Comparison of sampling bags for the analysis of volatile organic compounds in breath

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Abstract

Nalophan, Tedlar and Cali-5-Bond polymeric bags were compared to determine the most suitable type for breath sampling and storage when volatile organic compounds are to be determined. Analyses were performed by thermal desorption gas chromatography mass spectrometry.

For each bag, the release of contaminants and the chemical stability of a gaseous standard mixture containing eighteen organic compounds, as well as the CO_2 partial pressure were assessed. The selected compounds were representative of breath constituents and belonged to different chemical classes (i.e., hydrocarbons, ketones, aldehydes, aromatics, sulfurs and esters). In the case of Nalophan, the influence of the surface-to-volume ratio, related to the bag's filling degree, on the chemical stability was also evaluated.

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

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,

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

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

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

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

Based on this background information, the aim of the present study was to determine the most appropriate bag material for breath sampling. A critical evaluation was then carried out by comparing Tedlar, the most commonly used material, with Nalophan and Cali-5-Bond, whose numerous applications in environmental monitoring [23, 24, 25] suggest that them could also be used for breath analysis. The comparison was performed by testing, up to 72 hours, the release of interfering compounds from the material itself, the stability of CO_2 partial pressure (pCO_2) and the chemical stability of a standard gaseous mixture, containing eighteen volatile organic compounds (VOCs) at known concentrations (~~150~~180–~~450~~ 420 ppbv). The selected compounds were representative of breath constituents and belonged to different chemical classes (i.e., hydrocarbons, ketones, aldehydes, aromatics, sulfurs and esters) [26, 27].

2. Materials and methods

2.1. Sampling bags

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

1. *Nalophan*™: 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*™: 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~~ 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.

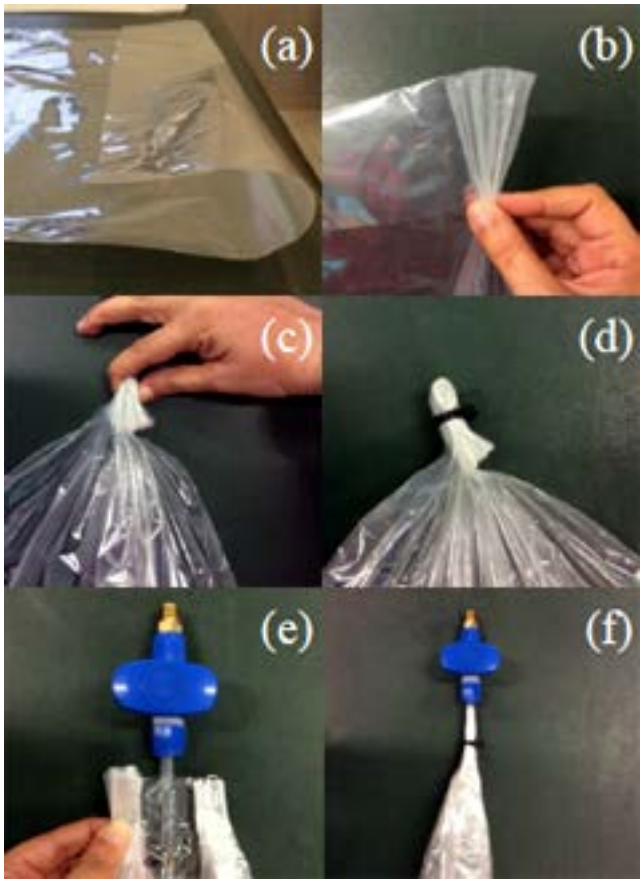

Figure 1. Assembly of a Nalophan sampling bag.

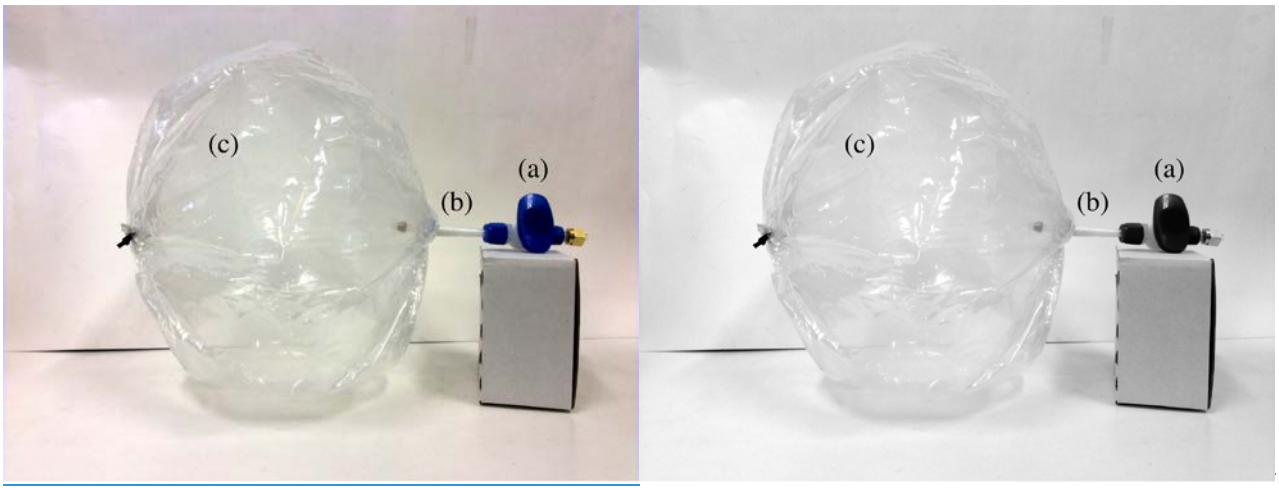

Figure ~~1~~2. Nalophan sampling bag composed of a) stopcock, b) PTFE tube, and c) Nalophan bag.

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,

dimethyldisulfide and toluene were purchased from Fluka, Sigma-Aldrich (Italy). All the compounds were GC grade standard with a higher than 99% purity. Labeled toluene-D8 was purchased at a purity of 99.8% from ARMAR Chemicals (Switzerland). All chemicals were used without any further purification.

Helium 5.6 IP and medical air (hydrocarbon free, purity of 99.95%) were purchased from Sol Group Spa (Italy).

A binary standard gaseous mixture consisting of 5% CO_2 in nitrogen, purchased from Sol Group Spa (Italy), was used to test the stability of pCO_2 (mmHg) in the sampling bags.

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

2.3. Preparation of standard gaseous mixtures

A liquid mixture was prepared by mixing 50 µL of eighteen pure liquid compounds in a glass vial equipped with a screw-cap mininert valve (Sigma Aldrich, Italy). ~~Then a~~A stock standard gaseous mixture (MIX 18) was then obtained by introducing 20 µL of the liquid mixture into a 2 L glass flask equipped with a screw-cap mininert valve (Sigma Aldrich, Italy) and pre-evacuated using a vacuum membrane pump. The glass flask was heated at 37 ± 1 °C to ensure complete evaporation of the liquid and subsequently balanced to ambient pressure. This gaseous mixture was kept in a 1.1 m^3 thermostat at 37 ± 1 °C. The storage time of the liquid solution, kept at 4 °C to minimize the risk of evaporation, and of the gaseous mixture was three and one month, respectively. The gaseous mixture was prepared once again if the amount of subtracted volume exceed 5% of the glass flask volume.

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

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

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 ~~mass~~ 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

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 pCO_2 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 pCO_2, the bags were filled with a standard gaseous mixture consisting of 5% CO_2 in nitrogen (500 mL/min). As previously reported, the volume of CO_2 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. CO_2 content was then measured 0.5, 1, 3, 6, 24, 29, 32, 48 and 72 hours after bag preparation. The measurement of pCO_2 (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.

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

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 cm^2, ~~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}).

To simulate the content of water vapor of real breath samples, humid gaseous mixtures were prepared by flowing medical air (500 mL/min) through a purge and trap glass system filled with 5 mL of fresh milli-Q water at room temperature (20 °C). After the half filling time, an aliquot of MIX 18 from the flask was injected into the flow of humidified medical air, during the filling of the three Nalophan bags, to obtain a 500-fold dilution. Once again, the volume of humidified medical air introduced into the bags was calculated from the flow and filling time as previously reported. All these bags were stored at a 37 ± 1 °C during the test. Three adsorption tubes were loaded with the sample (80 mL) at each observation time, namely 0.5, 3, 6, 24, 48 and 72 hours after filling the bags and ~~then~~ analyzed following the procedure described in section 2.4. The stability of pCO_2 in the Nalophan bag with different S/V ratios (0.3, 0.7 and 1.0 cm^{-1}) was also tested in humid conditions using the same analytical procedure used for testing CO_2 stability in dry condition. For this purpose, humid gaseous CO_2 samples were prepared by flowing different volumes (20, 10 and 7 L) of a standard gaseous CO_2 mixture (500 mL/min) through a purge and trap glass system filled with 5 mL of fresh milli-Q water at room temperature (20 °C).

Moreover, an additional test was carried out to simulate a real situation in which the sample is kept at ambient temperature for some time before being stabilized at 37 ° C. For this purpose, two Nalophan bags (20 L), having an S/V ratio of 0.3 cm^{-1}, were filled with humidified medical air (500 mL/min). During filling, an aliquot (40 mL) of MIX 18 from the flask was injected into the air flow. One bag was kept in the thermostat at 37 ± 1 °C and about 15 % RH for 15 hours, whereas the other was kept in the room at about 22 °C and 45 % RH, before being stabilized for half an hour in the thermostat. The content of each bag (250 mL) was then transferred into three adsorption tubes and analyzed according to the procedure described in section 2.4. The same experiment was performed using the humidified standard gaseous CO_2 mixture.

3. Results and discussion

3.1. Background test and effectiveness of cleaning procedure

Table ~~3~~ 4 shows the most abundant contaminants, released for each bag material.

Table ~~3~~4. 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 (3b)) 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).

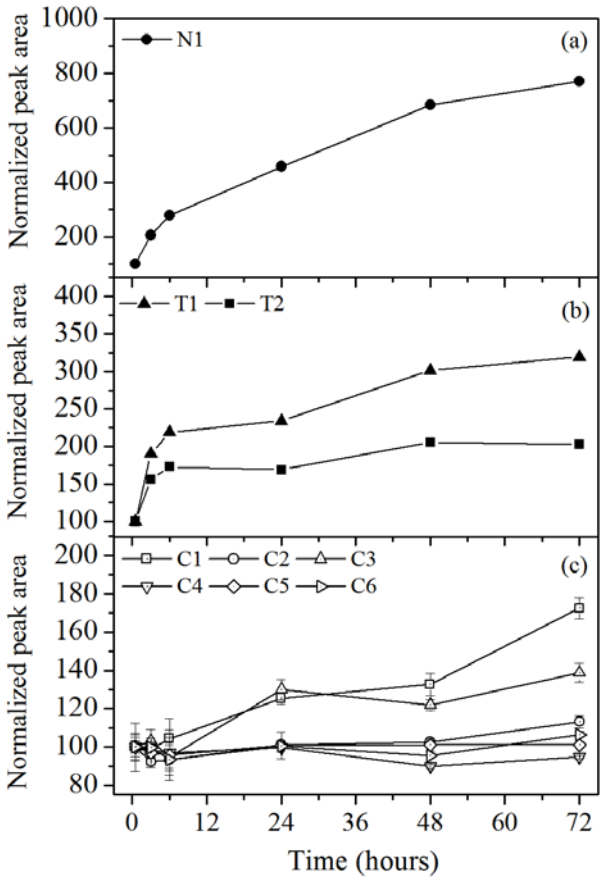

Figure ~~2~~3. Release over time of the compounds from Nalophan (a), Tedlar (b) and Cali-5-Bond (c) bags. Legends are explained in table ~~3~~4. Error bars correspond to the standard deviation of three replicates.

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.

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 ~~3~~4, ~~4~~ 5 and ~~5~~6. 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%.

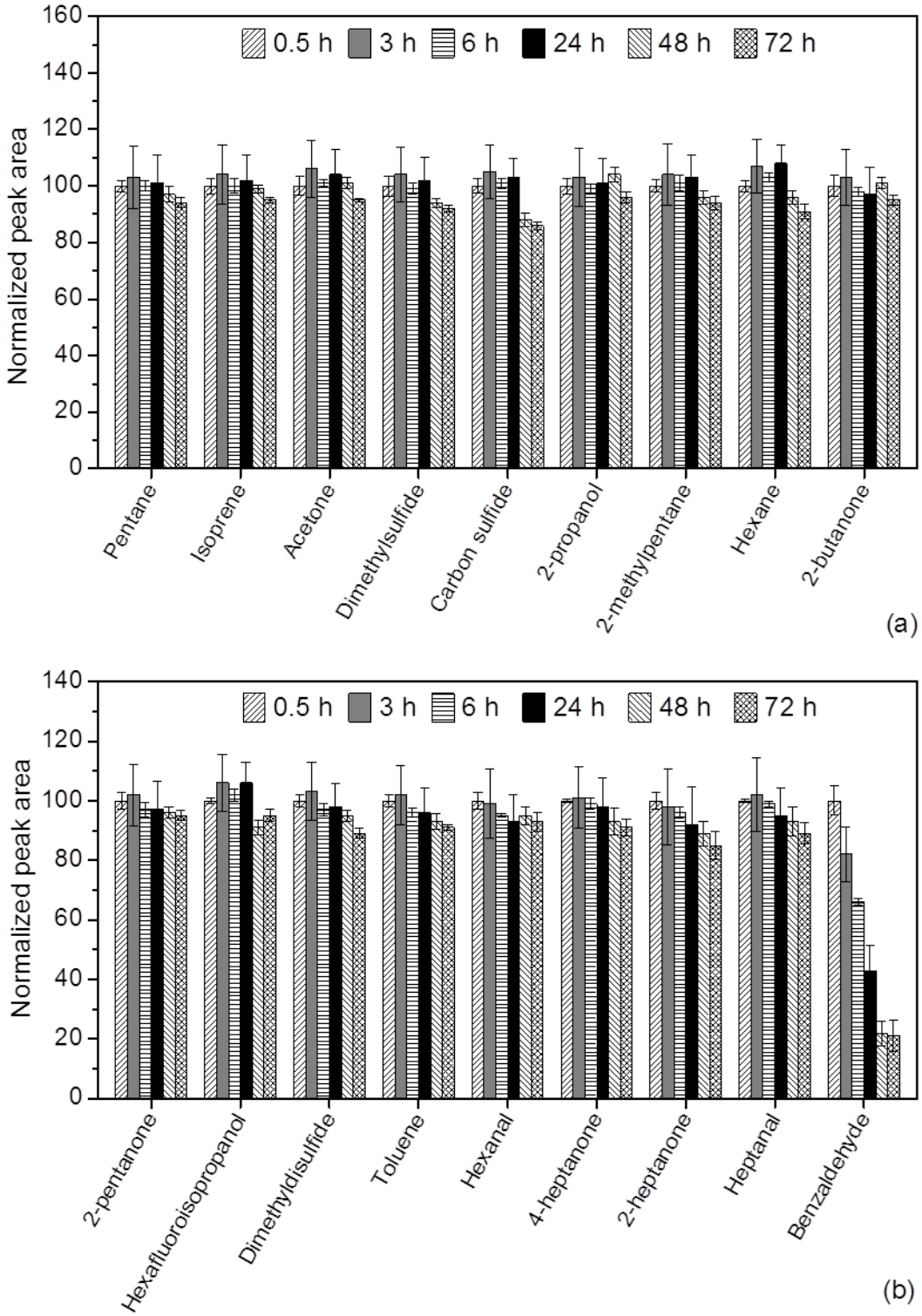

Figure 34. Average values of the peak areas of the compounds in the dry gaseous standard mixture measured in the Nalophan bag, normalized with respect to the toluene-D8 peak area and to the area of the peak corresponding to the first observation time (0.5 hours). Error bars correspond to the standard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

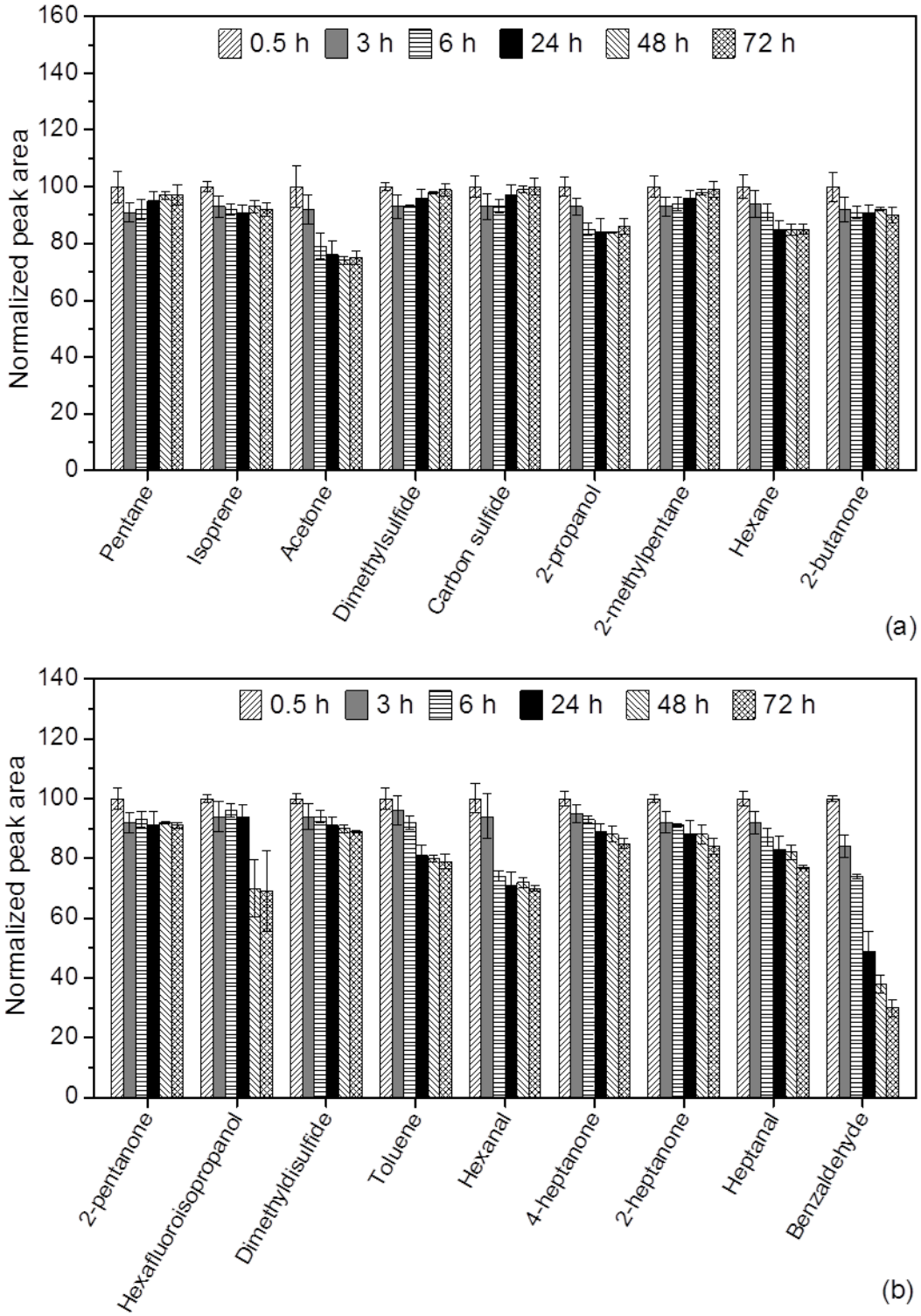

Figure 45. Average values of the peak areas of the compounds in the dry gaseous standard mixture measured in the Tedlar bag, normalized with respect to the toluene-D8 peak area and to the area of the peak corresponding to the first observation time (0.5 hours). Error bars correspond to the standard deviation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

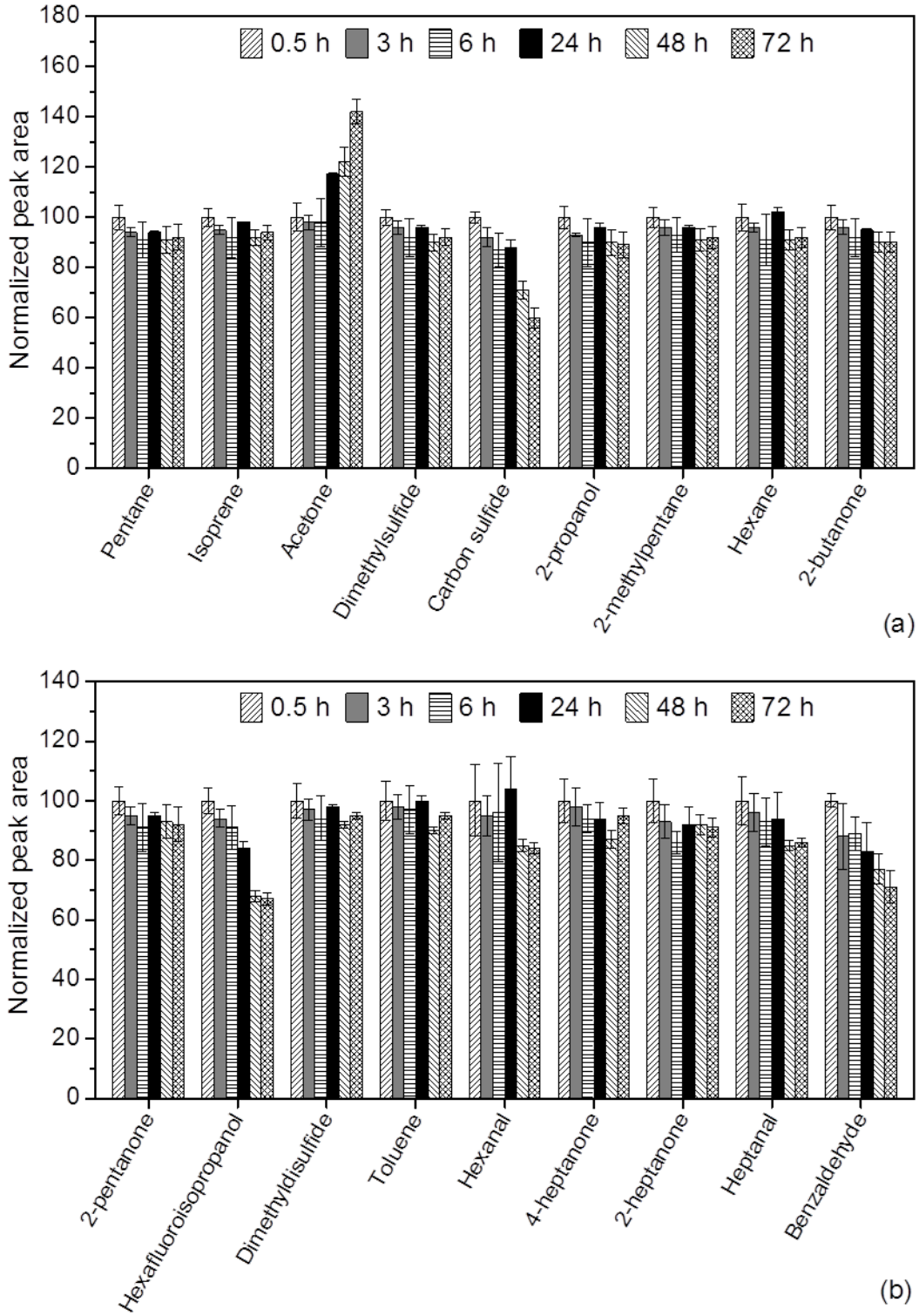

Figure 5 6. Average values of the peak areas of the compounds in the dry gaseous standard mixture measured in the Cali-5-Bond bag, normalized with respect to the toluene-D8 peak area and to the area of the peak corresponding to the first observation time (0.5 hours). Error bars correspond to the standard 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 CO_2 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 fig~~.~~ ure ~~6~~7, show that CO_2 was stable within 24 hours in all the bags, whereas it showed a moderate decrease (about 10%) at 72 hours in the Nalophan bag.

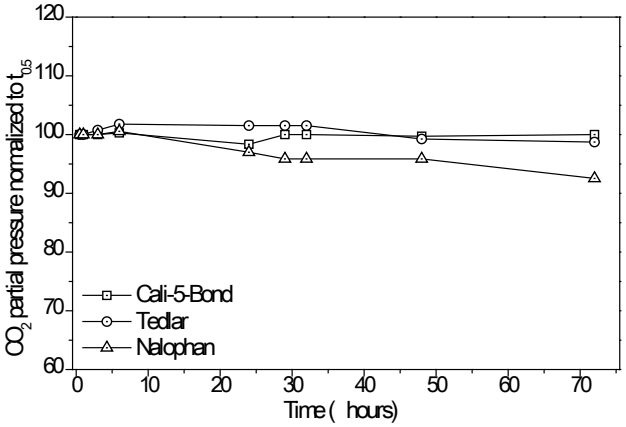

Figure ~~6~~7. CO_2 partial pressure values measured over time in Nalophan, Tedlar and Cali-5-Bond bags. Data were normalized to the value at the first observation time (0.5 hours).

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 CO_2, 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).

The influence of the (film) surface-to-(sample) volume ratio on the VOCs concentration and pCO_2 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%.

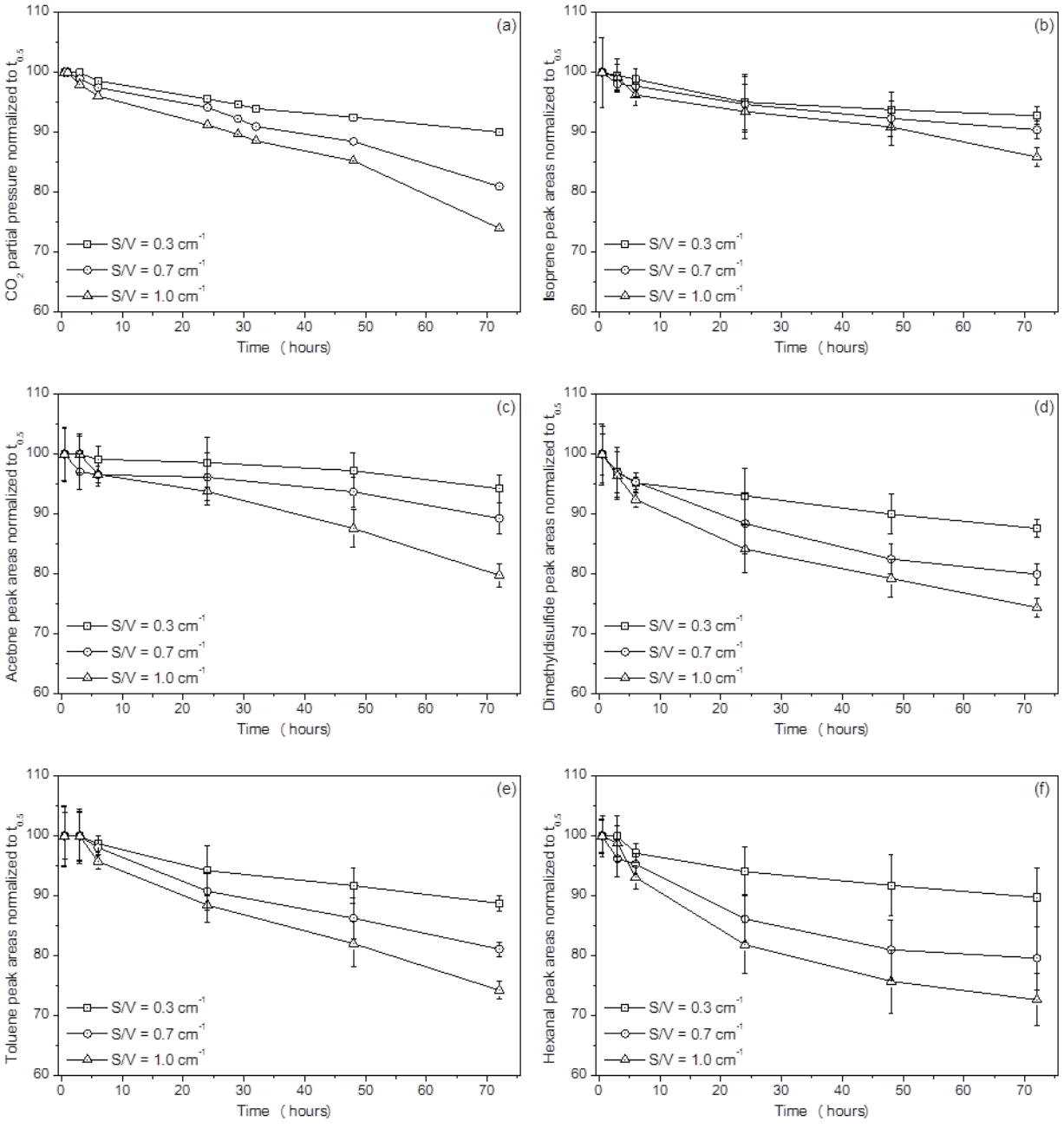

Figure ~~7~~8. Average values of CO_2 partial pressure (a) and of isoprene (b), acetone (c), dimethyldisulfide (d), toluene (e) and hexanal (f) peak areas in the humid gaseous standard mixture measured in the Nalophan bags (S/V of 0.3, 0.7 and 1.0 cm^{-1}). Peak areas were normalized with respect to the toluene-D8 peak area and to the area of the peak corresponding to the first observation time (0.5 hour). Error bars correspond to the standard deviation of three replicates.

~~The results of the bag~~ Data concerning stability ~~(S/V ratio of 0.3 cm^{-1})~~ over time ~~obtained in~~of the humid gaseous mixture prepared in the bag (S/V ratio of 0.3 cm^{-1}) confirmed the same trend observed in dry conditions. For the majority of investigated compounds, the difference between their stability in dry and humid mixtures was always smaller than 10%, which is in good agreement with the results observed for the Tedlar bag by Groves and Zellers [20]. Unlike Mochalski et al [17], we observed a good stability even for the heaviest compounds (e.g. hexanal) ~~and this suggests that the use of Nalophan bags enabled the possible interaction between the water vapor and such compounds to be minimized~~. ~~These~~ This result~~s~~ ~~are~~ is probably related to a more rapid decrease ~~in the amount~~ of the water vapour partial pressure by diffusion through the Nalophan bag walls at 37 °C compared to the bags used by Mochalski et al (i.e. Tedlar, Kynar and Flexifilm), which minimized the possible interaction between the water and such compounds. Also in this case, benzaldehyde confirmed its anomalous behavior, with a loss of about 60% at 24 hours. The results of pCO_2 stability over time confirmed the same trend observed in dry conditions, with a decrease of about 10% at 72 hours. The chemical stability of humid MIX 18 components in the Nalophan bag (S/V ratio of 0.3 cm^{-1}) was not significantly different (within the experimental error of 5%) when the bag was kept for 15 hours at ambient conditions (about 22 °C and 45 % RH), before being stabilized in the thermostat. The same result was obtained when the CO_2 mixture was used.

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% were observed for all the compounds, with the exception of benzaldehyde that showed a decrease of more than 80% in both bags. These findings prove how the stability of VOCs depends on the degree of bag filling (i.e., surface-to-volume ratio), confirming the results obtained for the Tedlar bag by Mochalski et al [17]. Also the pCO_2 was influenced by the bag's filling degree. In fact, the same variation of 10% was observed within 48 and 24 hours for the Nalophan bag with a S/V ratio of 0.7 and 1.0 cm^{-1}, respectively. These findings are not surprising since VOCs at high S/V ratio are more vulnerable to losses related to sorption or permeation.

4. Conclusions

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

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

The test we performed to assess the stability of samples in dry conditions highlighted slightly better performances of the Nalophan bags compared to the Tedlar and Cali-5-Bond bags, since losses of about 10% were observed within 72 hours for the majority of the compounds investigated. Benzaldehyde was only found to be not stable with a loss of about 60% at 24 hours. The pCO_2 was stable in Tedlar and Cali-5-Bond and decreased of about 10% within 72 hours in the Nalophan bag.

The presence of humidity in the mixture did not affect significantly the stability of the selected VOCs nor the pCO_2 in the Nalophan bag, since a rapid water diffusion through the bag walls was observed within 30 minutes at 37 °C. In the case of the Nalophan bag, the stability of VOCs as well as pCO_2 was influenced by the degree of bag filling (i.e., surface-to-volume ratio), and therefore it is strongly recommended to collect as large a volume of breath sample as possible in order to minimize the S/V ratio.

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

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~~funded by the European Commission under the H2020 programme.~~

References

[1] Ceccarini A, Di Francesco F, Fuoco R, Ghimenti S, Onor M, Tabucchi S and Trivella MG 2012 *Analytical Techniques for Clinical Chemistry: Methods and Applications*, ed S Caroli and G Z´aray (New York: Wiley) p 651

[2] Deng C, Zhang J, Yu X, Zhang W and Zhang X 2004 *J. Chromatog. B* **810** 269

[3] Cope KA, Watson MT, Foster WM, Sehnert and SS Risby TH 2004 *J. Appl. Physiol.* **96** 1371

[4] Hryniuk A and Ross BM 2009 *Int. J. Mass Spectrom.* **285** 26

[5] Smith D and Spanel P 2005 Mass Spectrom. Rev. **24** 661

[6] Thekedar B, Szymczak W, Höllriegl V, Hoeschen C and Oeh U 2009 *J. Breath Res.* 3:027007 doi:10.1088/1752-7155/3/2/027007

[7] Vautz W, Nolte J, Fobbe R and Baumbach JI 2009 *J. Breath Res.* 3:036004 doi: 10.1088/1752-7155/3/3/036004

[8] McCurdy MR, Bakhirkin Y, Wysocki G, Lewicki R and Tittel FK 2007 *J. Breath Res.* 1:014001 doi: 10.1088/1752-7155/1/1/014001

[9] Amann A, Miekisch W, Pleil JD, Risby T and Schubert JK 2010 *European Respiratory Society Monograph* vol 49, ed I Horvath and JC de Jongste (Leeds: Maney Publishing) p 96

[10] Thrall KD, Callahan PJ, Weitz KK, Edwards JA, Brinkman MC and Kenny DV 2001 *AIHAJ* **62** 28–35

[11] Prado C, Marin P and Periago JF 2003 *J. Chromatogr. A* **1011** 125

[12] Scott-Thomas AJ, Syhre M, Pattemore PK, Epton M, Laing R, Pearson J and Chambers ST 2010 *BMC Pulmonary Med.* **10** 56

[13] Lindstrom AB and Pleil, JD 2002 *Biomarkers* **7** 189

[14] Compendium Method TO-15 in Compendium of Methods for the Determination of Toxic Organic Compounds in Ambient Air, Second Edition (EPA/625/R-96/010b). Center for Environmental Research Information, US Environmental Protection Agency. Cincinnati, OH, USA, 1999

[15] Beauchamp J, Herbig J and Gutmann R 2008 *J. Breath Res.* **2** 1

[16] Mochalski P, Wzorek B, Sliwka I and Amann A 2009 *J. Chromatogr. B* **877** 189

[17] Mochalski P, King J, Unterkofler K and Amann A 2013 *Analyst* **138** 1405

[18] Kim YH, Kim KH, Jo SH, Jeon EC, Sohn JR and Parker DB 2012 *Anal. Chim. Acta* **712** 162

[19] Sironi S, Eusebio L, Capelli L, Boiardi E and Del Rosso R 2014 *Water Sci. Technol.* **69** 486

[20] Groves W and Zellers E 1996 *Am. Ind. Hyg. Assoc. J.* **57** 257

[21] Steeghs MML, Cristescu SM, Munnik P, Zanen P and Harren FJM 2007 *Physiol. Meas.* **28** 73

[22] Gilchrist FJ, Razavi C, Webb AK, Jones AM, Spanel P, Smith D and Lenney W 2012 *J. Breath Res.* 6:036004 doi: 10.1088/1752-7155/6/3/036004

[23] van Harreveld AP 2003 *J. Air Waste Manag. Assoc.* **53** 51

[24] Vollmer MK, Walter S, Mohn J, Steinbacher M, Bond SW, Rockmann T and Reimann S 2012 *Atmos. Chem. Phys.* **12** 6275–89

[25] Munksgaard NC, Davies K, Wurster CM, Bass AM and Bird MI 2013 *Isotopes Environ. Health Stud.* **49** 232

[26] de Lacy Costello B, Amann A, Al-Kateb H, Flynn C, Filipiak W, Khalid T, Osborne D and Ratcliffe NM 2014 *J. Breath Res.* 8:014001 doi:10.1088/1752-7155/8/1/014001

[27] Amann A, de Lacy Costello B, Miekisch W, Schubert J, Buszewski B, Pleil J, Ratcliffe N, Risby T 2014 *J. Breath Res.* 8:034001 doi:10.1088/1752-7155/8/3/034001

[28] Ghimenti S, Tabucchi S, Lomonaco T, Di Francesco F, Fuoco R, Onor M, Lenzi S and Trivella MG 2013 *J. Breath Res.* 7:017115 doi:10.1088/1752-7155/7/1/017115

[29] Woolfenden E 2010 *J. Chromatogr. A* **1217** 2685

[30] Ohkado Y, Kawamura Y, Mutsuga M, Tamura HO and Tanamoto K 2005 *J. food hyg soc jpn* **46** 13–20

[31] Shostakovskii MF, Atabin AS, Trofimov BA and Gusarov AV 1965 Russian Chemical Bulletin **14** 1032

[32] Trabue SL, Anhalt JC and Zahn JA 2006 *J. Environ. qual.* **35** 1668

[33] Lomonaco T, Salvo P, Ghimenti S, Biagini D, Bellagambi F, Fuoco R and Di Francesco F 2015 *Proc. The 37th Annual Int. Conf. of the IEEE Engineering in Medicine and Biology Society*. Milan. Accepted