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6 1 **Comparison of sampling bags for the analysis of volatile organic**
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29 15 **Keywords**

30 16 Breath analysis, VOCs, Sampling bag, Nalophan, Tedlar, Cali-5-bond

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3 35 **Abstract**
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8 38 Nalophan, Tedlar and Cali-5-Bond polymeric bags were compared to determine the most suitable type for
9 39 breath sampling and storage when volatile organic compounds are to be determined. Analyses were
10 40 performed by thermal desorption gas chromatography mass spectrometry.

11 41 For each bag, the release of contaminants and the chemical stability of a gaseous standard mixture containing
12 42 eighteen organic compounds, as well as the CO₂ partial pressure were assessed. The selected compounds
13 43 were representative of breath constituents and belonged to different chemical classes (i.e., hydrocarbons,
14 44 ketones, aldehydes, aromatics, sulfurs and esters). In the case of Nalophan, the influence of the surface-to-
15 45 volume ratio, related to the bag's filling degree, on the chemical stability was also evaluated.

16 46 Nalophan bags were found to be the most suitable in terms of contaminants released during storage (only 2-
17 47 methyl-1,3-dioxalane), good sample stability (up to 24 hours for both dry and humid samples), and very
18 48 limited costs (about 1 € for a 20 liter bag). The (film) surface-to-(sample) volume ratio was found to be an
19 49 important factor affecting the stability of selected compounds, and therefore we recommended to fill the bag
20 50 completely.
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1. Introduction

The chemical characterization of volatile compounds in human breath is a potential tool for modern medicine in order to obtain clinically relevant information on ongoing body physiological processes in a non-invasive way [1]. Exhaled breath analysis is typically carried out off-line by collecting a sample in a suitable container/trap, concentrating the analytes of interest into a solid phase extraction device, and analyzing it by thermal desorption gas chromatography couple to mass spectrometry. [2, 3, 4] Analytical techniques that employ the direct injection of breath sample into the instrument, i.e. selected ion flow tube mass spectrometry, proton transfer reaction mass spectrometry, ion mobility spectrometry and laser-based spectroscopy [5, 6, 7, 8], are also available for real time measurements. However, on-line analysis has two major drawbacks: the cost of the instrumentation and a somewhat less certain identification of compounds. For example, in the case of proton transfer mass spectrometry, each detected ion can be associated to parent molecules, fragments of parent molecules, and water clusters, or to a combination of these. In addition, only compounds with a proton affinity higher than water can be detected.

Thus, off-line techniques are still the most used methods, although sampling and sample stability are the most critical steps in the entire analytical procedure. In fact, phenomena like interaction with the sampling container (adsorption/desorption processes and release from the container material itself), permeation through the container walls (loss of sample components and contamination of external pollutants), as well as chemical reactions facilitated by high humidity and highly reactive species can modify the original composition of the sample and lead to erroneous conclusions [9].

Several types of containers, such as gas tight syringes, glass bulbs, stainless steel canisters and sampling bags, can be used for sampling and storing of breath samples. Syringes and glass bulbs are cheap and easy to use and clean, but they are also fragile and with a limited volume [10, 11, 12]. Pre-evacuated canisters are robust and provide an optimum stability of the sample after a suitable treatment of the surfaces. However, they are relatively heavy, bulky, expensive and require an effective cleaning procedure for multiple use [13, 14]. Polymer bags e.g. Tedlar (PVF, polyvinyl fluoride), Teflon (PTFE, polytetrafluoroethylene), Nalophan (PET, polyethylene terephthalate) and metal-coated multilayer bags (Flexfoil and polyester-aluminum, PEA) as sampling containers in breath analysis have also been investigated as a possible alternative [5, 15, 16, 17, 18]. There are several problematic issues: (i) chemical stability of samples, (ii) cleaning procedures in the case of multiple use or in the presence of a non negligible background, and (iii) cost. Chemical stability is strongly affected by the film thickness of the bag's walls, the permeation coefficient of the compound related to the bag material, and the (film) surface-to-(sample) volume ratio (S/V), which in turn controls the permeation through the wall bags. [17, 19].

Several studies have investigated the suitability of various polymer bags for the storage of breath constituents. Groves and Zellers [20] studied the influence of high humidity on the recovery of six breath-related compounds (methanol, acetone, 2-butanone, m-xylene, trichloroethane and perchloroethylene) at the ppm level in Tedlar bags. Only methanol was slightly affected (10%) at breath humidity levels. Steeghs et al. [21] investigated the stability of a gaseous humid mixture, composed of seven compounds (methanol,

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3 109 acetaldehyde, acetone, isoprene, benzene, toluene and styrene) in the concentration range of 100–200 ppbv,
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5 110 over a period of 72 hours in black-layered Tedlar bags. The results showed that only the styrene
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7 111 concentration decreased more than 20%.

8 112 A more detailed study, on the storage capability of Tedlar bags for gaseous compounds, was performed by
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10 113 Beauchamp et al. [15]. They tested 12 classes of chemical compounds (including alcohol, nitrile, aldehyde,
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12 114 ketone, terpene and aromatic compounds) in the concentration range of 64–85 ppbv. After storage of 10
13 115 hours, losses were less than 20% for all the analytes investigated.

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15 116 The suitability of Tedlar, Nalophan, Flexfoil and Teflon bags for the storage of volatile sulfur compounds
16 117 (VSCs) was assessed by Mochalski et al. over a period of 48 hours [16]. Flexfoil bags were the best choice
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18 118 for the VSCs storage up to 24 hours (stability of about 90%), although the authors suggested that Tedlar bags
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20 119 represent a good alternative to Flexfoil. Gilchrist and co-workers [22] investigated the stability of breath
21 120 samples containing hydrogen cyanide in 25 and 70 μm thick Nalophan and Tedlar bags at 20 °C and 37 °C.
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23 121 Their results showed for all bags a better correlation between concentrations measured on-line and off-line at
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25 122 37 °C rather than at 20 °C. The correlation between hydrogen cyanide concentrations measured on-line and
26 123 off-line in breath samples stored at 37 °C was good up to 24 h for 70 μm thick Nalophan and Tedlar bags.
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28 124 These findings suggested that both sampling bags would be appropriate for the collection of breath samples
29 125 containing hydrogen cyanide.

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31 126 Mochalski and co-workers [17] investigated the stability of 41 breath constituents (including hydrocarbons,
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33 127 ketones, aldehydes, aromatics, sulfurs, esters, terpenes, etc.) at ppb levels in Tedlar, Kynar (polyvinylidene
34 128 difluoride, PVDF), and Flexfilm (SKC Inc., unknown polymer composition) sampling bags. They found that
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36 129 Tedlar bags were better in terms of background emission, reusability and stability (up to 7 days for dry
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38 130 samples), although the recovery from the Tedlar bags was influenced by the high content of water (losses up
39 131 to 10%). The authors also reported a more pronounced loss (20–40%) only for volatile compounds with
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41 132 molecular masses higher than 90 Da.

42 133 Based on this background information, the aim of the present study was to determine the most appropriate
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44 134 bag material for breath sampling. A critical evaluation was then carried out by comparing Tedlar, the most
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46 135 commonly used material, with Nalophan and Cali-5-Bond, whose numerous applications in environmental
47 136 monitoring [23, 24, 25] suggest that them could also be used for breath analysis. The comparison was
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49 137 performed by testing, up to 72 hours, the release of interfering compounds from the material itself, the
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51 138 stability of CO₂ partial pressure (pCO₂) and the chemical stability of a standard gaseous mixture, containing
52 139 eighteen volatile organic compounds (VOCs) at known concentrations (~~150~~180–~~450~~420 ppbv). The selected
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54 140 compounds were representative of breath constituents and belonged to different chemical classes (i.e.,
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56 141 hydrocarbons, ketones, aldehydes, aromatics, sulfurs and esters) [26, 27].

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58 143 **2. Materials and methods**

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60 145 *2.1. Sampling bags*

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Bags made of three different materials were compared to evaluate sample stability and the release of compounds from the bag walls, namely:

1. *Nalophan*TM: a polyethylene terephthalate (PET) film with a thickness of 20 μm . Dimensions of the deflated bag: 70 cm x 47 cm;
2. *Tedlar*[®]: a polyvinylfluoride (PVF) film with a thickness of 50 μm . Dimensions of the deflated bag: 60 cm x 76.5 cm;
3. *Cali-5-Bond*TM: five layers of different material assembled to form a single, flexible material (from inside to outside: a 75 μm high density polyethylene sheet, a 40 μm polyamide layer, a 12 μm aluminum foil, a 3–4 μm polyvinyl dichloride layer and a 12 μm polyester layer) with a thickness of about 140 μm . Dimensions of the deflated bag: 38.5 cm x 46 cm.

Nalophan bags were fabricated from a roll of Nalophan tube, with a diameter of 47 cm and a thickness of 20 μm , supplied by Kalle (Germany). [Figure 1 shows the step by step assembly of a Nalophan bag.](#) ~~To make a sampling bag, a~~ 70 cm long paring was cut from the roll and then an 8 cm strip from one cut was folded in half to obtain a dead end ([figure \(1a\)](#)). This folded edge was folded again in the orthogonal direction, starting from each border towards the middle of the bag, so that two series of superimposed ~~1-centimeter~~ cm large creases were obtained ([figure \(1b\)](#)). Finally, the resulting bundle of creases was folded in half ([figure \(1c\)](#)) and then tightened using a nylon cable tie ([figure \(1d\)](#)). A simplified procedure was used for the other end of the Nalophan paring, as in this case the first and last steps were not performed and the two series of creases ([figure \(1e\)](#)) were tightened around a PTFE tube (1/4 inch i.d., 6 cm length) connected to a stopcock (Nordival Srl, Italy) placing another nylon cable tie 2 cm from the bag end ([figure \(1f\)](#)). ~~Figure 1-2 shows our hand-made disposable Nalophan bag assembled according to the procedure described above once filled with the sample.~~

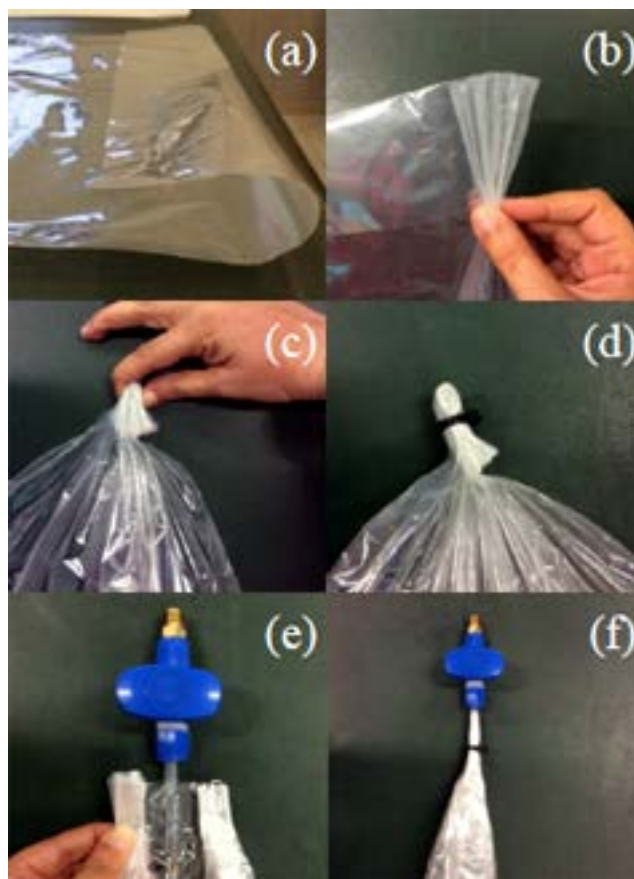


Figure 1. Assembly of a Nalophan sampling bag.

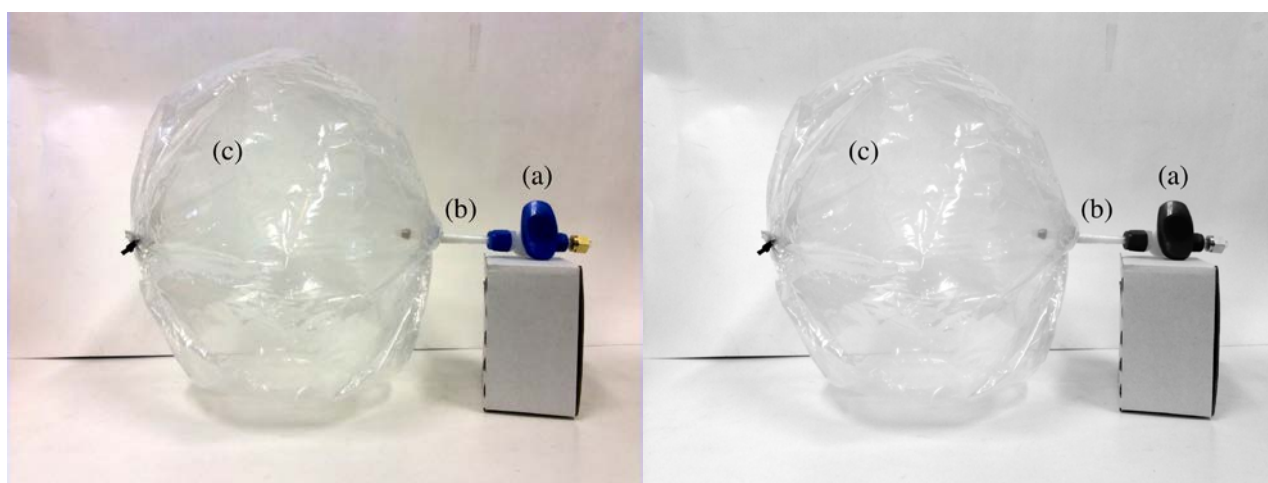


Figure 12. Nalophan sampling bag composed of a) stopcock, b) PTFE tube, and c) Nalophan bag.

Tedlar and Cali-5-Bond bags were purchased from SKC (USA) and Alltech (Italy), respectively.

2.2. Chemicals

Hexanal, 2-propanol, 2-butanone, 2-pentanone, 2-heptanone, 4-heptanone, heptanal and benzaldehyde were purchased from AccuStandard, Inc. Chemical Reference Standard (USA). Isoprene, acetone, pentane, 2-methylpentane, hexane, 1,1,1,3,3,3-hexafluoro-2-propanol, carbon sulfide, dimethylsulfide,

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3 182 dimethyldisulfide and toluene were purchased from Fluka, Sigma-Aldrich (Italy). All the compounds were
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5 183 GC grade standard with a higher than 99% purity. Labeled toluene-D8 was purchased at a purity of 99.8%
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7 184 from ARMAR Chemicals (Switzerland). All chemicals were used without any further purification.
8 185 Helium 5.6 IP and medical air (hydrocarbon free, purity of 99.95%) were purchased from Sol Group Spa
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10 186 (Italy).

11 187 A binary standard gaseous mixture consisting of 5% CO₂ in nitrogen, purchased from Sol Group Spa (Italy),
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13 188 was used to test the stability of pCO₂ (mmHg) in the sampling bags.

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15 189 Ultrapure water was obtained by a PureLab Classic Pro, USF Elga instrument (Italy).

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17 18 191 2.3. Preparation of standard gaseous mixtures

19 192 A liquid mixture was prepared by mixing 50 μL of eighteen pure liquid compounds in a glass vial equipped
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21 193 with a screw-cap mininert valve (Sigma Aldrich, Italy). ~~Then a~~ A stock standard gaseous mixture (MIX 18)
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23 194 was then obtained by introducing 20 μL of the liquid mixture into a 2 L glass flask equipped with a screw-
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25 195 cap mininert valve (Sigma Aldrich, Italy) and pre-evacuated using a vacuum membrane pump. The glass
26 196 flask was heated at 37 ± 1 °C to ensure complete evaporation of the liquid and subsequently balanced to
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28 197 ambient pressure. This gaseous mixture was kept in a 1.1 m³ thermostat at 37 ± 1 °C. The storage time of the
29 198 liquid solution, kept at 4 °C to minimize the risk of evaporation, and of the gaseous mixture was three and
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31 199 one month, respectively. The gaseous mixture was prepared once again if the amount of subtracted volume
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33 200 exceed 5% of the glass flask volume.

34 201 The concentration of the analytes in the glass flask is reported in table 1. This stock standard gaseous mixture
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36 202 was then used to prepare diluted standard gaseous mixtures in the bags.

37 203 A gaseous solution of labeled toluene-D8, for use as an internal standard, was prepared at a concentration of
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39 204 600 ppmv by evaporation of 5 μL of the liquid compound in a pre-evacuated 2 L glass flask equipped with a
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41 205 screw-cap mininert valve (Sigma Aldrich, Italy), heated at 37 ± 1 °C. This gaseous solution was stored in the
42 206 thermostat at 37 ± 1 °C for one month.
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Table 1. Concentration of 18 components in the glass flask calculated at 37 °C.

Analytes	Concentration in the glass flask (ppmv)
Pentane	110
Isoprene	130
Acetone	170
Dimethylsulfide	170
Carbon sulfide	210
2-propanol	210
2-methylpentane	100
Hexane	100
2-butanone	140
2-pentanone	120
1,1,1,3,3,3-hexafluoro-2-propanol	120
Dimethyldisulfide	140
Toluene	120
Hexanal	100
4-heptanone	90
2-heptanone	90
Heptanal	90
Benzaldehyde	130

2.4. Sample pre-concentration and analysis

Volatile organic compounds were analyzed by the method described elsewhere [26–28]. Sampling bags were stabilized for half an hour in the thermostat kept at 37 ± 1 °C to prevent water condensation on the bag walls.

An aliquot of sample (250 mL) was then flowed through a drying tube filled with 9 g of anhydrous sodium sulfate (SKC, USA) for water removal and transferred by a pocket pump into a glass adsorption tube pre-packed with 250 mg of 60/80 mesh Tenax GR phase (70% Tenax TA, 2,6-diphenyl-p-phenylene oxide and 30% graphite) purchased from Supelco (USA). The sample flow through the tubes (50 mL/min) was set up on the pocket pump, verified by a digital soap bubble flow meter (A. P. Buck Inc., USA) and continuously controlled by a rotameter (range 0-150 mm). During the sample transfer, the sampling bag and the drying tube were kept at 37 ± 1 °C, whereas the adsorption tube was at room temperature (about 20 °C).

The adsorption tubes were thermally desorbed by an automated STD 1000 two-stage thermal desorption unit (DANI Instruments, Italy) equipped with an internal focusing trap packed with 70 mg of Tenax GR (DANI Instruments, Italy) and connected to a Trace GC Ultra gas chromatograph (Thermo Electron Corporation, USA) coupled to a Trace DSQ quadrupole mass spectrometer (Thermo Electron Corporation, USA) operating in the positive electron impact ionization (70 eV) mode. The first desorption was carried out at 250 °C for 5 min under a helium splitless flow of 35 mL/min. The sample was concentrated into a 5 °C cold trap,

which was then rapidly heated to 250 °C. This second desorption allowed the fast transfer of the analytes to a DB-624 capillary column (60 m length, 0.25 mm internal diameter, 1.4 µm film thickness) composed of 6% cyanopropyl phenyl siloxane and 94% dimethylpolysiloxane (Agilent Technologies, USA). The temperature profile of the chromatographic oven was as follows: initial temperature 35 °C, isothermal for 10 min; 4 °C/min up to 130 °C and isothermal for 2 min, 20 °C/min up to 250 °C and isothermal for 10 min, 25 °C/min up to 260 °C and isothermal for 15 min. The inlet temperature was set at 200 °C. Helium 5.6 IP was used as a carrier gas at a constant pressure of 210 kPa with a split flow of 10 mL/min. The ion source and transfer line were kept at 250 °C and 260 °C, respectively. Chromatograms were collected both in Total Ion Current (TIC), with an m/z range set from 18 to 200.

Peak integration was based on the extracted ion chromatograms. The retention times of the investigated compounds for the applied chromatographic parameters as well as the quantifier ions used for the integration are presented in table 2.

The thermal desorption unit was controlled by TD Manager software (v. 3.2 DANI Instruments, Italy) and the GC-MS system was controlled by Xcalibur software (v. 1.4, Thermo Electron Corporation, USA). The unknown compounds, [released from the bag materials during the background test](#), were identified by the reference library (NIST MS search v. 2.0).

Table 2. Retention times and [characteristic m/z values of the quantification-quantifier ions](#) of the investigated compounds.

Compound	Retention Time (min)	Quantifier ion (m/z)
Pentane	6.27	43
Isoprene	6.98	67
Acetone	7.77	58
Dimethylsulfide	7.97	62
Carbon sulfide	8.27	76
2-propanol	8.38	45
2-methylpentane	9.55	43
Hexane	11.67	57
2-butanone	14.36	43
2-pentanone	20.01	43
1,1,1,3,3,3-hexafluoro-2-propanol	21.23	99
Dimethyldisulfide	22.59	94
Toluene-D8	23.24	98
Toluene	23.45	91
Hexanal	25.92	44
4-heptanone	29.73	71
2-heptanone	30.44	43
Heptanal	30.67	70

Benzaldehyde	32.69	105
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2.5. Background test

To identify the contaminants released from the various materials, a bag of each type was filled with dry medical air (20 L Nalophan, 30 L Tedlar and 10 L Cali-5-Bond) to obtain the same S/V ratio (0.3 cm⁻¹).

The volume of medical air introduced into the bags was calculated from the flow and filling time. The flow of 500 mL/min was verified by a digital soap bubble flow meter and continuously controlled during filling by a rotameter (range 0-150 mm) while the bag was being filled. For each bag, three adsorption tubes were loaded with the sample (250 mL) at [each observation time, namely](#) 0.5, 3, 6, 24, 48 and 72 hours after filling the bags. These tubes were then analyzed following the procedure described in section 2.4.

To test the effectiveness of the cleaning procedure, a volume of 20 and 60 mL of standard gaseous mixture (MIX18) from the flask was injected into the flow of medical air during the filling of Tedlar and Cali-5-Bond bags with 30 and 10 L of air, respectively. After 1 hour of storage at 37 °C, the bags were alternatively deflated and inflated ten times with dry medical air at 37 °C. All the filled bags were then kept at 37 °C for 24 hours. The effectiveness of the cleaning procedure was checked by comparing the concentration levels of MIX18 components measured 1 hour after filling the bags with a standard mixture and 24 hours after performing the cleaning procedure.

2.6. Dry standard stability test

The concentrations of the MIX18 components and the pCO₂ were monitored over a period of 72 hours to assess the [compounds](#) stability in the different bags with an S/V ratio of 0.3 cm⁻¹.

The MIX18 components, with different polarities and volatilities, are all of potential interest both in breath and ambient air [\[26, 27\]](#). A volume of 40, 60 and 20 mL of MIX 18 from the flask was injected in the flow of medical air during the filling of Nalophan, Tedlar and Cali-5-Bond bags with 20, 30 and 10 L of air, respectively. [Table 3 reports the calculated analytes concentration in the bags resulting from dilution.](#) For each bag, three adsorption tubes were loaded with the bag content (250 mL) at [each observation time, namely](#) 0.5, 3, 6, 24, 48 and 72 hours after filling the bags. The tubes were then analyzed following the procedure described in section 2.4.

To test the stability of pCO₂, the bags were filled with a standard gaseous mixture consisting of 5% CO₂ in nitrogen (500 mL/min). As previously reported, the volume of CO₂ mixture introduced into the bags (20 L for Nalophan, 30 L for Tedlar and 10 L for Cali-5-Bond) was calculated from the flow and filling time as previously reported. Each bag was equilibrated at 37 ± 1 °C for 30 minutes. CO₂ content was then measured 0.5, 1, 3, 6, 24, 29, 32, 48 and 72 hours after bag preparation. The measurement of pCO₂ (mmHg) was carried out by flowing (100 mL/min) the gaseous mixture from the sampling bags for 5 seconds through a Capnostat® 5 fast mainstream infrared sensor (Respironics Inc., USA).

[Table 3. Concentration of 18 components in the sampling bags at 37 °C.](#)

<u>Analytes</u>	<u>Concentration in the bags (ppbv)</u>
<u>Pentane</u>	<u>220</u>
<u>Isoprene</u>	<u>260</u>
<u>Acetone</u>	<u>340</u>
<u>Dimethylsulfide</u>	<u>340</u>
<u>Carbon sulfide</u>	<u>420</u>
<u>2-propanol</u>	<u>420</u>
<u>2-methylpentane</u>	<u>200</u>
<u>Hexane</u>	<u>200</u>
<u>2-butanone</u>	<u>280</u>
<u>2-pentanone</u>	<u>240</u>
<u>1,1,1,3,3,3-hexafluoro-2-propanol</u>	<u>240</u>
<u>Dimethyldisulfide</u>	<u>280</u>
<u>Toluene</u>	<u>240</u>
<u>Hexanal</u>	<u>200</u>
<u>4-heptanone</u>	<u>180</u>
<u>2-heptanone</u>	<u>180</u>
<u>Heptanal</u>	<u>180</u>
<u>Benzaldehyde</u>	<u>260</u>

2.7. Effect of humidity and surface-to-volume ratio on the sample stability in Nalophan bags

Water vapor is a major component of exhaled breath, whose relative humidity (RH) is close to 100% at 37 °C. Since a high humidity content strongly affects the performance of the solid phase extraction (SPE) technique [27][29], tests were carried out to evaluate water vapor diffusion through Nalophan bag walls at 37 °C. For this purpose, a 20 L Nalophan bag (S/V ratio of 0.3 cm⁻¹), equipped with a polypropylene valve with an integrated septum, was filled with multiple breathes at room temperature (20 °C). A real breath sample was used in order to have an RH value close to 100% in the shortest time possible (about 4 min), thus preventing any loss of water vapor that could occur in the time required (40 min) to fill the bag with humid medical air. The RH (%) and temperature (°C) inside the bag were continuously measured (response time of 80 ms) up to 24 hours using a portable thermo-hygrometer (Delta Ohm, Italy) equipped with an immersion probe (o.d. 2 mm, 230 mm length) and operating between 5 and 98% of RH.

The role of the (film) surface-to-(sample) volume ratio on the VOCs concentration decay inside the Nalophan bag was evaluated in three bags, with a calculated surface area of about 7000 cm², ~~created~~ fabricated from a ~~piece of tubular film~~ 70 cm long paring of Nalophan tube. These bags were filled with different amounts (20, 10 and 7 L) of the humidified test mixture (MIX 18), thus producing bags with different S/V ratios (0.3, 0.7 and 1.0 cm⁻¹).

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3 309 To simulate the content of water vapor of real breath samples, humid gaseous mixtures were prepared by
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5 310 flowing medical air (500 mL/min) through a purge and trap glass system filled with 5 mL of fresh milli-Q
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7 311 water at room temperature (20 °C). After the half filling time, an aliquot of MIX 18 from the flask was
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9 312 injected into the flow of humidified medical air, during the filling of the three Nalophan bags, to obtain a
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11 313 500-fold dilution. Once again, the volume of humidified medical air introduced into the bags was calculated
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13 314 from the flow and filling time as previously reported. All these bags were stored at a 37 ± 1 °C during the
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15 315 test. Three adsorption tubes were loaded with the sample (80 mL) at each observation time, namely 0.5, 3, 6,
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17 316 24, 48 and 72 hours after filling the bags and ~~then~~ analyzed following the procedure described in section 2.4.
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19 317 The stability of pCO₂ in the Nalophan bag with different S/V ratios (0.3, 0.7 and 1.0 cm⁻¹) was also tested in
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21 318 humid conditions using the same analytical procedure used for testing CO₂ stability in dry condition. For this
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23 319 purpose, humid gaseous CO₂ samples were prepared by flowing different volumes (20, 10 and 7 L) of a
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25 320 standard gaseous CO₂ mixture (500 mL/min) through a purge and trap glass system filled with 5 mL of fresh
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27 321 milli-Q water at room temperature (20 °C).

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29 322 Moreover, an additional test was carried out to simulate a real situation in which the sample is kept at
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31 323 ambient temperature for some time before being stabilized at 37 ° C. For this purpose, two Nalophan bags
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33 324 (20 L), having an S/V ratio of 0.3 cm⁻¹, were filled with humidified medical air (500 mL/min). During filling,
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35 325 an aliquot (40 mL) of MIX 18 from the flask was injected into the air flow. One bag was kept in the
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37 326 thermostat at 37 ± 1 °C and about 15 % RH for 15 hours, whereas the other was kept in the room at about 22
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39 327 °C and 45 % RH, before being stabilized for half an hour in the thermostat. The content of each bag (250
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41 328 mL) was then transferred into three adsorption tubes and analyzed according to the procedure described in
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43 329 section 2.4. The same experiment was performed using the humidified standard gaseous CO₂ mixture.

3. Results and discussion

3.1. Background test and effectiveness of cleaning procedure

Table 34 shows the most abundant contaminants, released for each bag material.

Table 34. Compounds released in dry medical air from sampling bags.

Compound	Bag
2-methyl-1,3-dioxolane (N1)	Nalophan
N,N-dimethylacetamide (T1)	Tedlar
Phenol (T2)	Tedlar
Acetone (C1)	Cali-5-Bond
Ethylacetate (C2)	Cali-5-Bond
2-ethyl-3-methyl-1-pentene (C3)	Cali-5-Bond
Toluene (C4)	Cali-5-Bond
1-metoxi-2-propylacetate (C5)	Cali-5-Bond
2,2,4,6,6-penthamethylheptane (C6)	Cali-5-Bond

Remarkable differences were found among the three bags in terms of release of contaminants. In our conditions, Nalophan was the cleanest material as only 2-methyl-1,3-dioxolane was measured. This compound has been reported to be present among the volatiles emitted by recycled PET samples as coming from polymer impurities [28]-[30]. It can react with hydrogen sulfide, but the required conditions are unlikely to happen in gaseous samples [29][31]. N,N-dimethylacetamide and phenol were detected in Tedlar bags. These compounds are generally thought to be attributable to the bag manufacturing process [23,30-32]. A large number of compounds were identified in the Cali-5-Bond bag; probably related to the solvent used for the production of the polymeric films as well as the assembly procedures of the five films that make up the bag.

Figure 2-3 shows the trend of the compounds released over time from Nalophan (figure 3(a)), Tedlar (figure 3(b)) and Cali-5-Bond (figure (3c)). Data are reported as average values of the areas of the chromatographic signals of the compounds in the sample, normalized with respect to toluene-D8 peak area and the mean value at the first sampling time (0.5 hour after filling the bags).

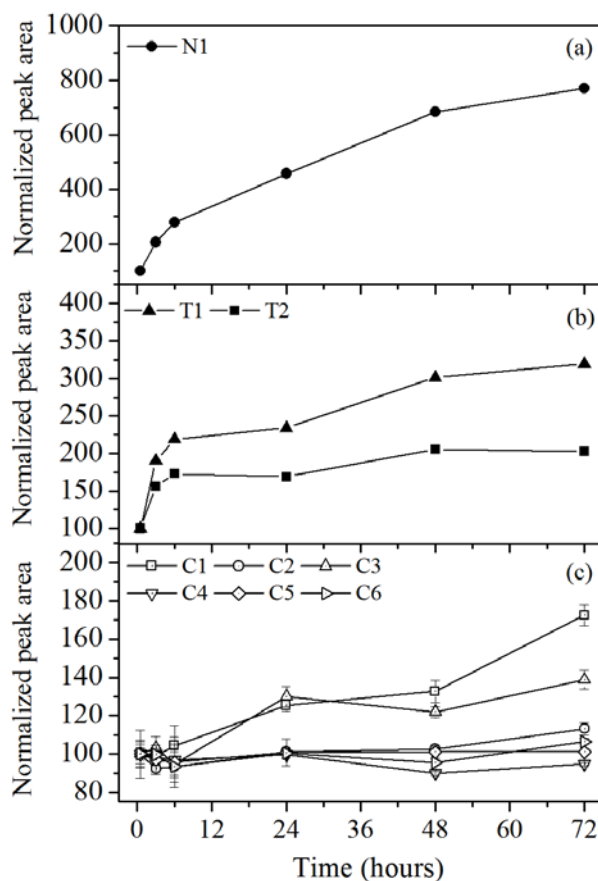


Figure 23. Release over time of the compounds from Nalophan (a), Tedlar (b) and Cali-5-Bond (c) bags. Legends are explained in table 34. Error bars correspond to the standard deviation of three replicates.

In the time-span of 72 hours, we observed a marked increase of up to 800%, 300% and 200% in the amount measured at the first sampling time for 2-methyl-1,3-dioxolane, N,N-dimethylacetamide and phenol, respectively. The amount of acetone and 2-ethyl-3-methyl-1-pentene released from Cali-5-Bond was constant for six hours and increased in the later hours reaching 170% and 140% at 72 hours, respectively. On the contrary, the content of the other compounds released from Cali-5-Bond never changed.

Neither the Tedlar nor the Cali-5-Bond bags are suitable for collecting breath samples without a preventive cleaning procedure for multiple use. Several authors have evaluated the possibility of using a cleaning procedure to minimize the background levels of compounds, both those released from the bag material and those from the previous sample collection, using a cleaning procedure [15, 17]. Thus, the possibility of reducing the background compounds of Tedlar and Cali-5-Bond bags was evaluated by carrying out ten cleaning cycles consisting in inflating the bags with dry medical air at 37 °C and then deflating them.

In both bags, a reduction of about 90% was observed for most of the compounds after the cleaning cycles. Nevertheless, 10% of carryover might be not negligible in the case of compounds at concentration levels close to the detection limit. In addition, considering that the decontamination procedures are tedious, time-consuming and do not always guarantee an acceptable reproducibility, the best solution for the breath sampling appears to be the use of disposable bags, with a low-cost material.

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3.2. Stability test of dry standard mixtures

The results of the stability test, carried out for the three types of bags with the same S/V ratio (0.3 cm^{-1}), are shown in figures [34](#), [45](#) and [56](#). For each observation time, the figures show the average values of the peak areas of the compounds present in the dry gaseous standard mixture. The peak areas were normalized with respect to the toluene-D8 peak area and to the area of the peak corresponding to the first observation time (0.5 hours after filling the bags). The chemical stability of the compounds, in the Nalophan, Tedlar and Cali-5-Bond bags, was evaluated by analysis of variance (ANOVA) at a confidence level of 95%.

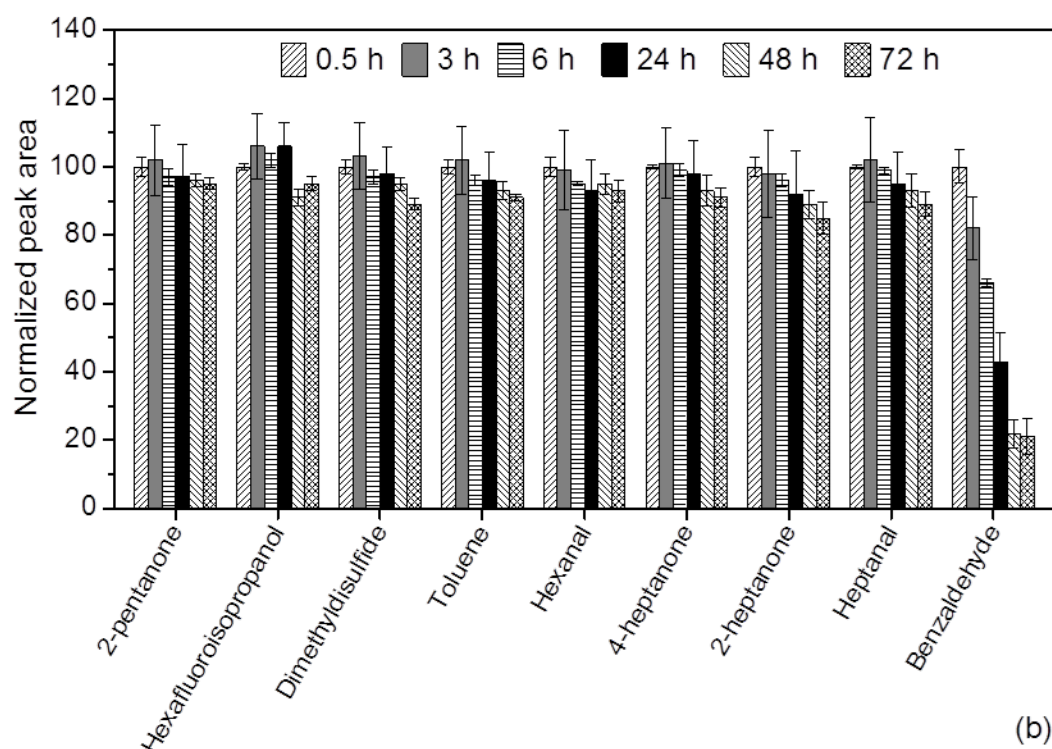
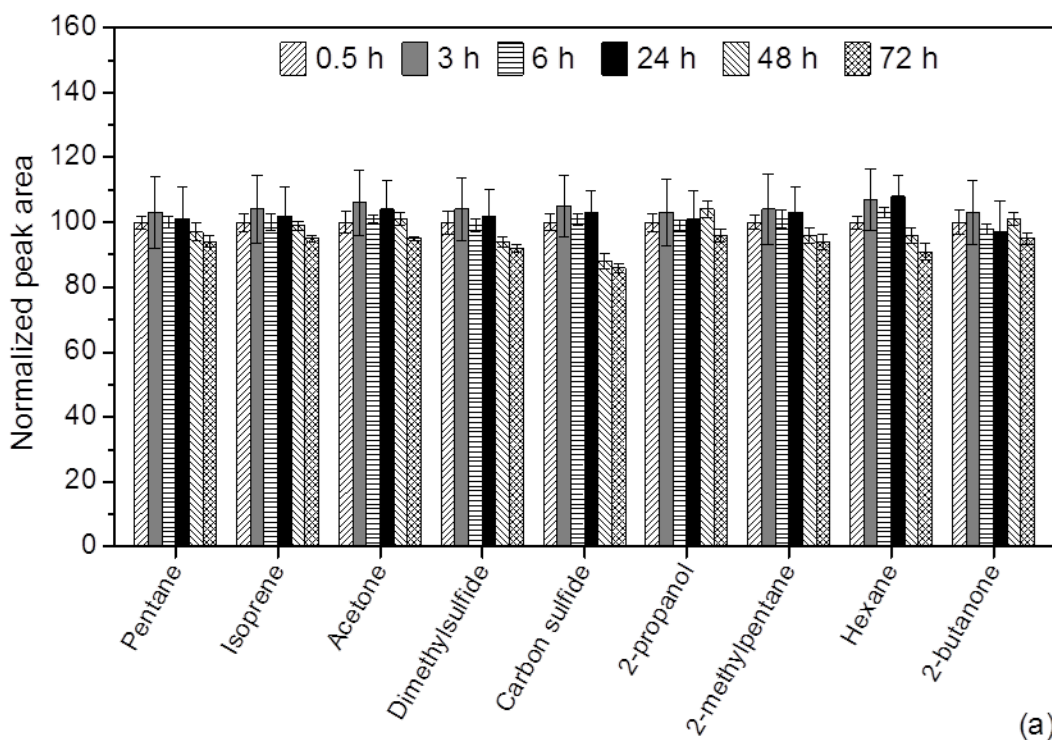
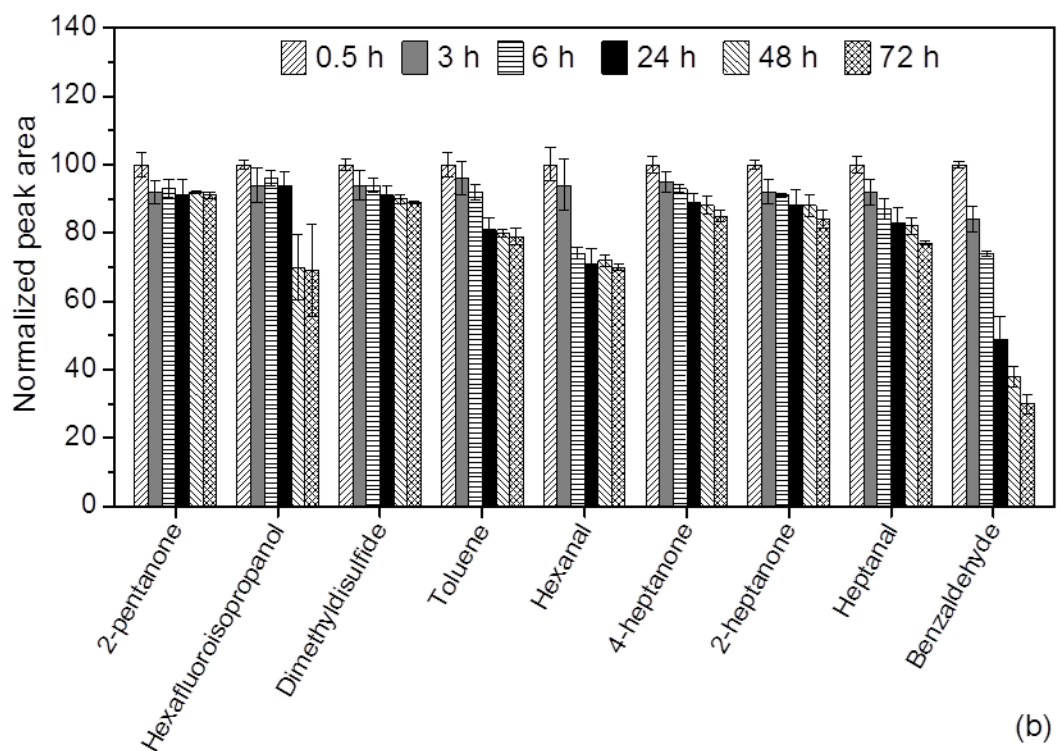
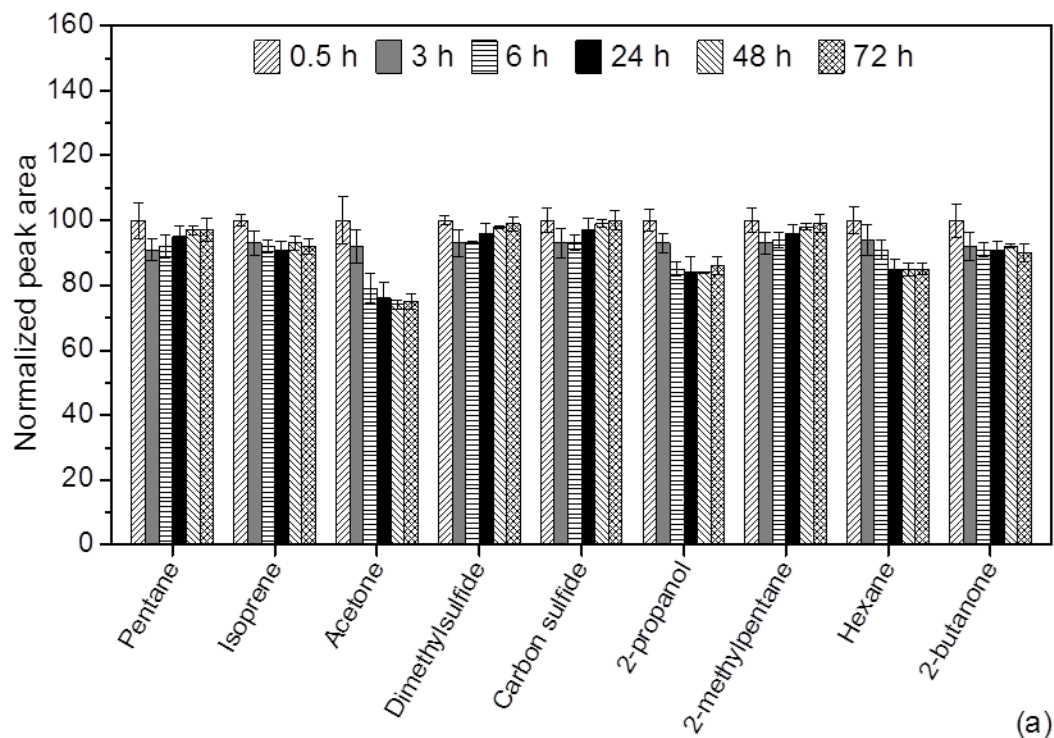


Figure 34. Average values of the peak areas of the compounds in the dry gaseous standard mixture measured in the Nalophan bag, normalized with respect to the toluene-D8 peak area and to the area of the peak corresponding to the first observation time (0.5 hours). Error bars correspond to the standard deviation of three replicates. Compounds reported according to the chromatographic elution: from 6-15 minutes (a) and from 20-35 minutes (b).



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Figure 45. Average values of the peak areas of the compounds in the dry gaseous standard mixture measured in the Tedlar bag, normalized with respect to the toluene-D8 peak area and to the area of the peak corresponding to the first observation time (0.5 hours). Error bars correspond to the standard deviation of three replicates. Compounds reported according to the chromatographic elution: from 6-15 minutes (a) and from 20-35 minutes (b).

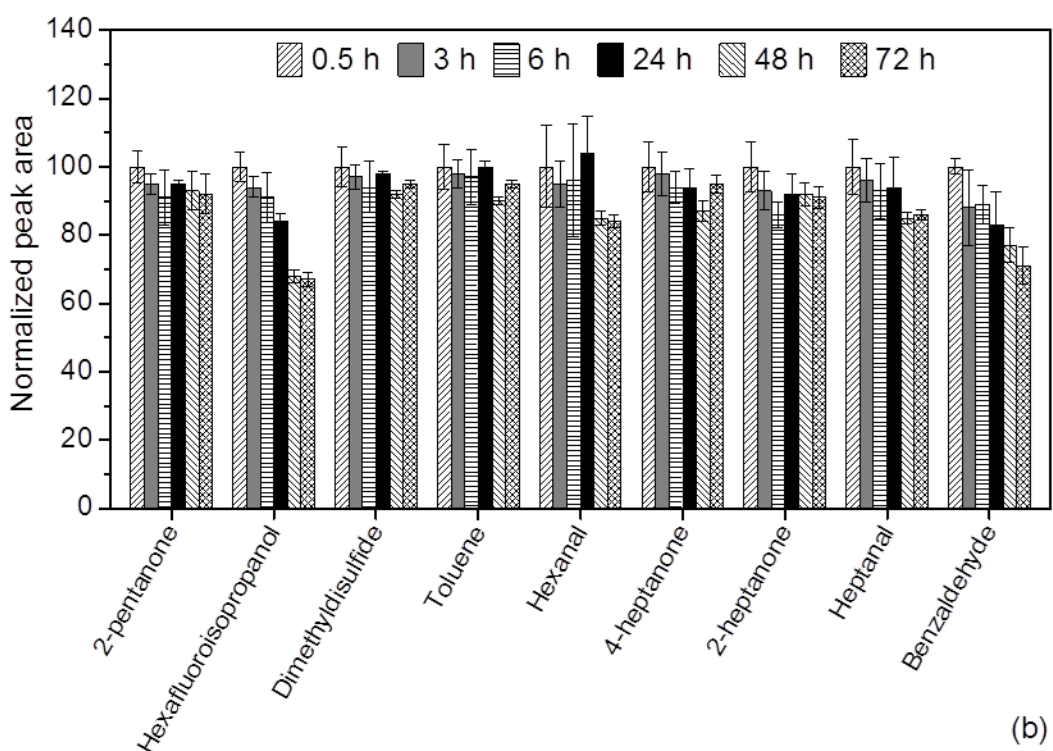
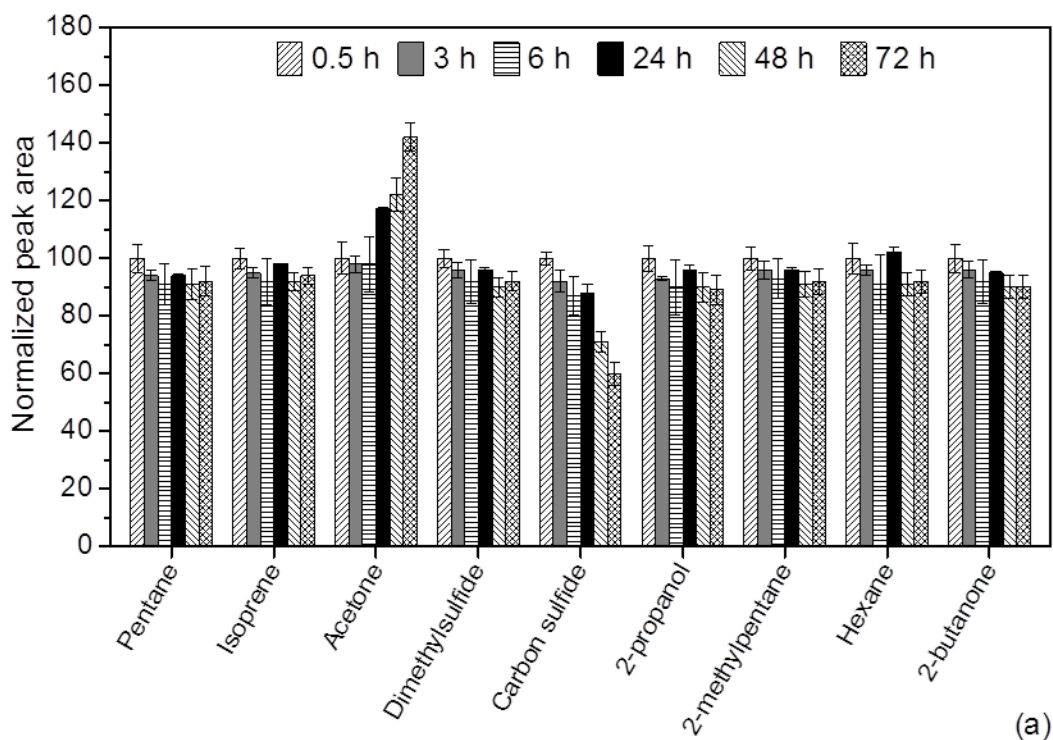


Figure 56. Average values of the peak areas of the compounds in the dry gaseous standard mixture measured in the Cali-5-Bond bag, normalized with respect to the toluene-D8 peak area and to the area of the peak corresponding to the first observation time (0.5 hours). Error bars correspond to the standard deviation of three replicates. Compounds reported according to the chromatographic elution: from 6-15 minutes (a) and from 20-35 minutes (b).

[The difference between the measured concentrations at the first observation time and the calculated ones resulted within the experimental error of 5%, thus excluding any loss related to bag filling and sample drying.](#)

For Nalophan bag, no significant variations were observed for any of the compounds within 24 hours; dimethyldisulfide, toluene and heptanal showed a 20% loss within 72 hours. The benzaldehyde content decreased quickly over time: loss was about 20% after 3 hours, 35% after 6 hours, 60% after 24 hours, 70% after 48 hours, and 80% after 72 hours. [This behavior could be related to the spontaneous oxidation of benzaldehyde to benzoic acid when exposed to air.](#)

For Tedlar bag, within 6 hours, acetone, 2-propanol and hexanal showed a 20% loss, which remained constant over the subsequent hours, whereas toluene had a variation of 20% within 24 hours, which remained constant till the end of the experiment. Hexafluoroisopropanol presented a 30% loss within 48 hours whereas benzaldehyde showed the same behavior as in Nalophan bag, although the variation within 6 hours was slightly less marked (4 vs 6%/h).

For Cali-5-Bond bag, within 24 hours there were no significant variations except for acetone, which showed a signal increase of about 20% and 40% after 24 and 72 hours, respectively. This increase is probably due to a release from the bag's wall, as already mentioned in section 3.1. Within 48 hours, carbon sulfide, hexafluoroisopropanol and benzaldehyde presented a variation of about 25%.

The stability of the CO₂ content in the three sampling bags was also evaluated since this parameter might be useful to normalize breath data collected from multiple breaths [31]-[33]. The data reported in figure 67, show that CO₂ was stable within 24 hours in all the bags, whereas it showed a moderate decrease (about 10%) at 72 hours in the Nalophan bag.

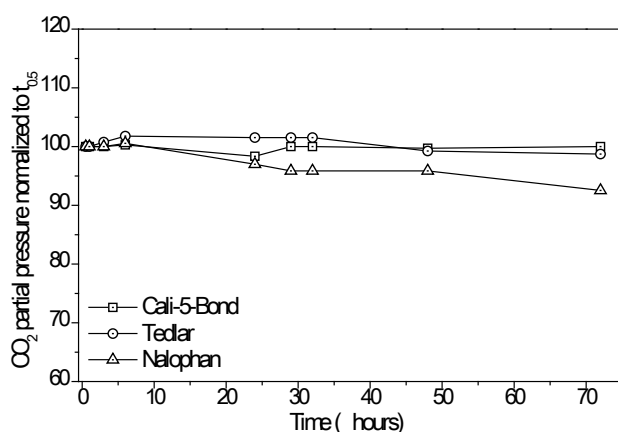


Figure 67. CO₂ partial pressure values measured over time in Nalophan, Tedlar and Cali-5-Bond bags. Data were normalized to the value at the first observation time (0.5 hours).

On the basis of these results, we selected Nalophan for our purposes. In fact, Nalophan has almost zero background contamination, good stability for all the compounds investigated, including CO₂, and low cost

(an estimated cost lower than 1 € for a disposable hand-made 20 liter bag). Moreover, Nalophan bag is suitable for disposable use, thus avoiding bag cleaning procedures.

3.3. Stability test of humid standard mixtures in Nalophan bags

The decay in sample humidity was evaluated at 37 °C, over a storage period of 24 hours, using a real breath sample in order to have a water vapor content close to 100%. Table 4-5 shows the RH and temperature values measured inside the Nalophan bag at each observation time. The first observation time refer to the measurement performed inside the thermostat.

Table 45. Relative humidity and temperature values measured in the Nalophan bag over time.

Observation time (min)	RH (%)	Temperature (°C)
0	12	37.1
2	91	35.6
10	54	36.9
20	43	37.1
30	37	37.2
40	34	37.1
50	29	37.0
60	26	37.1
180	14	36.9
360	13	37.2
480	11	37.1
1440	13	37.2

In the Nalophan bag, the RH rapidly decreases from a high humidity content (about 90% RH) to the approximate ambient air condition observed inside the thermostat (10% RH at 37 °C), within about 3 hours. Further reductions in water vapor were not observed in the later hours, suggesting that such decrease was probably due to the diffusion of water through the bag walls. Losses due to condensation could be ruled out since the Nalophan bag was kept at 37 °C. We confirmed our conclusion by measuring the RH values of the air contained in a cylindrical glass airtight vessel in which we inserted a Nalophan bag, with the same S/V ratio (0.3 cm⁻¹), filled with breath (90% RH). The glass container was filled with medical air (<10% RH) and immediately kept at 37 °C. We found (results not shown) an increase of RH in medical air until an equilibrium between the humidity in the Nalophan bag and in the air inside the glass container was reached (in about 30 minutes).

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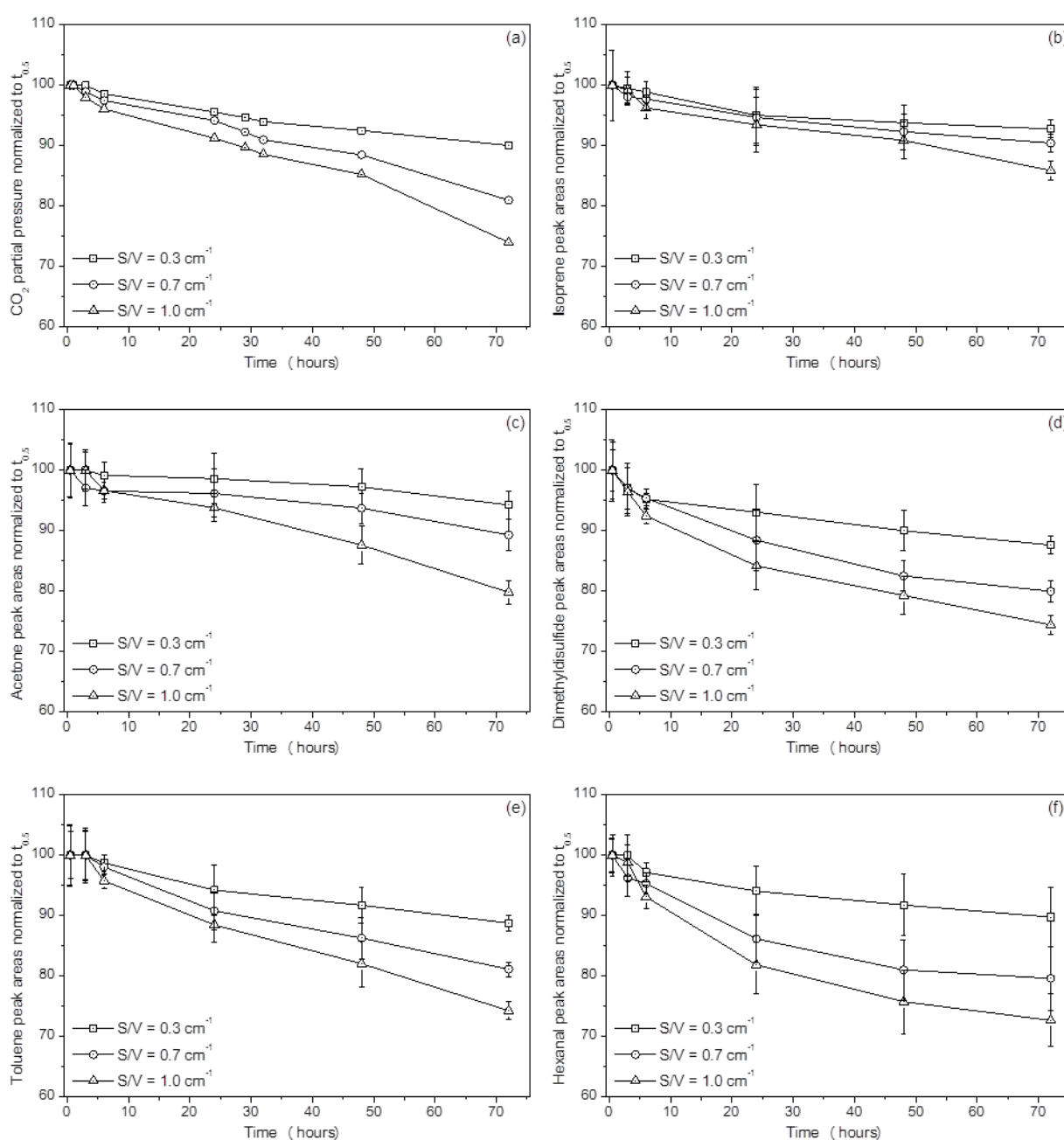
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The influence of the (film) surface-to-(sample) volume ratio on the VOCs concentration and pCO₂ decay inside the Nalophan bag was evaluated using a humid gaseous standard mixture by comparing different S/V ratios (0.3, 0.7 and 1.0 cm⁻¹).

For each observation time, figure 7–8 shows the average values of the peak areas of six compounds characterized by different chemical properties present in the Nalophan bags with S/V ratios of 0.3, 0.7 and 1.0 cm⁻¹. The peak areas were normalized with respect to the toluene-D8 peak area and the area of the peak corresponding to the first observation time (0.5 hours after filling the bags). Also in this case, the stability of the compounds in the Nalophan bag was evaluated by analysis of variance (ANOVA) at a confidence level of 95%.



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3 515 **Figure 78.** Average values of CO₂ partial pressure (a) and of isoprene (b), acetone (c), dimethyldisulfide (d),
4 516 toluene (e) and hexanal (f) peak areas in the humid gaseous standard mixture measured in the Nalophan bags
5 517 (S/V of 0.3, 0.7 and 1.0 cm⁻¹). Peak areas were normalized with respect to the toluene-D8 peak area and to
6 518 the area of the peak corresponding to the first observation time (0.5 hour). Error bars correspond to the
7 519 standard deviation of three replicates.

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10 521 ~~The results of the bag~~ Data concerning stability (S/V ratio of 0.3 cm⁻¹) over time ~~obtained in of~~ the humid
11 522 gaseous mixture prepared in the bag (S/V ratio of 0.3 cm⁻¹) confirmed the same trend observed in dry
12 523 conditions. For the majority of investigated compounds, the difference between their stability in dry and
13 524 humid mixtures was always smaller than 10%, which is in good agreement with the results observed for the
14 525 Tedlar bag by Groves and Zellers [20]. Unlike Mochalski et al [17], we observed a good stability even for
15 526 the heaviest compounds (e.g. hexanal) ~~and this suggests that the use of Nalophan bags enabled the possible~~
16 527 ~~interaction between the water vapor and such compounds to be minimized. These~~ This results are is probably
17 528 related to a more rapid decrease ~~in the amount of the~~ water vapour partial pressure by diffusion through the
18 529 Nalophan bag walls at 37 °C compared to the bags used by Mochalski et al (i.e. Tedlar, Kynar and
19 530 Flexifilm), which minimized the possible interaction between the water and such compounds. Also in this
20 531 case, benzaldehyde confirmed its anomalous behavior, with a loss of about 60% at 24 hours. The results of
21 532 pCO₂ stability over time confirmed the same trend observed in dry conditions, with a decrease of about 10%
22 533 at 72 hours. The chemical stability of humid MIX 18 components in the Nalophan bag (S/V ratio of 0.3 cm⁻¹)
23 534 was not significantly different (within the experimental error of 5%) when the bag was kept for 15 hours at
24 535 ambient conditions (about 22 °C and 45 % RH), before being stabilized in the thermostat. The same result
25 536 was obtained when the CO₂ mixture was used.

26 537 In the case of bags with higher S/V ratios (0.7 and 1.0 cm⁻¹), within 72 hours losses of about 25% and 30%
27 538 were observed for all the compounds, with the exception of benzaldehyde that showed a decrease of more
28 539 than 80% in both bags. These findings prove how the stability of VOCs depends on the degree of bag filling
29 540 (i.e., surface-to-volume ratio), confirming the results obtained for the Tedlar bag by Mochalski et al [17].
30 541 Also the pCO₂ was influenced by the bag's filling degree. In fact, the same variation of 10% was observed
31 542 within 48 and 24 hours for the Nalophan bag with a S/V ratio of 0.7 and 1.0 cm⁻¹, respectively. These
32 543 findings are not surprising since VOCs at high S/V ratio are more vulnerable to losses related to sorption or
33 544 permeation.

34 546 4. Conclusions

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36 548 We evaluated the most suitable bag to collect exhaled breath samples, by comparing three different
37 549 polymeric bags (i.e. Nalophan, Tedlar and Cali-5-Bond) in terms of possible contamination of the sample by
38 550 bag's material release and chemical stability of samples.

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3 551 In the field of breath analysis, the Nalophan and Tedlar bags seem to be the best choice since only a few
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5 552 chemicals were found to be released from these materials: 2-methyl-1,3-dioxolane from the former and N,N-
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7 553 dimethylacetamide and phenol from the latter. Cali-5-Bond bag seems not suitable for breath analysis due to
8 554 the presence of several contaminants.

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10 555 The test we performed to assess the stability of samples in dry conditions highlighted slightly better
11 556 performances of the Nalophan bags compared to the Tedlar and Cali-5-Bond bags, since losses of about 10%
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13 557 were observed within 72 hours for the majority of the compounds investigated. Benzaldehyde was only
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15 558 found to be not stable with a loss of about 60% at 24 hours. The pCO₂ was stable in Tedlar and Cali-5-Bond
16 559 and decreased of about 10% within 72 hours in the Nalophan bag.

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18 560 The presence of humidity in the mixture did not affect significantly the stability of the selected VOCs nor the
19 561 pCO₂ in the Nalophan bag, since a rapid water diffusion through the bag walls was observed within 30
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21 562 minutes at 37 °C. In the case of the Nalophan bag, the stability of VOCs as well as pCO₂ was influenced by
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23 563 the degree of bag filling (i.e., surface-to-volume ratio), and therefore it is strongly recommended to collect as
24 564 large a volume of breath sample as possible in order to minimize the S/V ratio.

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26 565 Finally, taking into consideration the low background, the good sample stability and the extremely low cost,
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28 566 which means it could be disposable (thus no need for cleaning), Nalophan bags represent in our view the best
29 567 choice for the collection of breath samples.
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32 569 **Acknowledgements**

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36 571 The work presented in this paper was supported by the ~~project PHC-643694-Hearten project~~, [which has](#)
37 572 [received funding from the European Union's Horizon 2020 research and innovation programme under grant](#)
38
39 573 [agreement No 643694](#).
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41 574 ~~funded by the European Commission under the H2020 programme.~~

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