (Annex 2).

Although a total of 94 individuals were recruited; 3 subjects did not complete the questionnaire and their data were, therefore excluded. Thus, 91 adult subjects were enrolled in the current study.

### **3.2 Exhaled breath collection**

A sampling protocol was optimised (Annex 3) to collect the exhaled breath air into a sterile 1 L Tedlar<sup>®</sup> bags (E.I. du Pont de Nemours and Company, Wilmington, DE, USA; SKC, Ltd, UK) following the procedural steps detailed in Annex 4. In brief, participants were asked to breathe through their mouth while using a nose clip and through a system made up of VOCs filter, anti-pollution directional valve, connector and nozzle piece (*Figure A4-2*) that allows to direct the first portion of exhaled breath inside the gas sampling bag without any environmental contamination. After 2 minutes of tidal breathing the valve of the Tedlar<sup>®</sup> bag was closed and the connection with the collecting system was removed, practical recommendations regarding the exhaled breath collection can be seen in Annex 4. Collection of all the exhaled breath samples (*Figure A4-3*) was undertaken consecutively from 25<sup>th</sup> May to 1<sup>st</sup> June 2022 under similar indoor and outdoor air conditions. Further, it shall be noticed that to avoid any risk of cross contamination, the room where breath samples were taken was spacious (69 m<sup>2</sup>) and naturally ventilated. All precautions to maintain the safety of all participants were also considered. Further description of the sampling site is provided in Annex 6.

From the 91 study participants, 13 were invited to provide additional breath samples with a one-day gap over the course of the breath air sampling campaign for future research (results not shown).

### **3.3. Analysis of exhaled breath**

The analysis of exhaled breath was carried out by an eNose (following the procedural steps detailed in Annex 4). This instrument has been widely used in applications such as detection and identification of complex chemical mixtures that constitute aromas, odours, fragrances, formulations, spills and leaks (Sensigent, 2013a). The eNose used in the present study was the Cyranose<sup>®</sup> 320 (Sensigent, Inc., Pasadena, CA), (*Figure 3-1*). It measures changes in resistivity for each of its 32 sensors, then the information is translated into a smellprint characteristic of

the sample taken. The equipment is first trained to distinguish a series of chemical vapours or classes after establishing a digital image or "smellprint" of each vapor. When presented with a new unfamiliar sample, the device transforms it to data and compares it to the smellprints hosted in its memory (Sensigent, 2013a).



Figure 3-1- Cyranose 320<sup>®</sup> device

The measurement relies on a change in resistivity of each chemical sensor (the Cyranose<sup>®</sup> 320 has 32-sensor NoseChip<sup>™</sup>). The sensor response (SR) is measured according to *Equation 1*, where baseline resistance ( $R_0$ ) is the resistance during baseline gas flow and maximum resistance ( $R_{max}$ ) is the maximum resistance obtained during the exposure to the sample vapour (Sensigent, 2013b).

$$\text{Equation 1} \quad SR = \frac{R_{max} - R_0}{R_0}$$

The analytical settings of Cyranose<sup>®</sup> 320 were based on manufacturer recommendations (Sensigent, (2013b) with further optimisation in the context of the exhaled breath procedure and the used material. Note that for the purpose of the current study a specific parametrization procedure was defined, all details are described in Annex 3, while *Table 3-1* presents a summary of key Cyranose<sup>®</sup> 320 settings.

*Table 3-1- Summary of Cyranose® 320 settings*

| Settings                                | Time (s) | Pump Speed |
|-----------------------------------------|----------|------------|
| <b>Baseline purge:</b>                  | 30       | Medium     |
| <b>Sample draw 1:</b>                   | 30       | Medium     |
| <b>Sample draw 2:</b>                   | 0        | Medium     |
| <b>Snout removal:</b>                   | 10       | ----       |
| <b>1<sup>st</sup> Sample gas purge:</b> | 10       | High       |
| <b>1<sup>st</sup> Air intake purge:</b> | 10       | High       |
| <b>2<sup>nd</sup> Sample gas purge:</b> | 40       | High       |
| <b>2<sup>nd</sup> Air purge intake:</b> | 30       | High       |

### 3.4 Indoor environmental monitoring

To better characterise the indoor environmental conditions during the exhaled breath collection, gaseous pollutants VOCs, carbon monoxide, CO<sub>2</sub> and physical parameters, namely temperature, and relative humidity were monitored concurrently in the room as described in subchapter 3.4.2.

#### 3.4.1 VOCs

VOC samples were collected by drawing air through a stainless-steel sampling tube containing Tenax TA using a personal air sampling pump (SKC Pocket pump) at a flow rate of 0.05 L/min for a period of 4 hours. The pumps were calibrated and checked daily prior and after each sample using a Gilian Gilibrator-2 Air Flow Calibrator. Before sampling, each tube was conditioned at 250°C, 300°C and 330°C for 30 minutes consecutively in the helium carrier gas flow. So far, due time constraints, analysis of total VOCs and families of specific VOCs will be performed later in stage by automatic thermal desorption coupled with capillary gas chromatography using a Perkin Elmer ATD 400 and AutoSystem GC fitted with flame ionisation detector (FID) and an SE30 column, according to ISO 16000, part 6 (ISO, 2004), and an internal method following ECA Report 19 (Jialei, 1997).

### 3.4.2 CO<sub>2</sub>, carbon monoxide, temperature and relative humidity

CO<sub>2</sub>, carbon monoxide, temperature, and relative humidity levels were continuously recorded using 7545 IAQ-Calc Indoor Air Quality Meter (TSI, Incorporated, Shoreview, USA) with logging intervals of 5 minutes over an average of 8 hours each day. The equipment included an infrared non-dispersive sensor for CO<sub>2</sub> (range: 0-5000 ppm; accuracy:  $\pm 3\%$ ), an electrochemical sensor for CO measures (range: 0-500 ppm; accuracy:  $\pm 3.0$  ppm); an thermistor for temperature (range: 0-60 C; accuracy:  $\pm 0.6$  C) and a thin-film capacitive sensor for relative humidity (range: 5-95%; accuracy:  $\pm 3.0\%$ ). The instrument was placed approximately at the breathing height zone, i.e., 1.0 m above the floor. Prior to the monitoring the equipment was calibrated at the manufacturer. The overall statistics are summarised in *Table 3-2*.

*Table 3-2- Data collected from monitoring indoor air quality assessment*

| Day                          |         | May 25 <sup>th</sup> | May 26 <sup>th</sup> | May 27 <sup>th</sup> | May 30 <sup>th</sup> | May 31 <sup>st</sup> | June 1 <sup>st</sup> |
|------------------------------|---------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| <b>CO<sub>2</sub> (ppm)</b>  | Average | 798                  | 808                  | 647                  | 564                  | 475                  | 675                  |
|                              | Maximum | 1009                 | 928                  | 806                  | 722                  | 606                  | 913                  |
|                              | Minimum | 489                  | 509                  | 499                  | 435                  | 421                  | 470                  |
| <b>Temperature (°C)</b>      | Average | 22.0                 | 23.2                 | 24.1                 | 24.5                 | 26.1                 | 24.1                 |
|                              | Maximum | 20.2                 | 24.3                 | 23.3                 | 25.1                 | 26.4                 | 24.4                 |
|                              | Minimum | 23.0                 | 21.7                 | 24.8                 | 24.0                 | 25.5                 | 23.4                 |
| <b>Relative humidity (%)</b> | Average | 48.3                 | 46.2                 | 39.2                 | 50.8                 | 53.8                 | 56.9                 |
|                              | Maximum | 51.3                 | 49.0                 | 37.4                 | 53.2                 | 50.4                 | 60.0                 |
|                              | Minimum | 46.5                 | 44.5                 | 40.4                 | 48.5                 | 56.5                 | 54.8                 |
| <b>CO (ppm)</b>              | Average | 0.2                  | 0.2                  | 0.5                  | 0.1                  | 0.1                  | 0.4                  |
|                              | Maximum | 0.6                  | 0.4                  | 1.2                  | 0.5                  | 0.5                  | 0.8                  |
|                              | Minimum | <0.1                 | <0.1                 | <0.1                 | <0.1                 | <0.1                 | <0.1                 |

### 3.5 Outdoor environmental monitoring

In order to provide an overall outdoor environmental characterization between different days of exhaled breath collection, levels of PM<sub>2.5</sub>, PM<sub>10</sub>, NO<sub>2</sub> and O<sub>3</sub> (over the period of sampling) was also retrieved from national monitoring network (operated under Portuguese Environmental Agency) for "Sobreiras-Lordelo do Ouro" (defined as urban background) and "Francisco Sá Carneiro-Campanhã (urban traffic)", as those that are the two only stations available in the city of Porto (QualAR, 2022).

The overall statistics (based on 24 hours registering) is summarised in Annex 6a. Conclusions concerning the air quality, can be found in Annex 6b.

### **3.6 Statistical analysis**

The main characteristics of the study population were expressed as means, standard deviations, minimum and maximum values, or as counts and percentages.

For the purposes of this work body mass index (BMI) was calculated on the basis of height and weight, was used to categorize subjects as: “underweight” ( $<18.5 \text{ kg/m}^2$ ), “normal weight” ( $18.5\text{-}24.9 \text{ kg/m}^2$ ), “overweight” ( $>25.0 \text{ kg/m}^2$ ), according WHO approach (Organization, 2010).

All hypotheses tested were conducted assuming a 0.05 significance level. Spearman correlation was used to evaluate relationships involving ordinal variables. All targeted statistical analyses were performed using Statistical Package for the Social Sciences, version 27.0.1 (SPSS Inc., Chicago, IL, USA).

Resistance measurements from the 32 sensors, for all samples were imported from the Cyranose<sup>®</sup> 320 to the software Pcnose+<sup>®</sup> (provided by the manufacturer with the equipment). Subsequently the raw data was exported to an excel database as a .CSV file (the procedure for data extraction and export is shown in Annex 4). Cluster analysis was performed using the software R *Studio* version 4.1.2 (R Foundation, Vienna, Austria) and MultyVariomics version 1.11 (João Rufo, Porto, Portugal) as presented in Annex 7. In total 97 variables were explored and evaluated for each individual, represented in Annex 7.

## 4. Results and discussion

### 4.1 Characteristics of study participants

In total, 91 adult subjects were enrolled in this cross-sectional study. A general characterisation of the study population is presented in *Table 4-1* (non-stratified characteristics).

Male gender was more prevalent (57.1% males vs 42.9% female) and the age of subjects ranged between 21.9 and 74.3 years. The mean BMI was  $24.4 \pm 3.7$  kg/m<sup>2</sup> indicating an overall majority of the participants with “normal weight” according to the WHO categorization (Organization, 2010).

The majority of participants had Portuguese nationality (80.2%), most of the study participants (>59%) were researchers or students. Within their occupational responsibilities, 30.8% of the study population was exposed to solvents, 18.7% to airborne particles and 12.1% to microbiological hazards.

Current smokers represented 23.1% of the participants and 11.0% were former smokers. According to the questionnaires, out of the 91 participants, 54.9% self-reported that they were exposed to ETS in leisure places, a percentage that decreased in the workplace (37.4%) and at home (24.2%). In this regard, information provided in literature is often incomplete, but it is still evident that criteria used to define ETS exposure is repeatedly different among studies, both in terms of frequency and dose (times/week, hours/week (Moore et al., 2019), and

considered exposure sources (at work, at home or in leisure time outside the home, or based on smoking habits of close relatives and co-workers (Sunyer et al., 2012).

Finally, 8.8% of the study population reported having respiratory problems while 56% had been infected by SARS-CoV-2.

Table 4-1- General characteristics of the study population (n= 91)

|                                                         |                                 | Total<br>n (%)          |
|---------------------------------------------------------|---------------------------------|-------------------------|
| <b>Sociodemographic information:</b>                    |                                 |                         |
| Gender                                                  | Female                          | 39 (42.9%)              |
|                                                         | Male                            | 52 (57.1%)              |
| Age <sup>1</sup> (years)                                |                                 | 37.5 ± 12.1 (21.9–74.3) |
| BMI <sup>1</sup> (kg/m <sup>2</sup> )                   |                                 | 24.4 ± 3.7 (17.9–39.7)  |
| Nationality                                             | Portuguese                      | 73 (80.2%)              |
|                                                         | Other                           | 18 (19.8%)              |
| Working status:                                         | Professor                       | 11 (12.1%)              |
|                                                         | Researcher                      | 25 (27.5%)              |
|                                                         | Lab technician                  | 8 (8.8%)                |
|                                                         | Administrative                  | 8 (8.8%)                |
|                                                         | Student                         | 29 (31.9%)              |
|                                                         | Other                           | 10 (11.0%)              |
| Number of years at university <sup>1</sup>              |                                 | 12.5 ± 10.0 (0–46)      |
| Daily time spent in university (hours/day) <sup>1</sup> |                                 | 7.5 ± 2.1 (0–11)        |
| Current occupational exposures                          | Solvents                        | 28 (30.8%)              |
|                                                         | Airborne particles              | 17 (18.7%)              |
|                                                         | Microbiological hazards         | 11 (12.1%)              |
|                                                         | Radiation                       | 2 (2.2%)                |
|                                                         | Other                           | 10 (11.0%)              |
| Past occupational exposures                             | Yes                             | 17 (18.7%)              |
|                                                         | No                              | 74 (81.3%)              |
| <b>Environmental exposure:</b>                          |                                 |                         |
| Pollution sources in the neighbourhood (~500 m)         | Factory                         | 11 (12.1%)              |
|                                                         | Landfill                        | 2 (2.2%)                |
|                                                         | Industrial area                 | 11 (12.1%)              |
| Scented candles/incense use                             | Yes                             | 39 (42.9%)              |
|                                                         | No                              | 52 (57.1%)              |
| Environmental tobacco smoke (home)                      | Yes                             | 22 (24.2%)              |
|                                                         | No                              | 68 (74.7%)              |
|                                                         | Missing values                  | 1 (1.1%)                |
| Environmental tobacco smoke (workplace)                 | Yes                             | 34 (37.4%)              |
|                                                         | No                              | 56 (61.5%)              |
|                                                         | Missing values                  | 1 (1.1%)                |
| Environmental tobacco smoke (leisure)                   | Yes                             | 50 (54.9%)              |
|                                                         | No                              | 40 (44.0%)              |
|                                                         | Missing values                  | 1 (1.1%)                |
| <b>Lifestyle behaviour:</b>                             |                                 |                         |
| Smoking habits                                          | Never smoker                    | 60 (65.9%)              |
|                                                         | Former smoker                   | 10 (11.0%)              |
|                                                         | Current smoker                  | 21 (23.1%)              |
|                                                         | Nº cigarettes/week <sup>1</sup> | 17.3 ± 40.8 (0–140)     |
| Coffee consumption                                      | Yes                             | 67 (73.6%)              |

|                                     |                |            |
|-------------------------------------|----------------|------------|
|                                     | No             | 23 (25.3%) |
|                                     | Missing values | 1 (1.1%)   |
| <b>Self-reported health status:</b> |                |            |
| Neoplasms                           | Yes            | 2 (2.2%)   |
|                                     | No             | 89 (97.8%) |
| Respiratory problems                | Yes            | 8 (8.8%)   |
|                                     | No             | 83 (91.2%) |
| Cardiac problems                    | Yes            | 4 (4.4%)   |
|                                     | No             | 87 (95.6%) |
| SARS-CoV-2 infection                | Yes            | 51 (56.0%) |
|                                     | No             | 40 (44.0%) |

<sup>1</sup> data reported as mean  $\pm$  standard deviation (minimum-maximum)

Subject characteristics according to their smoking status were also studied and are presented in *Table 4-2*. No statistically significant differences ( $p < 0.05$ ) were observed in all variables among non-smokers (i.e., never and former smokers) and current smoker participants; non-smokers were though more likely to be younger, and with high educational attainment in line with previous studies also using self-reported data (Tomioka et al., 2020). In addition, smokers were more likely to be underweight which is consistent with the findings reported by other authors (Wang, 2015).

*Table 4-2- General characterisation of study population (n=91) stratified by smoking habits*

|                                                            |                    | Non-smoker<br>(n=70)           | Current smoker<br>(n=21)       | Total<br>(n=91)                |
|------------------------------------------------------------|--------------------|--------------------------------|--------------------------------|--------------------------------|
| n (%)                                                      |                    |                                |                                |                                |
| <b>Sociodemographic information:</b>                       |                    |                                |                                |                                |
| Gender                                                     | Female             | 28 (40.0%)                     | 11 (52.4%)                     | 39 (42.9%)                     |
|                                                            | Male               | 42 (60.0%)                     | 10 (47.6%)                     | 52 (57.1%)                     |
| Age <sup>1</sup> (years)                                   |                    | 36.8 $\pm$ 11.8<br>(21.0–74.3) | 40.2 $\pm$ 13.0<br>(22.4–65.4) | 37.5 $\pm$ 12.1<br>(21.9–74.3) |
| BMI <sup>1</sup> (kg/m <sup>2</sup> )                      |                    | 24.5 $\pm$ 3.9<br>(17.9–39.7)  | 23.9 $\pm$ 3.3<br>(19.4–33.4)  | 24.4 $\pm$ 3.7<br>(17.9–39.7)  |
| Nationality                                                | Portuguese         | 56 (80.0%)                     | 17 (81.0%)                     | 73 (80.2%)                     |
|                                                            | Other              | 14 (20.0%)                     | 4 (19.0%)                      | 18 (19.8%)                     |
| Working status:                                            | Professor          | 9 (12.9%)                      | 2 (9.5%)                       | 11 (12.1%)                     |
|                                                            | Researcher         | 20 (28.6%)                     | 5 (23.8%)                      | 25 (27.5%)                     |
|                                                            | Lab technician     | 8 (11.4%)                      | 0 (0.0%)                       | 8 (8.8%)                       |
|                                                            | Administrative     | 5 (7.1%)                       | 3 (14.3%)                      | 8 (8.8%)                       |
|                                                            | Student            | 24 (34.3%)                     | 5 (23.8%)                      | 29 (31.9%)                     |
|                                                            | Other              | 4 (5.7%)                       | 6 (28.6%)                      | 10 (11.0%)                     |
| Number of years at university <sup>1</sup>                 |                    | 11.8 $\pm$ 10.0<br>(0.1–46)    | 15.2 $\pm$ 13.7<br>(0–43)      | 12.5 $\pm$ 10.0<br>(0–46)      |
| Daily time spent in university <sup>1</sup><br>(hours/day) |                    | 7.6 $\pm$ 1.8<br>(1–11)        | 7.3 $\pm$ 2.9<br>(0–10)        | 7.5 $\pm$ 2.1<br>(0–11)        |
|                                                            | Solvents           | 25 (35.7%)                     | 3 (14.3%)                      | 28 (30.8%)                     |
|                                                            | Airborne particles | 12 (17.1%)                     | 5 (23.8%)                      | 17 (18.7%)                     |

|                                               |                         |            |            |            |
|-----------------------------------------------|-------------------------|------------|------------|------------|
| Current occupational exposures                | Microbiological hazards | 9 (12.9%)  | 2 (9.5%)   | 11 (12.1%) |
|                                               | Radiation               | 1 (1.4%)   | 1 (4.8%)   | 2 (2.2%)   |
|                                               | Other                   | 6 (8.6%)   | 4 (19.0%)  | 10 (11.0%) |
| Past occupational exposures                   | Yes                     | 14 (20.0%) | 3 (14.3%)  | 17 (18.7%) |
|                                               | No                      | 56 (80.0%) | 18 (85.7%) | 74 (81.3%) |
| <b>Environmental exposure:</b>                |                         |            |            |            |
| Pollution sources in the neighbourhood (~500) | Factory                 | 9 (12.9%)  | 2 (9.5%)   | 11 (12.1%) |
|                                               | Landfill                | 1 (1.4%)   | 1 (4.8%)   | 2 (2.2%)   |
|                                               | Industrial area         | 9 (12.9%)  | 2 (9.5%)   | 11 (12.1%) |
| Scented candles/incense use                   | Yes                     | 29 (41.4%) | 10 (47.6%) | 39 (42.9%) |
|                                               | No                      | 41 (58.6%) | 11 (52.4%) | 52 (57.1%) |
| Environmental tobacco smoke (home)            | Yes                     | 11 (15.7%) | 11 (52.4%) | 22 (24.2%) |
|                                               | No                      | 58 (82.9%) | 10 (47.6%) | 68 (74.7%) |
|                                               | Missing values          | 1 (1.4%)   | -          | 1 (1.1%)   |
| Environmental tobacco smoke (workplace)       | Yes                     | 26 (37.1%) | 8 (38.1%)  | 34 (37.4%) |
|                                               | No                      | 43 (61.4%) | 13 (61.9%) | 55 (60.4%) |
|                                               | Missing values          | 1 (1.4%)   | -          | 1 (1.1%)   |
| Environmental tobacco smoke (leisure)         | Yes                     | 36 (51.4%) | 14 (66.7%) | 50 (54.9%) |
|                                               | No                      | 33 (47.1%) | 7 (33.3%)  | 40 (44.0%) |
|                                               | Missing values          | 1 (1.4%)   | -          | 1 (1.1%)   |
| <b>Lifestyle behaviour:</b>                   |                         |            |            |            |
| Coffee consumption                            | Yes                     | 48 (68.6%) | 19 (90.5%) | 67 (73.6%) |
|                                               | No                      | 22 (31.4%) | 1 (4.8%)   | 23 (25.3%) |
|                                               | Missing values          | -          | 1 (4.8%)   | 1 (1.1%)   |
| <b>Self-reported health status:</b>           |                         |            |            |            |
| Neoplasms                                     | Yes                     | 1 (1.4%)   | 1 (4.8%)   | 2 (2.2%)   |
|                                               | No                      | 69 (98.6%) | 20 (95.2%) | 89 (97.8%) |
| Respiratory problems                          | Yes                     | 7 (10.0%)  | 1 (4.8%)   | 8 (8.8%)   |
|                                               | No                      | 63 (90.0%) | 20 (95.2%) | 83 (91.2%) |
| Cardiac problems                              | Yes                     | 3 (4.3%)   | 1 (4.8%)   | 4 (4.4%)   |
|                                               | No                      | 67 (95.7%) | 20 (95.2%) | 87 (95.6%) |
| SARS-CoV-2 infection                          | Yes                     | 42 (60.0%) | 9 (42.9%)  | 51 (56.0%) |
|                                               | No                      | 28 (40.0%) | 12 (57.1%) | 40 (44.0%) |

<sup>1</sup> data reported as mean  $\pm$  standard deviation (minimum-maximum)

The characterisation of the population stratified by gender is presented in *Table 4-3*. There was no difference between two gender groups, except for BMI. Furthermore, the results indicate that for female subjects, the obtained mean ( $23.5 \pm 3.9 \text{ kg/m}^2$ ) was within the “normal weight” range ( $18.5\text{-}24.9 \text{ kg/m}^2$ ); while male population was classified as “overweight” due to the obtained mean BMI ( $25.0 \pm 3.5 \text{ kg/m}^2$ ), possibly indicating starting obesity for the respective study subgroup.

Table 4-3- General characterisation of study population (n=91) stratified by gender

|                                                         |                         | Female<br>(n=39)           | Male<br>(n=52)             | Total<br>(n=91)            |
|---------------------------------------------------------|-------------------------|----------------------------|----------------------------|----------------------------|
| n (%)                                                   |                         |                            |                            |                            |
| Sociodemographic information:                           |                         |                            |                            |                            |
| Age <sup>1</sup> (years)                                |                         | 38.1 ± 11.4<br>(21.9–66.6) | 37.2 ± 12.7<br>(21.9–74.3) | 37.5 ± 12.1<br>(21.9–74.3) |
| BMI <sup>1</sup> (kg/m <sup>2</sup> )                   |                         | 23.5 ± 3.9<br>(19.0–39.7)* | 25.0 ± 3.5<br>(17.9–33.9)* | 24.4 ± 3.7<br>(17.9–39.7)  |
| Nationality                                             | Portuguese              | 35 (89.7%)                 | 38 (73.1%)                 | 73 (80.2%)                 |
|                                                         | Other                   | 4 (10.3%)                  | 14 (26.9%)                 | 18 (19.8%)                 |
| Working status:                                         | Professor               | 2 (5.1%)                   | 9 (17.3%)                  | 11 (12.1%)                 |
|                                                         | Researcher              | 11 (28.2%)                 | 14 (26.9%)                 | 25 (27.5%)                 |
|                                                         | Lab Technician          | 5 (12.8%)                  | 3 (5.8%)                   | 8 (8.8%)                   |
|                                                         | Administrative          | 6 (15.6%)                  | 2 (3.8%)                   | 8 (8.8%)                   |
|                                                         | Student                 | 12 (30.8%)                 | 17 (32.7%)                 | 29 (31.9%)                 |
|                                                         | Other                   | 3 (7.7%)                   | 7 (13.5%)                  | 10 (11.0%)                 |
| Number of years at university <sup>1</sup>              |                         | 12.9 ± 8.7<br>(2–36.3)     | 12.2 ± 10.8<br>(0–46)      | 12.5 ± 10.0<br>(0–46)      |
| Daily time spent in university <sup>1</sup> (hours/day) |                         | 7.9 ± 2.0<br>(1–11)        | 7.3 ± 2.2<br>(0–10)        | 7.5 ± 2.1<br>(0–11)        |
| Current occupational exposures                          | Solvents                | 15 (38.5%)                 | 13 (25.0%)                 | 28 (30.8%)                 |
|                                                         | Airborne particles      | 9 (23.1%)                  | 8 (15.4%)                  | 17 (18.7%)                 |
|                                                         | Microbiological hazards | 6 (15.44%)                 | 5 (9.6%)                   | 11 (12.1%)                 |
|                                                         | Radiation               | 2 (5.1%)                   | -                          | 2 (2.2%)                   |
|                                                         | Other                   | 5 (12.8%)                  | 5 (9.6%)                   | 10 (11.0%)                 |
| Past exposures                                          | Yes                     | 7 (17.9%)                  | 10 (19.2%)                 | 17 (18.7%)                 |
|                                                         | No                      | 32 (82.1%)                 | 42 (80.8%)                 | 74 (81.3%)                 |
| Environmental exposure:                                 |                         |                            |                            |                            |
| Pollution sources neighbourhood (~500)                  | Factory                 | 4 (10.3%)                  | 7 (13.5%)                  | 11 (12.1%)                 |
|                                                         | Landfill                | 1 (2.6%)                   | 1 (1.9%)                   | 2 (2.2%)                   |
|                                                         | Industrial area         | 3 (7.7%)                   | 8 (15.4%)                  | 11 (12.1%)                 |
| Scented candles/incense use                             | Yes                     | 25 (64.1%)                 | 14 (26.9%)                 | 39 (42.9%)                 |
|                                                         | No                      | 14 (35.9%)                 | 38 (73.1%)                 | 52 (57.1%)                 |
| Environmental tobacco smoke (home)                      | Yes                     | 10 (25.6%)                 | 12 (23.1%)                 | 22 (24.2%)                 |
|                                                         | No                      | 29 (74.4%)                 | 39 (75.0%)                 | 68 (74.7%)                 |
|                                                         | Missing values          | -                          | 1 (1.9%)                   | 1 (1.1%)                   |
| Environmental tobacco smoke (workplace)                 | Yes                     | 11 (28.2%)                 | 23 (44.2%)                 | 34 (37.4%)                 |
|                                                         | No                      | 28 (71.8%)                 | 28 (53.8%)                 | 55 (60.4%)                 |
|                                                         | Missing values          | -                          | 1 (1.9%)                   | 1 (1.1%)                   |
| Environmental tobacco smoke (leisure)                   | Yes                     | 25 (64.1%)                 | 25 (48.1%)                 | 50 (54.9%)                 |
|                                                         | No                      | 14 (35.9%)                 | 26 (50.0%)                 | 40 (44.0%)                 |
|                                                         | Missing values          | -                          | 1 (1.9%)                   | 1 (1.1%)                   |
| Lifestyle characterisation:                             |                         |                            |                            |                            |
| Smoking habits                                          | Never smoked            | 25 (64.1%)                 | 35 (67.3%)                 | 60 (65.9%)                 |
|                                                         | Former smoker           | 3 (7.7%)                   | 7 (13.5%)                  | 10 (11.0%)                 |
|                                                         | Current smoker          | 11 (28.2%)                 | 10 (19.2%)                 | 21 (23.1%)                 |
| Nº cigarettes/week <sup>1</sup>                         |                         | 21.0 ± 43.9<br>(0-140)     | 14.5 ± 38.5<br>(0-140)     | 17.3 ± 40.8<br>(0-140)     |
| Coffee consumption                                      | Yes                     | 31 (79.5%)                 | 36 (69.2%)                 | 67 (73.6%)                 |
|                                                         | No                      | 7 (17.9%)                  | 16 (30.8%)                 | 23 (25.3%)                 |
|                                                         | Missing values          | 1 (2.6%)                   | -                          | 1 (1.1%)                   |
| Medical status:                                         |                         |                            |                            |                            |
| Neoplasms                                               | Yes                     | 2 (5.1%)                   | -                          | 2 (2.2%)                   |
|                                                         | No                      | 37 (94.9%)                 | 52 (100%)                  | 89 (97.8%)                 |
|                                                         | Yes                     | 1 (2.6%)                   | 7 (13.5%)                  | 8 (8.8%)                   |

|                      |     |            |            |            |
|----------------------|-----|------------|------------|------------|
| Respiratory problems | No  | 38 (97.4%) | 45 (86.5%) | 83 (91.2%) |
| Cardiac problems     | Yes | 2 (5.1%)   | 2 (3.8%)   | 4 (4.4%)   |
|                      | No  | 37 (94.9%) | 50 (96.2%) | 87 (95.6%) |
| SARS-CoV-2 infection | Yes | 20 (51.3%) | 31 (59.6%) | 51 (56.0%) |
|                      | No  | 19 (48.7%) | 21 (40.4%) | 40 (44.0%) |

\*statistically significant difference at  $p \leq 0.05$ ; <sup>1</sup>data reported as mean  $\pm$  standard deviation (minimum-maximum)

Table 4-4 summarised key personal habits of the study population (such as physical activity, teeth cleaning, smoking, intake of food, liquids, or medicine consumption) on the day prior to exhaled breath sample collection.

The results have shown that 97% of the participants ingested food or liquids (water being the most frequent drink-71.4%) prior (1.7-2.8 hours) to the exhaled breath collection or cleaned their teeth independently of the hour of the sample collection.

Table 4-4- Personal habits, food and liquids ingestion of the study population (n=91) prior to the exhaled breath collection

|                                                                                            |                       | Total<br>(n=91)          |
|--------------------------------------------------------------------------------------------|-----------------------|--------------------------|
| <b>Sampling:</b>                                                                           |                       |                          |
| Day period                                                                                 | Morning               | 38 (41.8%)               |
|                                                                                            | Afternoon (>12:00 pm) | 53 (58.2%)               |
| <b>Food prior to the exhaled breath collection on the day:</b>                             |                       |                          |
| Meal                                                                                       | Yes                   | 88 (96.7%)               |
|                                                                                            | No                    | 3 (3.3%)                 |
| Duration between last meal and exhaled breath collection <sup>1</sup> (hours)              |                       | 2.9 $\pm$ 2.2 (0.2-13.8) |
| <b>Drinks prior to the exhaled breath collection on day:</b>                               |                       |                          |
| Water                                                                                      | Yes                   | 65 (71.4%)               |
|                                                                                            | No                    | 26 (28.6%)               |
| Duration between water intake and exhaled breath collection <sup>1</sup> (hours)           |                       | 1.7 $\pm$ 1.9 (0-10.5)   |
| Coffee                                                                                     | Yes                   | 55 (60.4%)               |
|                                                                                            | No                    | 36 (39.6%)               |
| Duration between coffee ingestion and exhaled breath collection <sup>1</sup> (hours)       |                       | 3.3 $\pm$ 2.3 (0-10.1)   |
| Tea                                                                                        | Yes                   | 7 (7.7%)                 |
|                                                                                            | No                    | 84 (92.3%)               |
| Duration between tea ingestion and exhaled breath collection <sup>1</sup> (hours)          |                       | 4.0 $\pm$ 2.4 (1.7-7.7)  |
| Caffeinated soft drink                                                                     | Yes                   | 3 (3.3%)                 |
|                                                                                            | No                    | 88 (96.7%)               |
| Duration between caffeinated soft drink and exhaled breath collection <sup>1</sup> (hours) |                       | 3.4 $\pm$ 1.0 (2.7-4.6)  |
| Alcoholic drink                                                                            | Yes                   | 2 (2.2%)                 |
|                                                                                            | No                    | 89 (97.8%)               |

|                                                                                       |     |                      |
|---------------------------------------------------------------------------------------|-----|----------------------|
| Duration between alcoholic drink and exhaled breath collection <sup>1</sup> (hours)   |     | 2.8 ± 0.1 (2.7-2.9)  |
| Other drink                                                                           | Yes | 25 (27.5%)           |
|                                                                                       | No  | 66 (72.5%)           |
| Duration between a drink ingestion and exhaled breath collection <sup>1</sup> (hours) |     | 3.5 ± 2.3 (0.6-8.7)  |
| <b>Other daily routines in sampling day:</b>                                          |     |                      |
| Tooth brushing                                                                        | Yes | 88 (96.7%)           |
|                                                                                       | No  | 3 (3.3%)             |
| Duration between tooth brushing and exhaled breath collection <sup>1</sup> (hours)    |     | 5.1 ± 3.0 (0.9-14.2) |
| Smoke                                                                                 | Yes | 16 (17.6%)           |
|                                                                                       | No  | 75 (82.4%)           |
| Duration between smoke and exhaled breath collection <sup>1</sup> (hours)             |     | 1.2 ± 0.7 (0.2-2.2)  |
| <b>Health-related data:</b>                                                           |     |                      |
| Intake of medication on the day                                                       | Yes | 32 (35.2%)           |
|                                                                                       | No  | 59 (64.8%)           |
| Feeling sick on the day                                                               | Yes | 7 (7.7%)             |
|                                                                                       | No  | 84 (92.3%)           |
| <b>Occupational exposure:</b>                                                         |     |                      |
| Time spent at work on the day <sup>1</sup>                                            |     | 3.9 ± 2.6 (0-10.0)   |

<sup>1</sup> data reported as mean ± standard deviation (minimum-maximum)

The summary of Spearman correlation between continuous variables is summarised in *Table 4-5*. The obtained results have shown a statistically significant ( $p < 0.05$ ) and positive correlation between the subject age and BMI. These findings suggest an increase of BMI throughout the life of the study population, probably due to the adoption of a less active life with age. However, when stratified by gender (*Table 4-6*), significant (positive) correlation between age and BMI was obtained among the male subjects only.

*Table 4-5- Spearman correlations between continuous variables*

|                 | BMI (kg/m <sup>2</sup> ) |       |              | Coffee consumption (cups/day) |      |                   |
|-----------------|--------------------------|-------|--------------|-------------------------------|------|-------------------|
|                 | n                        | r     | p            | n                             | r    | p                 |
| Age (years)     | 91                       | 0.24  | <b>0.02*</b> | 90                            | 0.11 | 0.28              |
| Cigarettes/week | 89                       | -0.03 | 0.72         | 88                            | 0.39 | <b>&lt;0.001*</b> |

\* $p < 0.05$ ; bold values are statistically significant; n = number of subjects; r = Spearman coefficient; BMI: Body Mass Index

Concerning the personal habits, positive correlation was obtained between coffee consumption and smoking (*Table 4-5*), thus indicating the associations between these two habits as has been reported by several authors (Bjørngaard et al., 2017). Further, the correlation remained statistically significant even when stratified by gender (*Table 4-6*), both for women and men participants.

Table 4-6- Spearman correlations stratified by gender

|                 | Female                   |       |      |                               |      |              | Male                     |      |              |                               |      |                  |
|-----------------|--------------------------|-------|------|-------------------------------|------|--------------|--------------------------|------|--------------|-------------------------------|------|------------------|
|                 | BMI (kg/m <sup>2</sup> ) |       |      | Coffee consumption (cups/day) |      |              | BMI (kg/m <sup>2</sup> ) |      |              | Coffee consumption (cups/day) |      |                  |
|                 | n                        | r     | p    | n                             | r    | p            | n                        | r    | p            | n                             | r    | p                |
| Age (years)     | 39                       | 0.26  | 0.11 | 38                            | 0.17 | 0.31         | 52                       | 0.31 | <b>0.03*</b> | 52                            | 0.08 | 0.59             |
| Cigarettes/week | 38                       | -0.06 | 0.74 | 37                            | 0.36 | <b>0.03*</b> | 51                       | 0.06 | 0.64         | 51                            | 0.43 | <b>&lt;0.01*</b> |

\*p<0.05; bold values are statistically significant; n = number of subjects; r = Spearman coefficient; BMI: Body Mass Index

## 4.2 Hierarchical cluster analysis

Cluster analysis was made to verify how volatile patterns can be divided out using standard resistivity values from the 32 sensors. Since Hopkins value is close to the unit (0.88), this data was considered as clusterable.

The heatmap (*Figure 4-2*) represents the contribution of the 32 sensors to both clusters (sensor values in z-scores). Red colour represents positive correlations while blue colours represent inverse correlations. It is possible to observe in the bar plot that the number of hours since last water intake is generally higher in cluster 1. This suggests that water intake, by increasing the humidity in exhaled air, has a notable effect on the majority of sensors, with the exception of sensor 8, which does not follow the same tendency.

Average silhouette width methods confirmed the tendency of data to form two clusters ( $K=2$ ) (Figure 4-1).

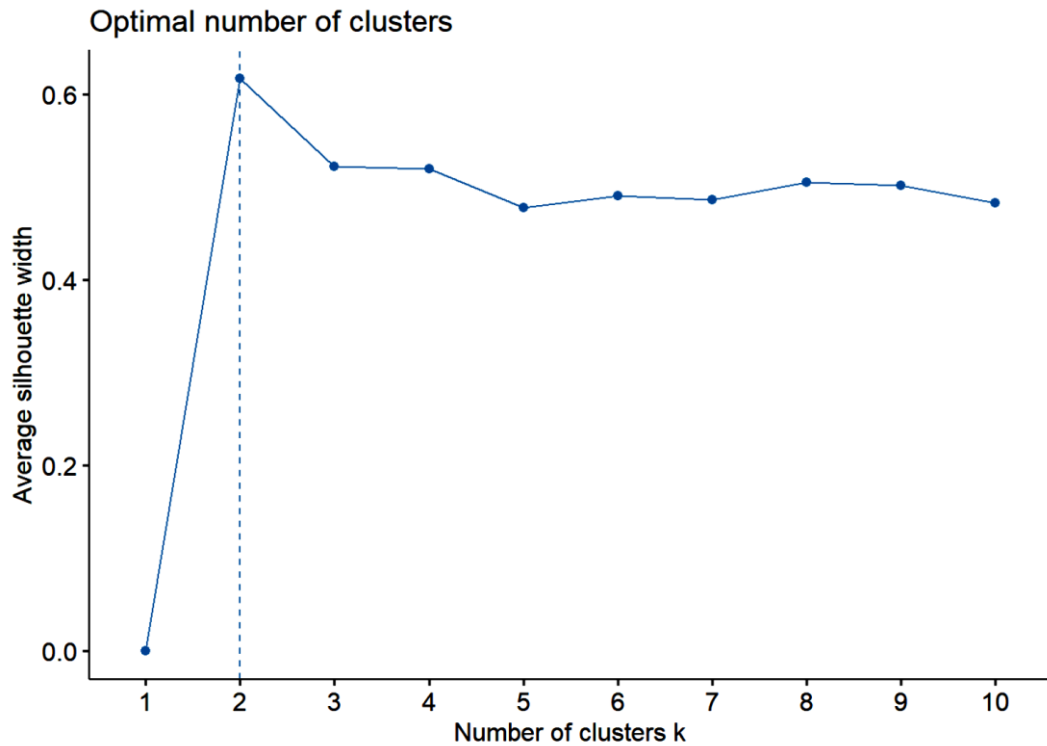


Figure 4-1- Average silhouette width to assess the optimal number of clusters,  $K=2$

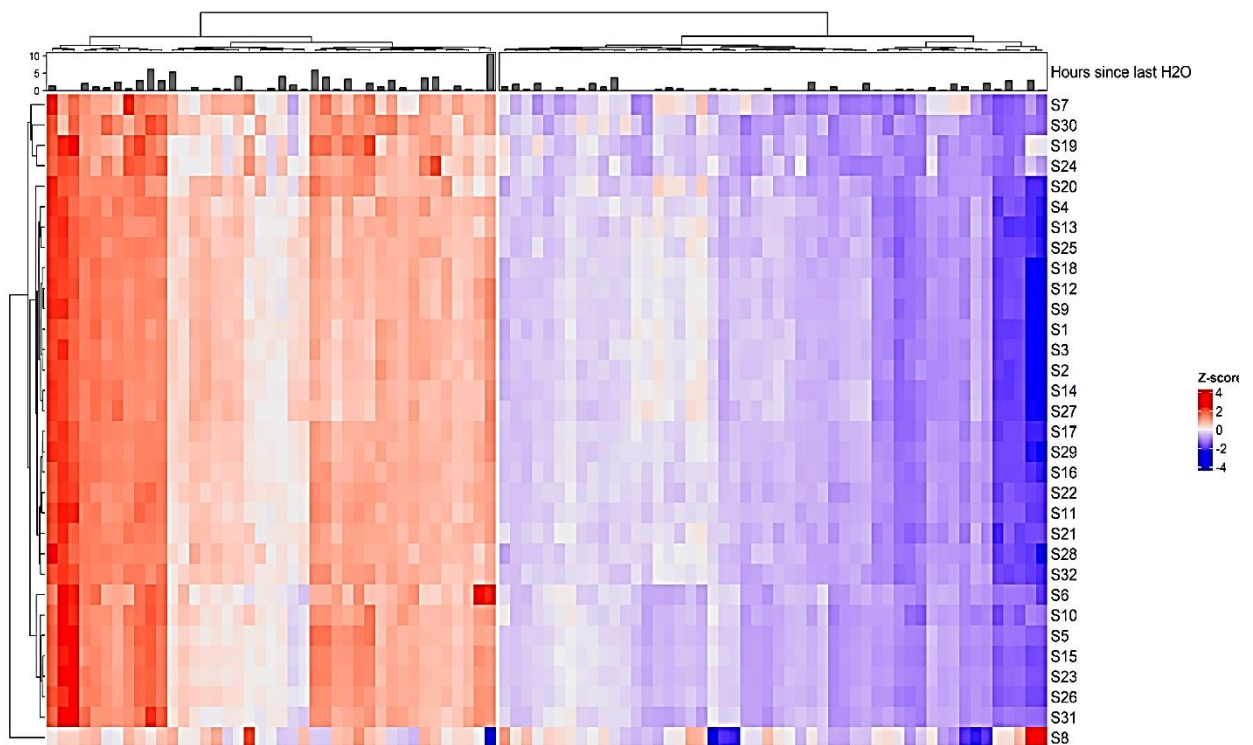


Figure 4-2- Heat map

The hierarchical cluster analysis dendrogram volatile profiles is represented in *Figure 4-3*.

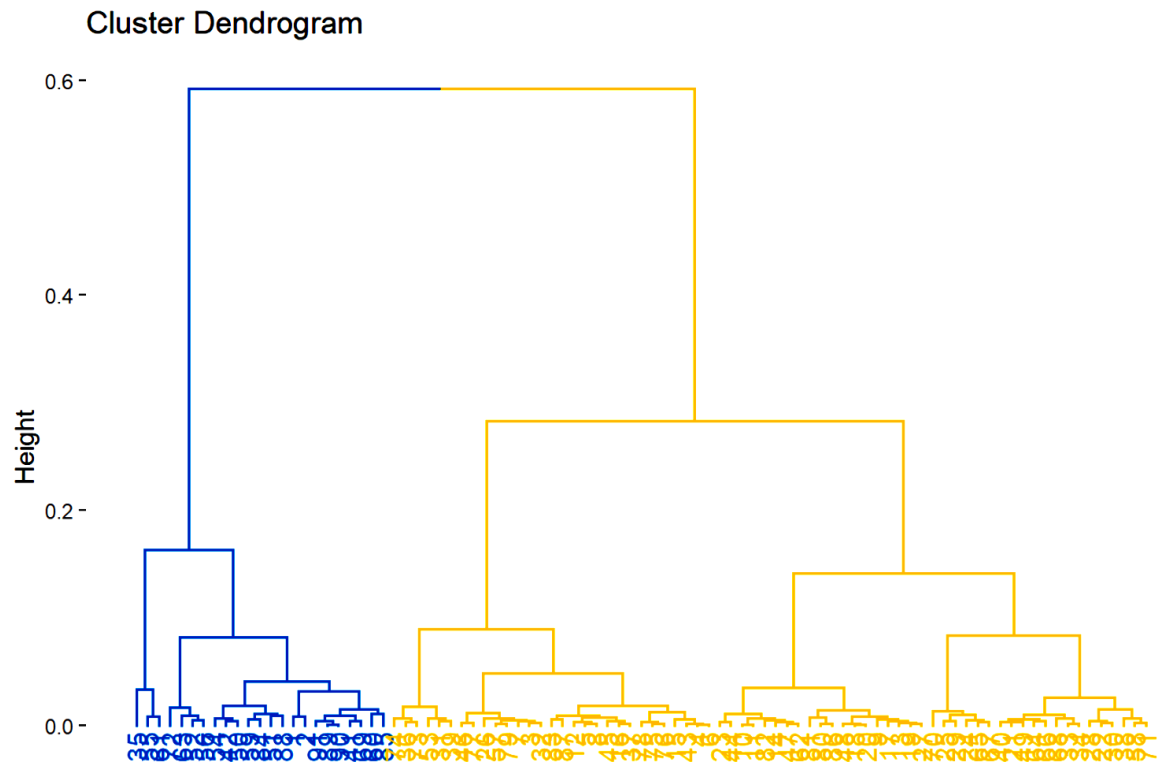


*Figure 4-3- Ward's minimum variance hierarchical clustering model. Yellow: cluster 1; Blue: cluster 2*

Accordingly, characteristics of the two clusters formed through the hierarchical model are represented in *Table 4-7*. Indeed, the two clusters were distinctly separated, but significant differences between the two clusters ( $p < 0.05$ ) were identified for drinks consumption, namely water prior to exhaled breath test.

*Table 4-7- Differences between the hierarchical model clusters according to participants' characteristics*

|                                              |                   | Cluster 1                        | Cluster 2                        | p     |
|----------------------------------------------|-------------------|----------------------------------|----------------------------------|-------|
|                                              |                   | n=23                             | n=68                             |       |
| Gender                                       | Male (%)          | 43.5                             | 42.6                             | 0.944 |
|                                              | Female (%)        | 56.5                             | 57.4                             |       |
| Age (years, mean $\pm$ SD (minimum-maximum)) |                   | 35.9 $\pm$ 10.4<br>(22.5 – 63.1) | 38.1 $\pm$ 12.6<br>(21.9 – 74.3) | 0.456 |
| BMI (kg/m <sup>2</sup> )                     | Under-weight (%)  | 0.0                              | 1.5                              | 0.705 |
|                                              | Normal-weight (%) | 69.6                             | 61.8                             |       |
|                                              | Over-weight (%)   | 30.4                             | 36.8                             |       |
| Past occupational exposure                   | No (%)            | 82.6                             | 80.9                             | 0.854 |
|                                              | Yes (%)           | 17.4                             | 19.1                             |       |
| Current occupational exposure                | No (%)            | 91.3                             | 75.0                             | 0.096 |
|                                              | Yes (%)           | 8.7                              | 25.0                             |       |
| Exposure to solvents                         | No (%)            | 69.6                             | 69.1                             | 0.968 |
|                                              | Yes (%)           | 30.4                             | 30.9                             |       |
| Exposure to airborne particles               | No (%)            | 69.6                             | 85.3                             | 0.094 |
|                                              | Yes (%)           | 30.4                             | 14.7                             |       |

|                                                                        |                    |                     |                     |               |
|------------------------------------------------------------------------|--------------------|---------------------|---------------------|---------------|
| Exposure to microbiological hazardous                                  | No (%)             | 87.0                | 88.2                | 0.871         |
|                                                                        | Yes (%)            | 13.0                | 11.8                |               |
| Exposure to radiation                                                  | No (%)             | 95.7                | 98.5                | 0.416         |
|                                                                        | Yes (%)            | 4.3                 | 1.5                 |               |
| Exposure to other environmental risks                                  | No (%)             | 91.3                | 88.2                | 0.684         |
|                                                                        | Yes (%)            | 8.7                 | 11.8                |               |
| Current smoker                                                         | No (%)             | 73.9                | 77.9                | 0.692         |
|                                                                        | Yes (%)            | 26.1                | 22.1                |               |
| Ever smoked                                                            | No (%)             | 65.2                | 64.7                | 0.965         |
|                                                                        | Yes (%)            | 34.8                | 35.2                |               |
| Former smoker                                                          | No (%)             | 91.3                | 86.8                | 0.564         |
|                                                                        | Yes (%)            | 8.7                 | 13.2                |               |
| Coffee consumption                                                     | No (%)             | 21.7                | 26.9                | 0.627         |
|                                                                        | Yes (%)            | 78.3                | 73.1                |               |
| Feeling sick on the day                                                | No (%)             | 91.3                | 92.6                | 0.835         |
|                                                                        | Yes (%)            | 8.7                 | 7.4                 |               |
| Medication take on the day                                             | No (%)             | 65.2                | 64.7                | 0.965         |
|                                                                        | Yes (%)            | 34.8                | 35.3                |               |
| Smoked on the day                                                      | No (%)             | 82.6                | 82.4                | 0.978         |
|                                                                        | Yes (%)            | 17.4                | 17.6                |               |
| Brushed teeth on the day                                               | No (%)             | 91.3                | 98.5                | 0.093         |
|                                                                        | Yes (%)            | 8.7                 | 1.5                 |               |
| Infected with SARS-CoV-2                                               | No (%)             | 43.5                | 44.1                | 0.957         |
|                                                                        | Yes (%)            | 56.5                | 55.9                |               |
| Neoplasm problems                                                      | No (%)             | 95.7                | 98.5                | 0.416         |
|                                                                        | Yes (%)            | 4.3                 | 1.5                 |               |
| Respiratory problems                                                   | No (%)             | 91.3                | 91.2                | 0.985         |
|                                                                        | Yes (%)            | 8.7                 | 8.8                 |               |
| Cardiac problems                                                       | No (%)             | 100                 | 94.1                | 0.234         |
|                                                                        | Yes (%)            | 0.0                 | 5.9                 |               |
| Period of sampling                                                     | Morning (%)        | 34.8                | 44.1                | 0.433         |
|                                                                        | Afternoon (%)      | 65.2                | 55.9                |               |
| Teeth cleaning prior breath collection (time; hh:mm (minimum-maximum)) |                    | 05:10 (00:55-14:11) | 05:07 (01:07-11:07) | 0.057         |
| Last smoke prior breath collection (time; hh:mm (minimum-maximum))     |                    | 01:11 (00:12-02:10) | 01:07 (00:32-01:38) | 0.058         |
| Smoking habits                                                         | Never smoked (%)   | 65.2                | 65.7                | 0.877         |
|                                                                        | Current smoker (%) | 26.1                | 22.4                |               |
|                                                                        | Former smoker (%)  | 8.7                 | 11.9                |               |
| H <sub>2</sub> O ingestion prior to breath collection (time, hh:mm)    |                    | 02:39 (00:09-10:30) | 01:21 (00:05-05:21) | <b>0.008*</b> |
| Hours at the workplace on the day (time, hh:mm)                        |                    | 03:55 (00:00-08:30) | 03:51 (00:10-10:00) | 0.925         |

\*p<0.05; bold values are statistically significant; n = number of subjects

### 4.3 Logistic regression models

Logistic regression models were first constructed to evaluate differences in the associations between hours since last water ingestion and the two clusters. Results showed a significant association between the number of hours since last water ingestion and a decrease in the risk of being in cluster 2, with each hour passing since last drinking corresponding to an average of 42% decrease (OR [95% CI] = 0.68 [0.47 – 0.92], p = 0.021; *Table 4-8*, model 1). When

adjusted for potential confounders, (current smoking and brushing teeth prior to the test), (*Table 4-8*, model 1). The effect estimates remained practically unaltered.

*Table 4-8- Differences between the hierarchical model clusters according to the model 1*

| Predictors                               | Cluster |              |                   | Cluster |              |                   |
|------------------------------------------|---------|--------------|-------------------|---------|--------------|-------------------|
|                                          | OR      | CI           | p                 | OR      | CI           | p                 |
| (Intercept)                              | 5.16    | 2.33 – 13.01 | <b>&lt;0.001*</b> | 6.88    | 2.77 – 20.55 | <b>&lt;0.001*</b> |
| H <sub>2</sub> O ingestion prior to test | 0.68    | 0.47 – 0.92  | <b>0.021*</b>     | 0.65    | 0.45 – 0.90  | <b>0.017*</b>     |
| Brush teeth prior to test                |         |              |                   | 0.31    | 0.01 – 8.60  | 0.429             |
| Current smoker                           |         |              |                   | 0.41    | 0.10 – 1.71  | 0.212             |
| n                                        | 66      |              |                   | 66      |              |                   |
| R <sup>2</sup> Tjur                      | 0.103   |              |                   | 0.138   |              |                   |

\*p<0.05; bold values are statistically significant; OR: odds ratio; CI=confidence interval

Subsequently, a logistic regression model was used to estimate the association between current occupational exposure and the two clusters. Results showed that being exposed to a risk factor at work corresponds, on average, a 3.5 risk increase for being placed in cluster 2 (*Table 4-9*). While these results were not statistically significant, the observed tendency may suggest that a future study with a larger population might unravel a more pronounced association.

*Table 4-9- Differences between the hierarchical model clusters according to the model 2*

| Predictors                            | Cluster 2 |              |               | Cluster 2 |              |               |
|---------------------------------------|-----------|--------------|---------------|-----------|--------------|---------------|
|                                       | OR        | CI           | p             | OR        | CI           | p             |
| (Intercept)                           | 2.43      | 1.48 – 4.12  | <b>0.001*</b> | 4.06      | 1.78 – 10.51 | <b>0.002*</b> |
| Current occupational exposure         | 3.50      | 0.89 – 23.30 | 0.113         | 4.47      | 0.73 – 86.55 | 0.174         |
| H <sub>2</sub> O ingestion prior test |           |              |               | 0.69      | 0.47 – 0.94  | <b>0.033*</b> |
| n                                     | 91        |              |               | 65        |              |               |
| R <sup>2</sup> Tjur                   | 0.030     |              |               | 0.136     |              |               |

\*p<0.05; bold values are statistically significant; OR: odds ratio; CI= confidence interval;

*Figure 4-4* shows the trend between both clusters in relation to water intake prior to the exhaled breath collection. As demonstrated (in red) an inconsistent trend due to water intake is evident especially during the first 2 hours prior to the exhaled breath collection. These results suggests that water ingestion should be limited at least to 2.5 hours prior to exhaled breath test in order

to achieve unbiased findings. Moreover, these results also need to be considered and implemented in the future population study assessments.

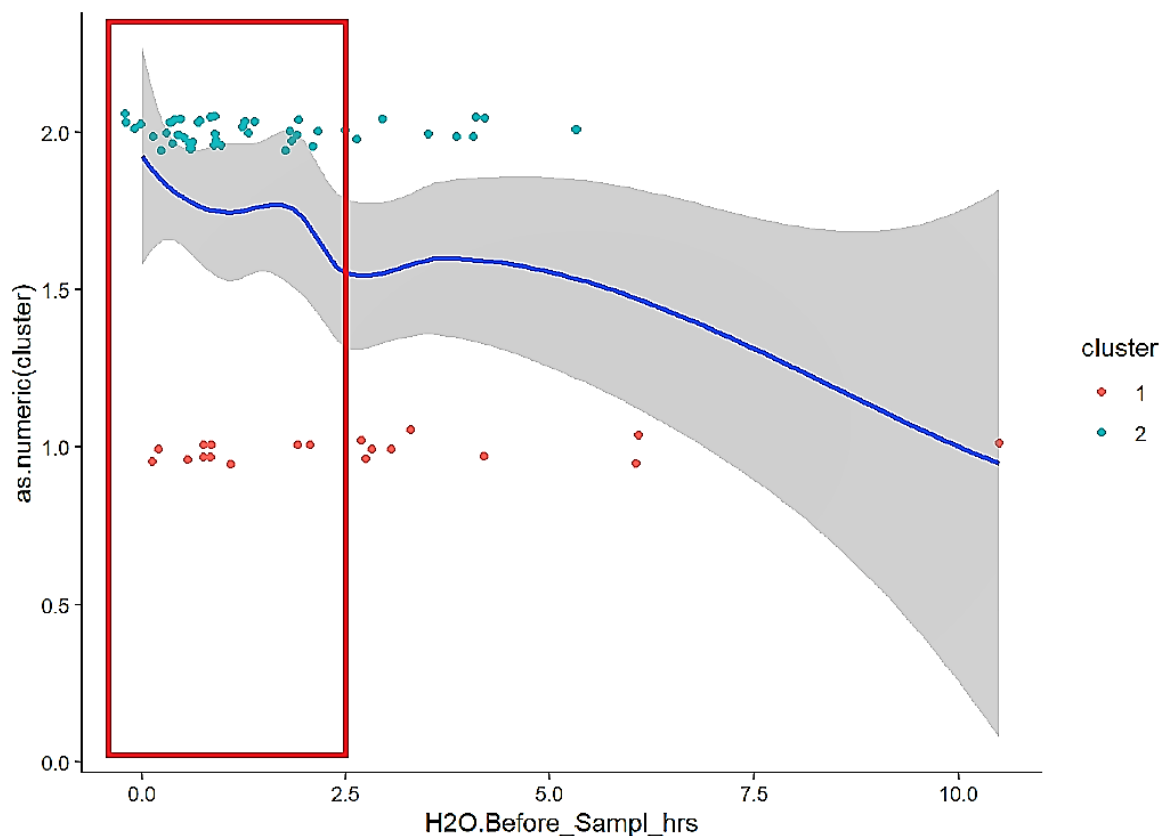


Figure 4-4- Effect of water intake prior to collection. Red border indicates period whit more consistent trend

#### 4.4 Limitations and future perspectives

This study assessed a general population in order to provide a reference benchmark for future exposure studies. Two detailed questionnaires were used to characterise the study population and to obtain all the relevant individual data for the exhaled breath profiles analysis via eNose.

The limitations of the conducted work are summarised as the following. First of all, the consumption of food or drinks prior to the exhaled breath collection were not controlled in the study population (as voluntary participants could not be forced to not consume food or beverages prior to the breath test). However, in view of the obtained results it would be useful to adapt the sampling protocol to liquids or food not being ingested at least 2.5 hours prior to the test. If such measures cannot be implemented, the use of Silica filters at the expiratory port or the heating of breath samples to reduce the humidity present in the samples can be

implemented as an alternative (Fens et al., 2013). In regard to fluid intakes, the impacts of specific drinks (such as caffeinated tea, coffee or soft drinks) versus water ingestion should be addressed. Secondly, administration of different pharmaceuticals prior to the test (as reported by several participants) should be evaluated in future. Thirdly, replicability of VOC profiles determination by eNose was not studied in this work. This step shall be considered in the future work as well as the inclusion of a larger number of study participants as demonstrated by some of the obtained results. According to previous literature, the influence of humidity is a more general factor affecting multisensor arrays, as extensively debated (Homer et al., 2004). Since the presence of water vapour might significantly change the chemistry of the interactions of other substances with the sensitive materials, eNose can behave differently depending on the humidity level. A possible way to overcome the impact of humidity is a calibration of the eNose to an application-specific range of humidity variations.

From the practical perspective, it should be noted that when collecting exhaled breath, the handling of the used material requires a care. Polymer bags are a fundamental piece of material used in the sampling process. The ideal collection bag should be cost and user-friendly, durable, inert and importantly allow neither ingress of environmental nor egress of breath VOCs. While Tedlar<sup>®</sup> bags that were used in this work are the most common, they are rather fragile and can be expensive. Thus, other polymer bags such as Mylar or and aluminium bags can be viable options for exhaled breath collection (Lawal et al., 2017). The possible variability of the breath sample during storage should also be considered, as Tedlar<sup>®</sup> bags may absorb some constituents of breath during storage (Beauchamp et al., 2008). To prevent gas leaks of the polymer bags that easily occurs after multiple perforations of septum in the closing valve, the use of a suitable and small-external diameter needle can be a viable solution. Tedlar<sup>®</sup> bags are the only model with stainless steel valves. Thus if possible, regular replacement of the spare parts (i.e., septum in a closing valve) should be provided to guarantee the scientific quality of the methods. Additionally, an alternative system with the possibility to perform the exhaled breath analysis directly, without the need for Tedlar bags could be employed (Fens et al., 2013).

To maintain the environmental and economical sustainability of the analytical method, the disposable material, whenever possible, should be re-utilised. A suitable environmental-friendly cleaning process and sterilisation is though required, avoiding any possible cross contamination of the material and/or subjects.

Finally, as stated by many authors (Kiselev et al., 2018) the “instability” of the eNose, mainly the loss of the ability to recognize target VOCs over time following an initial training, seems to be a limitation and should be taken into consideration in future works. Although the after-training tests of eNose usually show a quite good recognition of target objects, this degrades frequently in following tests under long-term operation. Additionally, there is one more related issue. Each multisensor array is unique as the primary “receptor/transducer”. It cannot be replaced in the same eNose unit without re-calibration (or re-training). If the arrays were reproducible like ordinary electronic components (i.e., resistors, capacitors, etc.), any cost required to calibrate an eNose versus any possible environment would be worth paying because the pattern recognition would not need to build a new knowledge database. In this case, the generated calibration model patterns corresponding to gases or gas mixtures in the eNose database could be applied to other multisensor arrays of that particular type. However, today’s multisensor arrays are rather individual, although the need to make them reproducible is frequently addressed. So far, there are only few reports that have considered producing comparable sensors. However, even these sensors are found to deviate in performance with time of eNose exploitation, which again refers to the temporal instability of the device operation. The possibility to transfer a calibration data set created by one eNose unit versus some chemically pure analytes to another eNose unit has been tested (Farraia et al., 2020). However, the effectiveness of the procedure needs further validation for practical applications (Kiselev et al., 2018).

## 5. Conclusions

Electronic nose technology is a pioneer application, but it has various advantages and a potential to be a useful tool for future non-occupational and occupational exposure studies.

Cluster analysis of VOCs patterns in exhaled breath resulted in two groups, with water intake prior to the test being the significant difference. Further, based on the obtained results, water intake shall be limited to at least 2.5 hours prior to the exhaled breath test. Other factors, such as occupational exposure to solvents and airborne particles, were observed to potentially impact the VOCs profiles of the study participants. Finally, the reproducibility of this analysis needs to be further explored in the foreseeable future. Moreover, the obtained findings should be implemented to the respective study designs, preferably with a larger population, in order to minimise possible errors and confounding factors, thus ensuring reliable results.

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## **ANNEX**

## **Annex. 1 Characterisation of Bragança region**

The region of Bragança (north of Portugal) has a population density of around 30 residents/km<sup>2</sup>. It is bounded to the east and north by Spain, west by the municipality of Vinhais and to the south by the municipality of Macedo de Cavaleiros. With a total area of 1174 km<sup>2</sup> (117 357 ha), the municipality is administratively subdivided into 49 “freguesias” (Gabinete Técnico Florestal de Bragança, 2015a). Most of its territory (about 85%) is located between 600 and 1000 metres above sea level. The altitude is an element that influences several parameters of high importance at the level of forest fire protection, such as humidity and temperature. The increase in altitude results in a decrease in temperature and in general, an increase in the humidity content, resulting in a decrease in the risk of wildfire and a lower speed of propagation of the fire. However, the increase in humidity can also affect the accumulation of available fuels to sustain a fire, so it will be necessary to bear in mind the type and distribution of vegetation, accesses, intervention times and also the gradient of the terrain that can represent another problem since the slope is considered one of the topographic elements with greater influence on fire propagation (Fernandes, 2004; Pereira, 2006).

Most adverse climatic conditions (those that cause the largest areas burnt in mainland Portugal) are often associated with hot and dry winds from the east and southeast, which accounts for 24% of the region (Gabinete Técnico Florestal de Bragança, 2015a). The climate is characterised by a continental climate, with a high intra annual variation in temperature and precipitation, with short hot summers and long cold winters and low precipitation (Gabinete Técnico Florestal de Bragança, 2015a).

Regarding temperature, the peak values are registered in July (38.8°C), August (38.4°C) and September (37.7°C). The temperature is generally high in the critical period of wildfires (July to September), which contributes to an easier ignition and speed of progression of the fire. Occurrence of extremes temperature are especially relevante since they influence the moisture content present in vegetable fuels, as well as their temperature and consequently, the energy required for combustion, raising the risk of fire (Gabinete Técnico Florestal de Bragança, 2015a).

In terms of soil use, brush and pasture correspond for 39% of the municipality and forest for 32% (seen in *Figure A1-1*), meaning that vegetation is the predominant occupation in the

municipality of Bragança, representing around 71% of the land area (83 112 ha. The characteristic vegetation present in Bragança includes Pyrenean oak (*Quercus pyrenaica*) and European white birch (*Betula alba*) in higher altitudes heather (*Ericas* spp.), Carqueja (*Chamaespartium tridentatum*), Montpellier cistus (*Halimium allyssoides*), wild rose (*Rosa* spp.), broom (*Cytisus* spp.) and gorse (*Ulex* spp.). In the lower parts, there are holm oak (*Quercus Ilex*), common oak (*Quercus faginea*), lavender (*Lavandula pendunculata*), cistus (*Cistus ladanifer*), spurge flax (*Daphne gum*) and thuriferous (*Daphne gnidium*) and cork oak (*Quercus suber*), as well as ash (*Fraxinus* spp.) in watercourse areas (Gabinete Técnico Florestal de Bragança, 2015a).

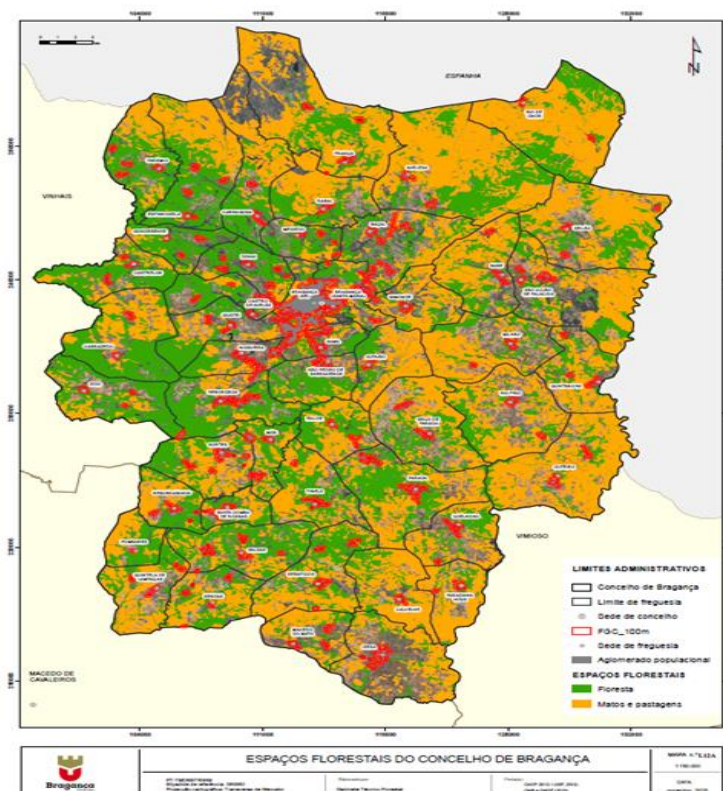


Figure A1- 1- Distribution of type space in Bragança, colours:  
(grey- Population, green-Forest, yellow- Pasture)

Meteorological conditions that favoured the occurrence of large fires in the region are well known, the relevance of the relative humidity of the air present and wind speed is significant for estimating the forest fire risk. Thus, the forest fire risk are very high whenever the relative humidity of the air is close to 30% and the wind speed higher than 20 km/h (Gabinete Técnico Florestal de Bragança, 2015b). Between 2010 and 2020, 6814 fires occurred with 241 incidents, this was the municipality with the one with the second-largest burned area in Portugal, 6522 ha (ICNF, 2020).

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## Annex. 2 Questionnaire “Routine and personal habits on the day”

Questionnaire to understand the routine of the individual so far into the day, *Figure A2-1*.

HORA: \_\_\_\_ : \_\_\_\_

### VII. QUESTÕES REFERENTES AO DIA DE HOJE

1. Exerceu esforços físicos hoje no decorrer do seu dia de trabalho? ☐ Não ☐ Sim

Se **sim**, o quê?

2. Desempenhou algum desporto físico no decorrer do dia até este momento? ☐ Não ☐ Sim, qual?

☐ Ginásio ☐ Corrida ☐ Caminhada ☐ Nataçã

☐ Outro

3. Quando foi a sua última refeição hoje?

Qual?

4. Quando foi a última vez hoje que ingeriu (indicar a hora, se não consumiu deixar em branco):

▶ Café \_\_\_\_:\_\_\_\_(hora/min) ▶ Chá \_\_\_\_:\_\_\_\_(hora/min) ▶ Refrigerante com cafeína \_\_\_\_:\_\_\_\_(hora/min)

▶ Refrigerante sem cafeína \_\_\_\_:\_\_\_\_(hora/min) ▶ Bebida alcoólica \_\_\_\_:\_\_\_\_(hora/min) ▶ Água \_\_\_\_:\_\_\_\_(hora/min)

▶ Outro: \_\_\_\_\_ (qual) \_\_\_\_:\_\_\_\_(hora/min)

5. Quando lavou os dentes ou usou elixir bucal pela última vez?

Dia: \_\_\_\_\_ : \_\_\_\_ (hora/min)

6. Fumou hoje? ☐ Não ☐ Sim

Se **sim**, indicar quantidade (ex. cigarros, charuto, vaper)?

Quando foi a última vez? (se foi hoje, indicar hora)

7. Hoje sente-se doente/constipado? ☐ Não ☐ Sim

8. Hoje tomou alguma medicação? ☐ Não ☐ Sim

Quando foi a última vez? (se foi hoje, indicar hora)

Qual?

9. Quantas horas hoje permaneceu em média no seu local de trabalho (até ao momento)?

MUITO OBRIGADO !!! 😊

*Figure A2- 1- Individual questionnaire “Routine and personal habits on the day”*

### Annex. 3 Optimization of exhaled breath procedure and analysis

Different settings (regarding to purge time and draw sample) of the Cyranose<sup>®</sup> 320 were tested regarding the type of sample to be collected (exhaled breath) in the process of methodology optimization as represented in *Figure A3-1*. Each colour represents a specific sensor that was active (all 32 sensors represented). The majority of sensors are sensitive to humidity, being particularly considerable sensor 31 (light blue curve).

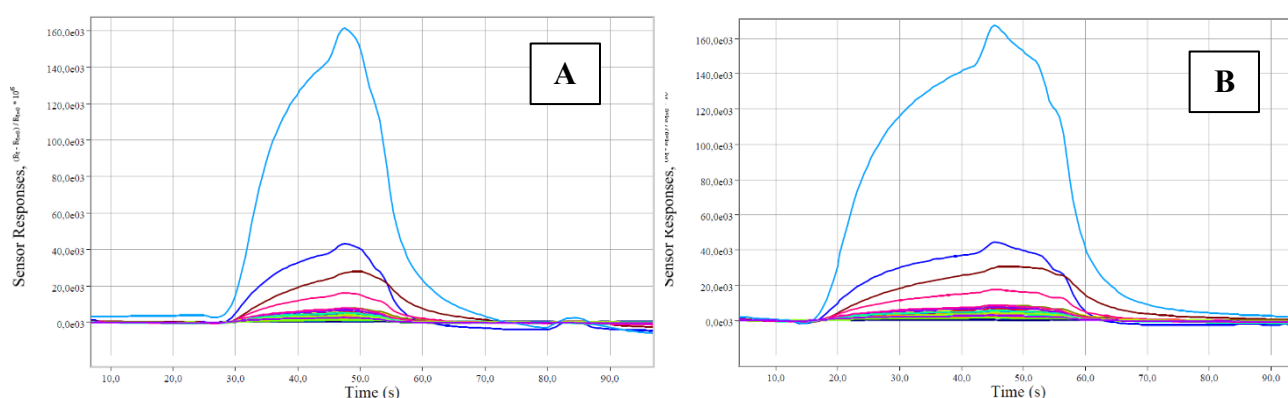


Figure A3- 1- Comparisons of equipment settings: (A) parameters value recommended by the manufacturer, Sensigent, (2013b); (B) values adapted from previous exhaled breath studies (Farraia et al., 2020)

The different durations of breathing through the VOCs filtering system prior to the collection was insufficient. Duration of 2 min was adapted, also in view of physical constraints for period of 5min, the results can be seen in *Figure A3-2*

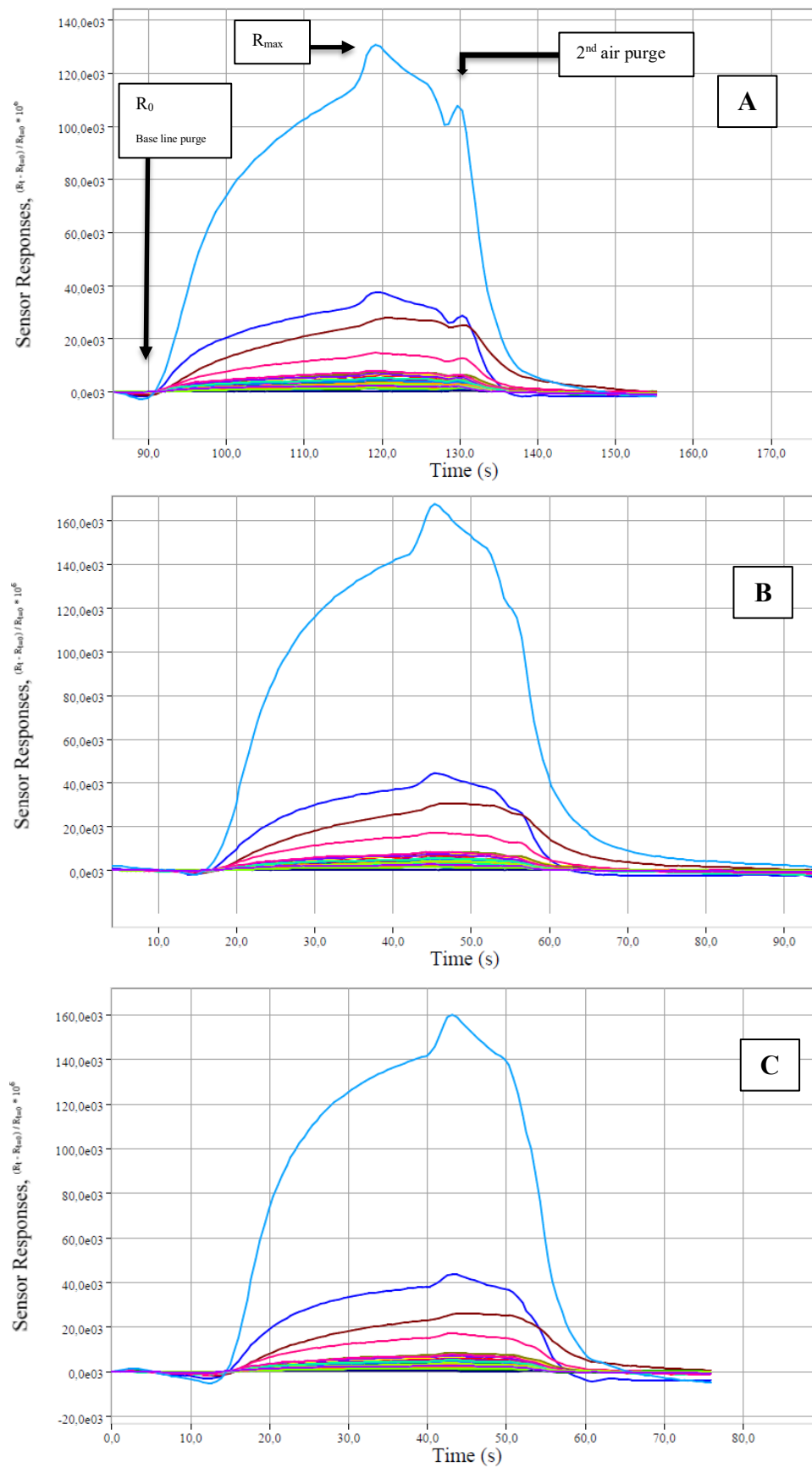
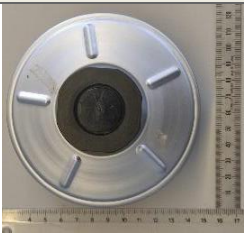
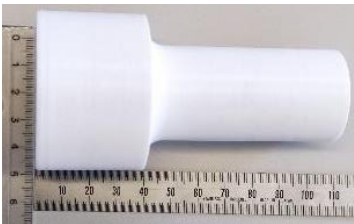
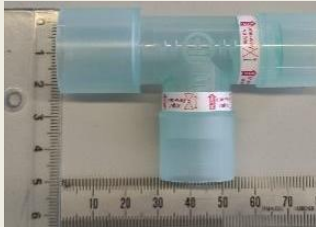
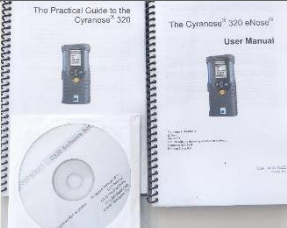


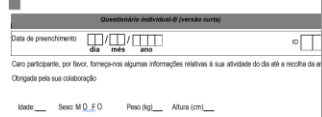
Figure A3- 2- Graphical representation of exhaled breath analysis: (A) 1 min; (B) 2 min; and (C) 5 min of breathing through VOCs filtering system prior to the test. Note all 32 sensors are included.

## Annex. 4 Methodology for collection and analysis of volatile organic compounds in exhaled breath

### Material Required:

|                                           |                                                                                                                                                                                                            |                                                                                      |                  |                                                                                                                                                               |                                                                                       |
|-------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| 1 un/participant                          | <p>Tedlar Bags® 1 L</p> <p>Supplier: Ambicontrol</p> <p>Brand: SKC</p> <p>Description: Tedlar bag-breath-gas-fitting 1L</p> <p>Reference: 249-01</p>                                                       |    | 1 un/participant | <p>Tedlar bag nozzle</p> <p>Supplier: Ambicontrol</p> <p>Brand: SKC</p> <p>Description: Mouthpieces for breath-gas analysis bags</p> <p>Reference: P20054</p> |    |
| <p>1 un</p> <p>(approx. 100 max uses)</p> | <p>VOC filter</p> <p>Supplier: sintimex</p> <p>Brand: Honeywell</p> <p>Reference: Rd40 aluminium 250 A2 for VOC</p>                                                                                        |    | 1 un             | 3D adapter piece                                                                                                                                              |   |
| 1 un/participant                          | <p>Directional valve anti-pollution T 22F – 22M (flow inlet) – 22M (flow outlet)</p> <p>Supplier: Intersurgical</p> <p>Description: One-way valve 22F-22M – 22M</p> <p>Reference: 1953000 CDM 45333661</p> |  | 1 un/participant | <p>Straight connector 22F- 15M</p> <p>Supplier: Intersurgical</p> <p>Description: 22F-15M connector</p> <p>Reference: 1962000 CDM: 15088278</p>               |  |

|      |                                                                                                                     |                                                                                     |      |                                                                                                                                                                                                  |                                                                                       |
|------|---------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| 1cx  | Disposable needles<br>Brand: Terumo<br>Description: Neolus<br>1.1x40mm<br>Reference: NN-1938R                       |    | 2 un | Nose clip                                                                                                                                                                                        |    |
| 1 un | Cyranose® 320<br>Publisher: Sensigent LLC<br>Brand: Sensigent<br>Description: Cyranose® 320 w/<br>disposable needle |    | 1 un | Equipment for continuous<br>measurement of temperature and<br>relative humidity of the air<br><br>Publisher: Amberg<br>Brand: TSI<br>Description: TSI 7545 IAQ-Calc Indoor Air<br>Quality Meters |    |
| 2 un | Ethyl alcohol 70%                                                                                                   |   | 1cx  | Dry paper towels                                                                                                                                                                                 |    |
| 1 un | RS-232 cable (4-wire serial<br>communications cable) with the RS-<br>232 9-pin adapter                              |  | 2 un | 320 Practical Guide Rev H w /CD<br>+<br>320 User Manual Rev I                                                                                                                                    |  |

|                                                             |                                                                                                                                   |                                                                                    |                                                             |                                                                                                                          |                                                                                      |
|-------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| 1 un/participant                                            | - Questionnaire BioFirEx- -D -<br>População_Controlo (if not yet done)<br>- Questionnaire – “Questions of the day” (pre-analysis) |  | 1 un<br>1 cx<br>1 cx                                        | Lab coat<br>Gloves<br>Surgical mask                                                                                      | (generalized)                                                                        |
| 1 un                                                        | Stopwatch (undifferentiated)                                                                                                      |   | 1 pack                                                      | Labels                                                                                                                   |   |
| 1 cx                                                        | Garbage bags<br>(for waste and to store disposable material)                                                                      |   | 5 un (minimum)<br><br>1 un                                  | Transport bags (IKEA type)<br><br>Or<br>Thermal bag for transporting samples<br>(if not analysed immediately afterwards) |   |
| Miscellaneous<br>(to be distributed among the participants) | Pens/ ball point pens                                                                                                             |  | Miscellaneous<br>(to be distributed among the participants) | Block holder / clipboard                                                                                                 |  |

## I. Before the collection of exhaled breath sample

1. Monitor the temperature (°C) and relative humidity (%) conditions of the site in the indoor space/room where the exhaled breath sampling of each of the participants takes place throughout the day using the TSI 7545 IAQ-Calc equipment.
2. Ask participants to wait, if possible sitting, for at least 10 minutes in the room where the breath sampling will take place; and fill in:
  - a. Individual questionnaire “*BioFirEx-D-População\_Controlo*” just in case this questionnaire has not been completed by the participant in any of the previous phases of the BioFirEx project;
  - b. Individual questionnaire “*Routine and personal habits on the day*” (pre-analysis);

**Note that for tracking and confidentiality purposes, both questionnaires identified above will have the same code (following BioFirEx project nomenclature).**

3. Identify the Tedlar® 1 L bag with a label with the individual code of the participant and connect the “nozzle” piece to the Tedlar® 1 L bag at the stainless-steel opening present in the bag (as shown in *Figure A4-1*).



*Figure A4- 1- Insert nozzle*

4. Assemble the harvesting system as illustrated in *Figure A4-2*, starting with:
  - a. Screw the 3D adapter piece into the VOC filter (keep the base of this VOC filter covered until the breath sample is collected);
  - b. Connect the directional anti-pollution valve T-piece 22F – 22M (flow inlet) – 22M (flow outlet) and the straight connector 22F- 15M;

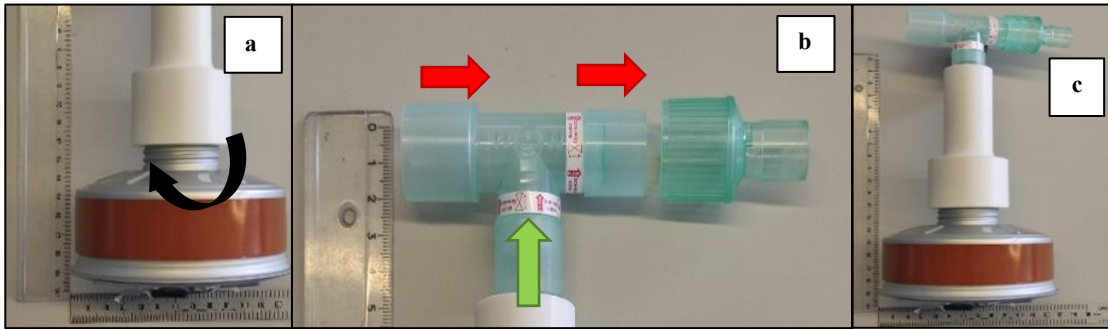


Figure A4- 2 - Exhaled breath system: (a) placement of 3D piece; (b) anti- pollution valve flow; (c) complete assembled system

5. Explain and exemplify the method of collecting the breath sample to each of the participants, namely:
  - a. Identify the end of the sampling system where the mouthpiece is placed;
  - b. Point out that the mouth should remain sealed and can't be open during the process (so that the air collected in the bag is only coming from the participant's lungs);
  - c. Breathe normal/regular/continuous manner for 2 minutes through the assembled system;
  - d. After 2 minutes (timed), insert the Tedlar bag at the end of the assembly system, and the participant must continue to breathe in a normal/regulation/continuous manner until filling half the capacity of the Tedlar® 1 L bag;

## II. During the collection of the exhaled breath sample

1. Place the nose clip on the participant;
2. Request the participant to start normal/regular/continuous breathing for 2 minutes, as seen in Figure A4- 3 (time it) through the system represented in Figure A4-2;



Figure A4- 3- Sample collection

3. When missing approximately 10 seconds, prepare the Tedlar<sup>®</sup> 1 L bag, opening the valve (2 full turns counter clockwise) as illustrated in *Figure A4-4*;



*Figure A4- 4- Turn counter clockwise*

4. At the end of these 2 minutes, insert the Tedlar<sup>®</sup> 1 L bag into the stainless-steel opening of the Tedlar bag which makes up the mounting system and ask the participant to continue normal/regular/continuous breathing until half the capacity of the Tedlar<sup>®</sup> 1L bag is filled;
5. Then remove the Tedlar<sup>®</sup> 1 L bag and close the valve by turning 2 full turns clockwise (seen in *Figure A4-5*).



*Figure A4- 5- Turn clockwise*

### **III. After collection of the exhaled breath sample**

1. Once the sample collection is complete, proceed with the sample analysis if the location allows it; if not, the Tedlar bag should be placed in a cooler which should be kept at 4°C (Rodríguez-Aguilar et al., 2020) to ensure the quality of the sample for up to 5 hours;
2. Cover VOC filter with proper lid;
3. Discard into the appropriate sack for subsequent sterilisation:
  - a. Nozzle
  - b. Anti-pollution directional valve
  - c. Straight connector
  - d. Disinfect collection site with dry paper towels and 70% ethyl alcohol.

#### IV. Information relevant to the settings do Cyranose® 320

1. The settings chosen for Cyranose®320 were based on those recommended by the manufacturer in the practical guide (Sensigent, 2013b) and optimised for this procedure is represented in *Table A4-1*.

These settings are optimised in the context of the present procedure and the material specified for this methodology (indicated in material required, mentioned above).

*Table A4- 1- Settings defined for analysis*

| Settings                     | Time (s) | Pump Speed |
|------------------------------|----------|------------|
| <b>Baseline Purge:</b>       | 30       | Medium     |
| <b>Sample Draw 1:</b>        | 30       | Medium     |
| <b>Sample Draw 2:</b>        | 0        | Medium     |
| <b>Snout Removal:</b>        | 10       | ----       |
| <b>1st Sample Gas Purge:</b> | 10       | High       |
| <b>1st Air Intake Purge:</b> | 10       | High       |
| <b>2nd Sample Gas Purge:</b> | 40       | High       |
| <b>2nd Air Purge Intake:</b> | 30       | High       |

Figure A4-6, below is shown a representation of the settings using the Pcnose+® software. These are relevant as the values will be transferred and stored on the equipment (Cyrano® 320).

**Flow Settings - (WARNING: Changes to this section may require retraining)**

|                        | Time (s) | Low                   | Medium                           | High                             |
|------------------------|----------|-----------------------|----------------------------------|----------------------------------|
| <b>Baseline</b>        |          |                       |                                  |                                  |
| Baseline Purge :       | 30       | <input type="radio"/> | <input checked="" type="radio"/> | <input type="radio"/>            |
| <b>Sample</b>          |          |                       |                                  |                                  |
| Sample Draw 1 :        | 30       | <input type="radio"/> | <input checked="" type="radio"/> | <input type="radio"/>            |
| Sample Draw 2 :        | 0        | <input type="radio"/> | <input checked="" type="radio"/> | <input type="radio"/>            |
| <b>Purge</b>           |          |                       |                                  |                                  |
| Snout Removal :        | 10       | <input type="radio"/> | <input type="radio"/>            | <input type="radio"/>            |
| 1st Sample Gas Purge : | 10       | <input type="radio"/> | <input type="radio"/>            | <input checked="" type="radio"/> |
| 1st Air Intake Purge : | 10       | <input type="radio"/> | <input type="radio"/>            | <input checked="" type="radio"/> |
| 2nd Sample Gas Purge : | 40       | <input type="radio"/> | <input type="radio"/>            | <input checked="" type="radio"/> |
| 2nd Air Intake Purge : | 30       | <input type="radio"/> | <input type="radio"/>            | <input checked="" type="radio"/> |

Digital Filtering (id/training/calibration):

Substrate Heater: ☒ On / Off  °C

Training Repeat Count:

Identifying Repeat Count:

---

**Data Processing - (Changes to this section will not require retraining)**

Active Sensors:

|                                        |                                        |                                        |                                        |                                        |                                        |                                        |                                        |
|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| <input checked="" type="checkbox"/> 1  | <input checked="" type="checkbox"/> 2  | <input checked="" type="checkbox"/> 3  | <input checked="" type="checkbox"/> 4  | <input checked="" type="checkbox"/> 5  | <input checked="" type="checkbox"/> 6  | <input checked="" type="checkbox"/> 7  | <input checked="" type="checkbox"/> 8  |
| <input checked="" type="checkbox"/> 9  | <input checked="" type="checkbox"/> 10 | <input checked="" type="checkbox"/> 11 | <input checked="" type="checkbox"/> 12 | <input checked="" type="checkbox"/> 13 | <input checked="" type="checkbox"/> 14 | <input checked="" type="checkbox"/> 15 | <input checked="" type="checkbox"/> 16 |
| <input checked="" type="checkbox"/> 17 | <input checked="" type="checkbox"/> 18 | <input checked="" type="checkbox"/> 19 | <input checked="" type="checkbox"/> 20 | <input checked="" type="checkbox"/> 21 | <input checked="" type="checkbox"/> 22 | <input checked="" type="checkbox"/> 23 | <input checked="" type="checkbox"/> 24 |
| <input checked="" type="checkbox"/> 25 | <input checked="" type="checkbox"/> 26 | <input checked="" type="checkbox"/> 27 | <input checked="" type="checkbox"/> 28 | <input checked="" type="checkbox"/> 29 | <input checked="" type="checkbox"/> 30 | <input checked="" type="checkbox"/> 31 | <input checked="" type="checkbox"/> 32 |

Algorithm:

Preprocessing:

Normalization:

Identification Quality:

Acceptance Threshold:  %

Figure A4- 6- Settings according to Pcnose+ ® software

2. The analyses are made and recorded using one of the 4 available methods, always with the settings mentioned to in the first point, the “Train” mode will be used to record the results; Each “Method” has 6 classes each one with the capacity to register 10 samples, which allows to collect 60 samples for each method. If the sample number is greater than 60, continue sampling by selecting the next blank method (without any records), as illustrated in the following Figure A4-7.

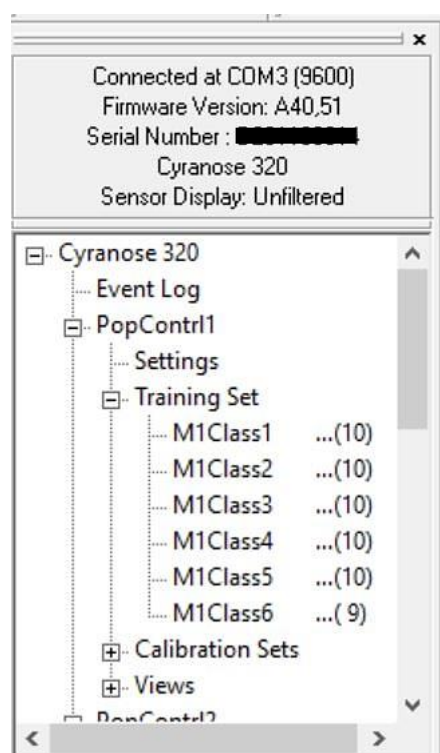


Figure A4- 7- Train mode description

3. Using a record sheet (Table A4-2) identify participant and its corresponding test/analysis, example:

Table A4- 2- Record sheet

|            |           |            |                              |
|------------|-----------|------------|------------------------------|
| Method (1) | Class (1) | Sample (1) | Corresponding code (ID ____) |
| Method (1) | Class (1) | Sample (2) | Corresponding code (ID ____) |
| .          | .         | .          | .                            |
| Method (1) | Class (2) | Sample (1) | Corresponding code (ID ____) |

## V. Programming Cyranose® 320

1. Install the Pcnose+® software on a computer, it is available on the CD that comes with the equipment and its manuals (follow the installation instructions as described in the (Sensigent, 2013a).
2. Connect the Cyranose® 320 to the power grid and press the right-side button “On/Off” (shown in Figure A4-8) and wait for it to “warm up” as indicated on the display (approximately 360 sec depending on ambient conditions);



Figure A4- 8- On/Off Button

3. Connect the Cyranose<sup>®</sup> 320 to the computer using the RS-232 cable (4-wire serial communications cable) with the RS-232 9-pin adapter, *Figure A4-9*;

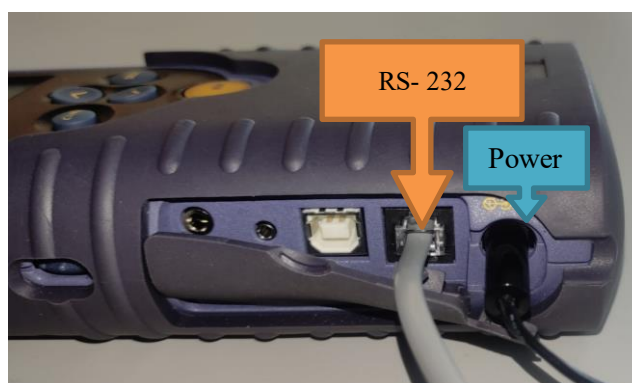


Figure A4-9 - Connection of the RS-232 cable and power cord

4. Open the Pcnose<sup>+</sup><sup>®</sup> software and connect it to the Cyranose<sup>®</sup> 320 to verify that the equipment is recognized (see upper left corner of the window, see *Figure A4-10*);

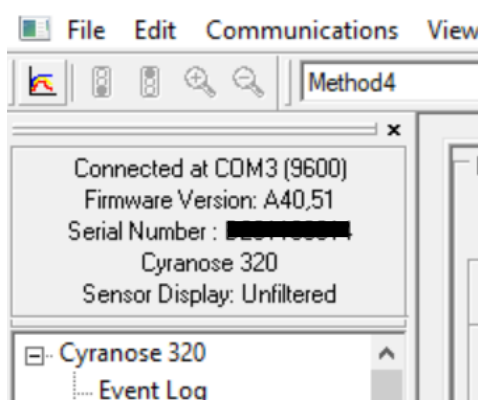


Figure A4-10 - Connection established between Cyranose 320<sup>®</sup> and Computer

5. Set the settings mentioned in *Table A4-1* by double-clicking “settings” within the chosen method. When all the parameters of the settings have been set according to *Table A4-1*, save them by clicking the “Save to Cyranose 320” button (see *Figure A4-6*);
6. From this moment the analyses can start. During the analyses the Cyranose<sup>®</sup> 320 does not need to be connected to the computer and the data will be saved on the device and can be

downloaded at the end, to disconnect go to “Communications” and click on “Disconnect” and then disconnect the RS-232 cable;

## VI. Analyses of exhaled breath samples using the Cyranose® 320

1. Before performing the first sample and/or whenever the Cyranose® 320 is left in stand-by for more than 30 min, perform a double burn/cleaning using the “Identify” option. To do this select the “Identify” option followed by “Select” button and then “Run”. Once the two runs are complete click “Cancel”;

**IMPORTANT:** Note that the Cyranose® 320 has two vents on the sides (inlet and outlet) designated “Purge Inlet” and “Exhaust” where the air is pumped through and must always be unobstructed.

2. Confirm that the desired method is selected (see the top of screen, as shown in *Figure A4-11*). If you need to change method, select “Change Method” and choose the desired method from the available list press “Select” button, once selected press “Cancel”;

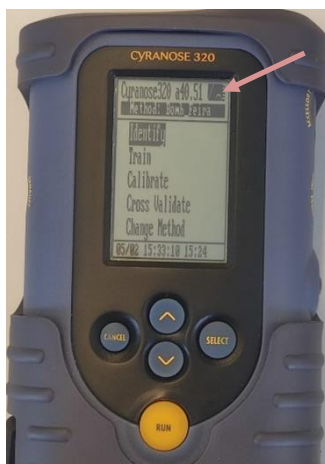


*Figure A4-11- Cyranose® 320 display*

3. Once the clean-up is complete, we can start collecting data, to do so use the arrows until you find “Train” option click on the “Select” button. Select the first class “M1Class1” (where the data from the first 10 analyses will be save, after the first 10, change to the next class;

4. Screw the disposable needle into the Cyranose<sup>®</sup> 320 and pierce the Tedlar<sup>®</sup> 1 L bag with it in the stainless-steel opening (*Figure A4-12*);
5. Then select “Run”. Once “Sample Draw” is complete, quickly remove the bag and needle from the Cyranose<sup>®</sup> 320. With the settings that have been set you will have 10 sec to do



*Figure A4- 12- Thread needle on Cyranose<sup>®</sup> 320*

this, indicated as “Snout Removal” (the Cyranose<sup>®</sup> 320 will beep a warning before starting the count);

6. After “Snout Removal” let the Cyranose<sup>®</sup> 320 finish purging the system “Purge” and the data collection to Cyranose<sup>®</sup> 320 is completed;
7. To perform a new analysis, start again from point 4;
8. Once all analyses are complete return to the home screen using the “Cancel” button as many times as necessary;
9. To switch off the Cyranose<sup>®</sup> 320 press the On/Off button;

## **VII. Data extraction**

1. Once all samples have been taken, export the data to the computer through the Pcnose+<sup>®</sup> software;
2. Open the Pcnose+<sup>®</sup> software and establish the connection to Cyranose<sup>®</sup> 320, once connected, double click on “Training Set”, within the chosen method, a window will appear with the collected data, to save it click on “Save method to PC” the file will be stored as .met format (the same can only be read in the Pcnose+<sup>®</sup> software with the Cyranose<sup>®</sup> 320 connected to the computer);
3. The data can be read in the computer through a new Excel file, go to “Open” and do “search on this PC” select “all files” the settings of the “Test Import Wizard” should not be changed. At the end save the file as .csv format to facilitate further reading/analysis;

## VIII. Cleaning of the material

1. To clean the Tedlar<sup>®</sup> 1 L bags pure nitrogen is used, according to the system depicted in *Figure A4-13*.



*Figure A4- 13- Cleaning site*

2. The nozzle piece, anti-pollution directional valve and straight connector should be discarded. Tedlar<sup>®</sup> 1 L bag and the used needles (to pierce the septum of the bag) underwent a cleaning process with pure nitrogen. The cleaning procedure should be conducted within a laboratory exhauster to ensure safe removal of the vapours.



*Figure A4- 14- Cleaning Tedlar® 1 L bags*

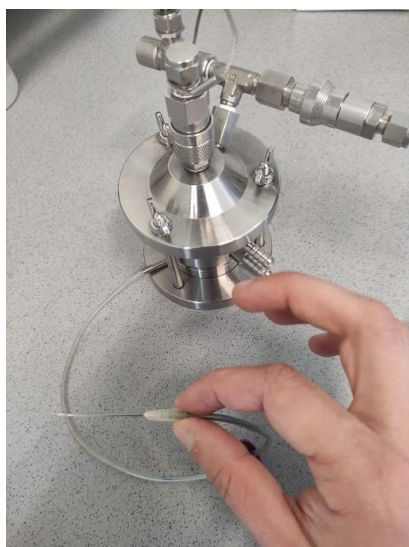


*Figure A4- 15- Cleaning needles used in the analysis*

3. The cleaning of the Tedlar® 1 L bags done with nitrogen (*Figure A4-14*). To ensure effective cleaning and without any cross-contamination, the cleaning procedure is each bag should be filled three times (Rodríguez-Aguilar et al., 2020). The used needles (*Figure A4-15*) must be passed through a stream of nitrogen for about 5 seconds.
4. Treatment and disposable of material (nozzle, anti-pollution directional valve, straight Connector) should be done appropriately for medical waste. Whenever possible, material should be sterilised and reused according INSA internal procedure. To store them for, keep the 3 pieces together in a sterile bag;

## Annex. 5 Practical recommendations in the course of exhaled breath sample collection

During the course of exhaled breath collection, some obstacles were experienced. Firstly, the Tedlar® 1 L bags which were rather costly are easily worn out (and consequently torn) near the valve section (stainless steel opening) of the bag (*Figure A5-1 (a)*). The tear was found to be simply caused by gentle contact with other bags (during transport) where the thread on the valve damaged the surface of the bag. This problem was resolved by the coating of the parts more susceptible to damage using two layers of adhesive tape (*Figure A5-1 (b)*). It may be a temporary solution but allowed for damage prevention of the bags.

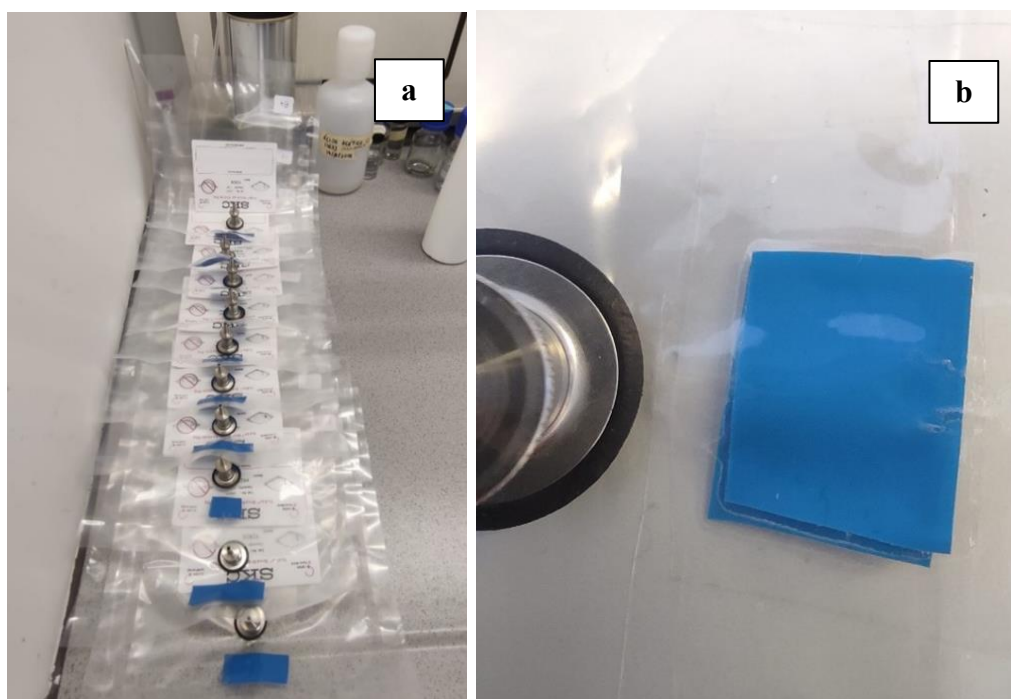


*Figure A5- 1- (a) Organization of the clean bags (b) Protection of the bags against wear and tear*

Secondly, the septum placed inside the bag began to fail after very few samples taken, resulting in leaks of the exhaled breath. These leaks were caused by the combination of two factors: a pierced septum and valve being closed extremely tightly (*Figure A5-2*) resulting in a spreading of the septum too thinly. Therefore, attention must be given to proper the valve closing (not over tightening) while at the same time ensuring that quality of the septum.

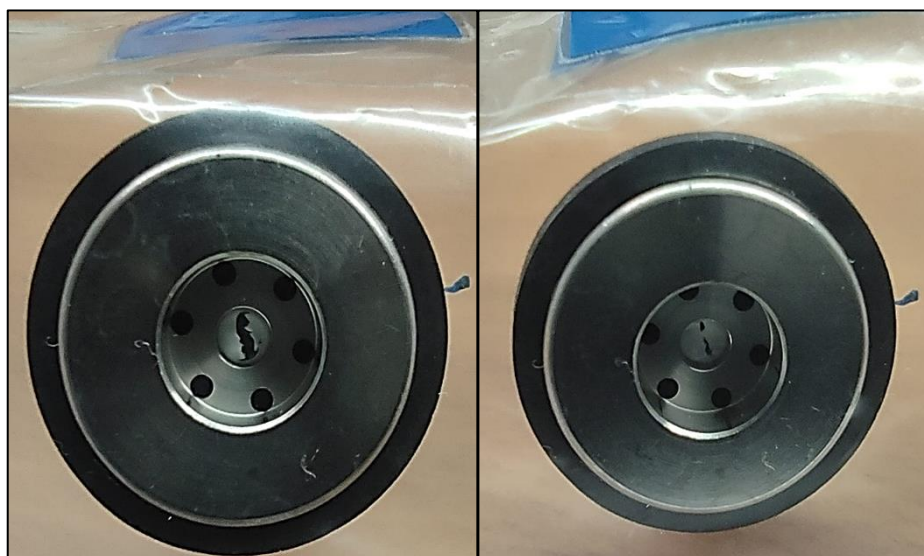


*Figure A5- 2- Detail of pierced septum with valve fully closed (left); valve slightly released (right)*

#### Annex references:

Rodríguez-Aguilar, M., Díaz De León-Martínez, L., Gorocica-Rosete, P., Padilla, R. P., Thirión-Romero, I., Ornelas-Rebolledo, O., & Flores-Ramírez, R. (2020). Identification of breath-prints for the COPD detection associated with smoking and household air pollution by electronic nose. *Respiratory Medicine*, 163, 105901. <https://doi.org/10.1016/j.rmed.2020.105901>

## Annex. 6a Sampling site statistics

The physical parameters of the “site” as well as environmental condition during the sampling days was documented to provide more complex characterisation for the study. Indoors, only natural ventilation was provided in the room through door opening (*Figure A6-1*). Air conditioning system was switched off and windows were always maintained closed.



Figure A6- 1- Sample collection room

Data on air pollutants retrieved from national monitoring network available in the city of Porto (operated under Portuguese Environmental Agency) for "Sobreiras-Lordelo do Ouro" (defined as urban background) are summarized in *Table A6-1* and "Francisco Sá Carneiro-Campanhã (urban traffic)" in *Table A6-2*.

Table A6- 1- Concentrations of air pollutants measured at ambient air quality monitoring station Sobreiras-Lordelo do Ouro

| Sobreiras-Lordelo do Ouro           |         |                      |                      |                      |                      |                      |                      |
|-------------------------------------|---------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| Day                                 |         | May 25 <sup>th</sup> | May 26 <sup>th</sup> | May 27 <sup>th</sup> | May 30 <sup>th</sup> | May 31 <sup>st</sup> | June 1 <sup>st</sup> |
| O <sub>3</sub> µg/m <sup>3</sup>    | Average | 54.1                 | 55.6                 | 67.3                 | 68.7                 | 41.6                 | 64.0                 |
|                                     | Maximum | 87.0                 | 88.0                 | 117.0                | 96.0                 | 58.0                 | 85.0                 |
|                                     | Minimum | 10.0                 | 8.0                  | 9.0                  | 30.0                 | 8.0                  | 34.0                 |
| NO <sub>2</sub> µg/m <sup>3</sup>   | Average | 18.1                 | 29.7                 | 21.9                 | 12.0                 | 11.5                 | 7.8                  |
|                                     | Maximum | 53.0                 | 88.0                 | 72.0                 | 35.0                 | 33.0                 | 19.0                 |
|                                     | Minimum | 7.0                  | 4.0                  | 4.0                  | 2.0                  | 4.0                  | 2.0                  |
| PM <sub>10</sub> µg/m <sup>3</sup>  | Average | 19.6                 | 28.4                 | 23.5                 | 19.5                 | 13.9                 | 19.6                 |
|                                     | Maximum | 46.0                 | 69.0                 | 39.0                 | 27.0                 | 23.0                 | 34.0                 |
|                                     | Minimum | 11.0                 | 8.0                  | 9.0                  | 11.0                 | 6.0                  | 6.0                  |
| PM <sub>2.5</sub> µg/m <sup>3</sup> | Average | 3.3                  | 5.7                  | 7.4                  | 3.1                  | 2.3                  | 1.6                  |
|                                     | Maximum | 7.0                  | 19.0                 | 15.0                 | 8.0                  | 8.0                  | 5.0                  |
|                                     | Minimum | <0.1                 | <0.1                 | 1.0                  | <0.1                 | <0.1                 | <0.1                 |

Table A6- 2- Data collected from monitoring air quality station Francisco Sá Carneiro-Campanhã

| Francisco Sá Carneiro-Campanhã     |         |                      |                      |                      |                      |                      |                      |
|------------------------------------|---------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| Day                                |         | May 25 <sup>th</sup> | May 26 <sup>th</sup> | May 27 <sup>th</sup> | May 30 <sup>th</sup> | May 31 <sup>st</sup> | June 1 <sup>st</sup> |
| NO <sub>2</sub> µg/m <sup>3</sup>  | Average | 28.4                 | 39.4                 | 43.8                 | 42.9                 | 33.4                 | 33.2                 |
|                                    | Maximum | 67.0                 | 98.0                 | 142.0                | 78.0                 | 63.0                 | 64.0                 |
|                                    | Minimum | <0.1                 | 10.0                 | 9.0                  | 11.0                 | 13.0                 | 11.0                 |
| PM <sub>10</sub> µg/m <sup>3</sup> | Average | 18.1                 | 22.4                 | 25.1                 | 25.3                 | 23.4                 | 18.5                 |
|                                    | Maximum | 26.0                 | 32.0                 | 36.0                 | 31.0                 | 31.0                 | 24.0                 |
|                                    | Minimum | 13.0                 | 11.0                 | 19.0                 | 21.0                 | 14.0                 | 13.0                 |

## Annex. 6b Sampling site conclusions

Based on the existing Portuguese legislation (Portaria n.º 138-G/2021 of 1<sup>st</sup> July), the limit for CO<sub>2</sub> in indoor air is 1250 ppm and 8.7 ppm for CO in accordance with Article n.º 4 "*Protection limits and reference conditions*". During the period of the study, the obtained 8 hours means (CO<sub>2</sub>: 475–808 ppm; CO: 0.1–0.4 ppm) were below those guidelines.

Regarding the ambient pollutants, PM<sub>10</sub> concentrations were below the legislation limits of 50 µg/m<sup>3</sup> at both stations, while NO<sub>2</sub> concentrations fulfilled the 1 hour hourly maxima 200 µg/m<sup>3</sup> (Directive-2008/50/EC).

## Annex references:

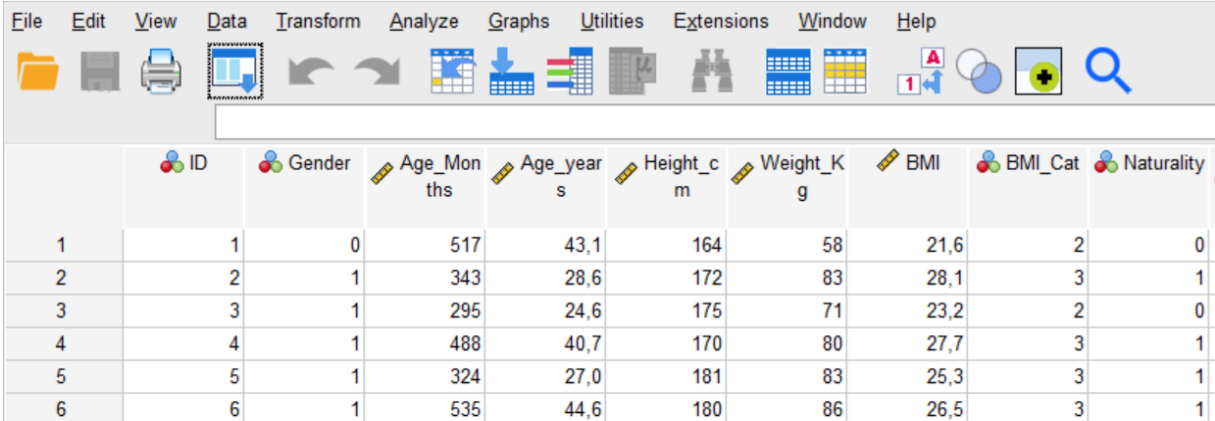
Directive-2008/50/EC. Directive 2008/50/EC of the European Parliament and of the Council of 21 May 2008 on ambient air quality and cleaner air for Europe. *L152*, 1-44. <http://data.europa.eu/eli/dir/2008/50/oj>

Portaria n.º 138-G/2021, de 1 de julho. *Diário da República n.º 126/2021, 2º Suplemento, Série I*. Ministerio do Ambiente. Lisboa

## Annex.7 Data analysis

Figure A7-1, picture of the software, Statistical Package for the Social Sciences, version 27.0.1, practical application.

Database eNose ControlGroup FEUP 2022.sav [DataSet1] - IBM SPSS Statistics Data Editor



|   | ID | Gender | Age_Months | Age_years | Height_cm | Weight_Kg | BMI  | BMI_Cat | Naturality |
|---|----|--------|------------|-----------|-----------|-----------|------|---------|------------|
| 1 | 1  | 0      | 517        | 43,1      | 164       | 58        | 21,6 | 2       | 0          |
| 2 | 2  | 1      | 343        | 28,6      | 172       | 83        | 28,1 | 3       | 1          |
| 3 | 3  | 1      | 295        | 24,6      | 175       | 71        | 23,2 | 2       | 0          |
| 4 | 4  | 1      | 488        | 40,7      | 170       | 80        | 27,7 | 3       | 1          |
| 5 | 5  | 1      | 324        | 27,0      | 181       | 83        | 25,3 | 3       | 1          |
| 6 | 6  | 1      | 535        | 44,6      | 180       | 86        | 26,5 | 3       | 1          |

Figure A7- 1- Database SPSS eNose Control Group, FEUP 2022

Graphical application of cluster analysis using MultyVariomics version 1.11 (João Rufo, Porto, Portugal), represented in Figure A7-2.

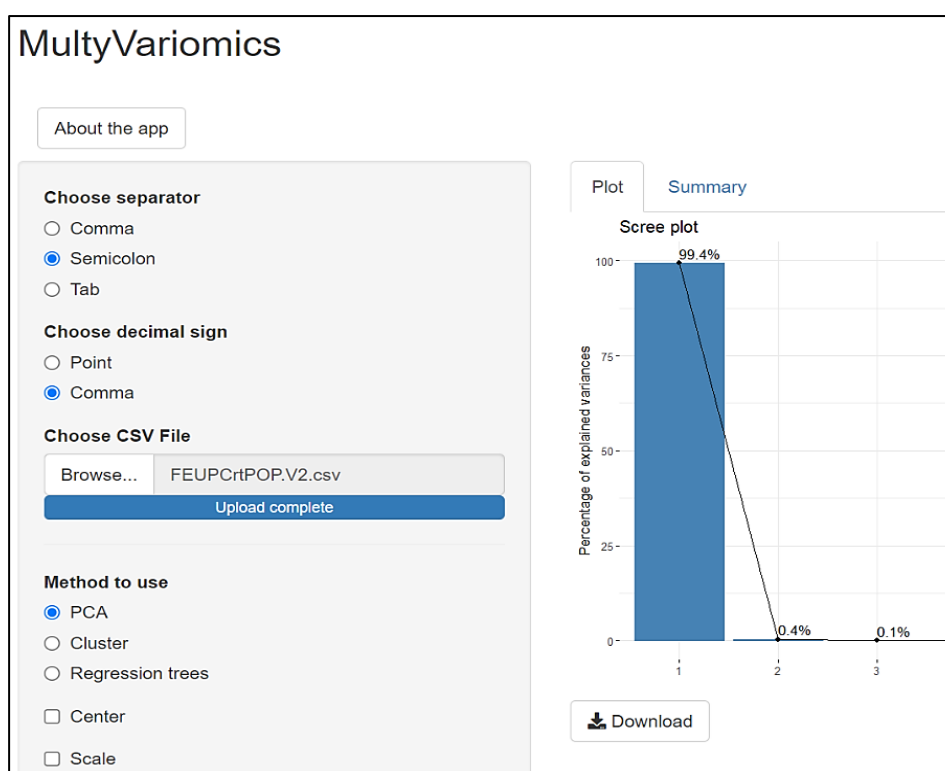


Figure A7- 2- MultyVariomics analysis of our data