Journal of Breath Research

PAPER

Determination of volatile organic compounds in exhaled breath of heart failure patients by needle trap micro-extraction coupled with gas chromatography-tandem mass spectrometry

D Biagini[1], T Lomonaco[1], S Ghimenti[1], F G Bellagambi[1], M Onor[2], M C Scali[3], V Barletta[3], M Marzilli[3], P Salvo[4], M G Trivella[4], R Fuoco[1] and F Di Francesco[1]

[1] Department of Chemistry and Industrial Chemistry, University of Pisa, Pisa, Italy
[2] Institute of Chemistry of Organometallic Compounds, CNR, Pisa, Italy
[3] Department of Surgical, Medical and Molecular Pathology and Critical Care Medicine, University of Pisa, Pisa, Italy
[4] Institute of Clinical Physiology, CNR, Pisa, Italy

E-mail: tommaso.lomonaco@unipi.it

Keywords: needle trap micro-extraction, GC-MS/MS, breath analysis, heart failure, VOCs

Supplementary material for this article is available online

OPEN ACCESS

RECEIVED
1 May 2017

REVISED
9 October 2017

ACCEPTED FOR PUBLICATION
20 October 2017

PUBLISHED
30 November 2017

Abstract

The analytical performances of needle trap micro-extraction (NTME) coupled with gas chromatography-tandem mass spectrometry were evaluated by analyzing a mixture of twenty-two representative breath volatile organic compounds (VOCs) belonging to different chemical classes (i.e. hydrocarbons, ketones, aldehydes, aromatics and sulfurs). NTME is an emerging technique that guarantees detection limits in the pptv range by pre-concentrating low volumes of sample, and it is particularly suitable for breath analysis. For most VOCs, detection limits between 20 and 500 pptv were obtained by pre-concentrating 25 ml of a humidified standard gas mixture at a flow rate of 15 ml min^{-1}. For all compounds, inter- and intra-day precisions were always below 15%, confirming the reliability of the method. The procedure was successfully applied to the analysis of exhaled breath samples collected from forty heart failure (HF) patients during their stay in the University Hospital of Pisa. The majority of patients (about 80%) showed a significant decrease of breath acetone levels (a factor of 3 or higher) at discharge compared to admission (acute phase) in correspondence to the improved clinical conditions during hospitalization, thus making this compound eligible as a biomarker of HF exacerbation.

1. Introduction

Needle trap micro-extraction (NTME) coupled with gas chromatography-mass spectrometry (GC-MS) represents an emerging and promising technique that guarantees detection limits in the pptv range by pre-concentrating a volume of breath less than 50 ml [1]. A needle trap device (NTD) consists of a stainless-steel needle (22 or 23 gauge) packed with few milligrams of different types of stationary phases. Such devices, introduced for the first time in the late nineties [2], allows combining the sample collection and the analyte pre-concentration in a single step requiring less than 3 min to be accomplished. The technique has been already applied to environmental [3–5] and clinical [1, 6] studies, even it is still evolving. Analyses by NTDs are technically easy and straightforward, since the sample desorption occurs directly into a standard GC injector without the necessity of an additional thermal desorption unit [7]. The simplest method to transfer the adsorbed analytes to the capillary column is the expansive flow technique, which exploits the gas flow generated by the thermal expansion of air inside the needle [7]. When working with water-saturated samples like breath, the desorption efficiency is enhanced by the additional flow generated from the expansion of the water vapor during the desorption step [1, 6, 8]. As recently reported [8], the presence of water does not represent a significant problem for the GC column and for the MS due to the very low amount retained by hydrophilic sorbents [8, 9].

Breath analysis by NTD can be a potential tool to diagnose diseases at an early stage or to monitor their evolution, especially in the case of chronic diseases such as heart failure (HF). The composition of the breath depends on several factors whose relative effect is very difficult to quantify [10, 11]. To improve the interpretation of the experimental findings, a more in-depth knowledge of the metabolic processes and their relationship with the presence of volatile organic compounds (VOCs) in the breath samples is needed. Currently, in our opinion, the most useful approach for breath monitoring analysis is to focus on single subjects acting as their own control.

HF is a complex clinical syndrome caused by a wide range of cardiovascular disorders leading to structural or functional abnormalities of the heart, and it represents a main cause of mortality and poor quality of life in the western societies [12]. According to the European Heart Failure Association, 26 million people experience HF globally and 3.6 million people are diagnosed with HF every year. Prevalence is expected to increase in the coming years, so that more than 8 million people aged over 18 are expected in 2030 [13]. The main HF symptoms are fatigue and dyspnea, which limit exercise tolerance, and fluid retention that may lead to pulmonary and/or splanchnic congestion and/or peripheral edema. HF is a condition very often associated with poor prognosis and frequent hospital admissions [12]. Nowadays, the common clinical approaches used to diagnose and monitor HF are based on (i) clinical history and physical examination, to provide information about familial cardiac diseases and severity degree, (ii) electrolyte (e.g. sodium and potassium), albumin and creatinine determination in blood and (iii) transthoracic Doppler 2D echocardiography [9]. Natriuretic peptides such as B-type or N-terminal pro-B-type natriuretic peptides have been intensively studied to monitor HF [14, 15]. Moreover, there is an increasing interest for the identification of biomarkers for this pathological condition, possibly in easy-to-collect biological fluids by minimally invasive procedures, to improve early identification of relapses that would reduce hospital admissions and improve prognosis, especially for stable HF patients [16].

Breath analysis is extraordinarily appealing for an effective, easy, painless and non-invasive monitoring of HF patients because the chemical composition of exhaled breath reflects in real time the physiological and pathophysiological processes occurring in the body [10, 17, 18]. Generally, the analysis of VOCs in exhaled breath is carried out off-line by (i) collecting the sample in sampling bags (e.g. Tedlar [19] or Nalophan [20]), (ii) pre-concentrating the compounds of interest using solid phase extraction [21] or solid phase micro-extraction (SPME) [22] and, finally, (iii) analyzing the sample by thermal desorption coupled to GC-MS. The real-time measurement of carbon dioxide by infrared sensors can allow the selection of specific breath fractions and improve the representativeness of samples [23, 24]. In addition, analytical approaches (e.g. selected ion flow tube MS and proton transfer reaction MS) based on the injection of breath samples directly into the instrument are also available. However, the cost of the instrumentation and the uncertain identification of analytes (e.g. isobaric compounds) are the main drawbacks of these procedures [25, 26], making the off-line methods the most commonly used in this field.

In the present work, the analytical performances of triple-bed NTDs, packed with 1 cm of each Divinylbenzene, Carbopack X and Carboxen 1000, were evaluated by analyzing a humidified standard gaseous mixture of twenty-two VOCs. The selected compounds are representative of breath composition belonging to different chemical classes (i.e. hydrocarbons, ketones, aldehydes, aromatics and sulfurs) [27, 28]. Finally, the proposed NTME-GC-MS/MS procedure was successfully applied to the determination of the chemical composition of exhaled breath samples collected from 40 patients suffering from HF during their hospitalization in the University Hospital of Pisa.

2. Materials and methods

2.1. Chemicals and materials

Ethanol, 2-propanol, 1-propanol, 1-butanol, butanal, pentanal, hexanal, heptanal, benzaldehyde, 2-butanone, 2-pentanone, 2-hexanone, 2-heptanone and 4-heptanone were from AccuStandard, Inc. Chemical Reference Standard (USA), whereas isoprene, acetone, pentane, hexane, dimethyl sulfide, dimethyl disulfide, carbon disulfide and toluene were from Fluka, Sigma-Aldrich (Italy). All compounds were GC grade standard with a purity higher than 99%. Labeled ^{8}D-toluene was purchased with a purity of 99.8% from ARMAR Chemicals (Switzerland). All chemicals were used without any further purification.

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

Helium 5.6 IP, medical air (hydrocarbon free, purity of 99.95%), nitrogen 5.0 IP and CO_2 (5% balanced in nitrogen) were purchased from Sol Group Spa (Italy). Helium, medical air, nitrogen and CO_2 were purified with a super clean filter from Agilent Technologies (USA) to further remove water, oxygen and hydrocarbon contaminants.

Nalophan bags were fabricated from a roll of Nalophan tube (diameter 47 cm, film thickness 20 μm) supplied by Kalle (Germany) according to the procedure described elsewhere [29].

2.2. Preparation of standards

A liquid solution was prepared by mixing 50 μl of the pure compounds in a 1 ml glass vial equipped with a screw-cap mininert valve (Sigma-Aldrich, Italy). A stock standard gaseous mixture (MIX22) was then

Table 1. Concentration of MIX22 components calculated at 37 °C and ambient pressure.

	Concentration in the glass flask (ppmv)
Ethanol	312
Acetone	168
2-propanol	238
Pentane	290
Isoprene	297
Dimethyl sulfide	228
Carbon disulfide	235
1-propanol	242
Butanal	128
2-butanone	193
Hexane	150
1-butanol	199
2-pentanone	202
Pentanal	206
Dimethyl disulfide	107
Toluene	193
2-hexanone	149
Hexanal	116
4-heptanone	171
2-heptanone	130
Heptanal	444
Benzaldehyde	353

obtained by introducing 20 μl of solution into a 2 l glass flask equipped with a screw-cap mininert valve and pre-evacuated using a vacuum membrane pump. The internal pressure was balanced to ambient pressure and the flask was then kept in a 1.1 m^3 thermostat chamber (37 °C ± 1 °C and RH < 15%) during use. The stock standard gaseous mixture was used for 1 month or prepared again when the amount of subtracted volume exceeded 10% of the flask volume. The concentrations of the mixture components are reported in table 1.

To simulate water vapor contained in real breath samples, humid working gaseous mixtures at about 90% RH were prepared at room conditions (25 °C ± 2 °C and 50% ± 5% RH) flowing medical air (500 ml min^{-1}) through a purge and trap glass system filled with 5 ml of fresh ultrapure water. An aliquot of MIX22 from the glass flask was injected into the flow of humidified medical air during the filling of a Nalophan bag. To minimize water diffusion through the Nalophan film [29], a double-walled bag having a (film) surface-to-(sample) volume ratio (S/V) equal to 0.3 cm^{-1} was prepared by fitting one Nalophan tube chunk inside another. This bag was equipped with a single polypropylene septum fitting (SKC, USA), which combines the hose/valve and the septum holder. A long life non-stick septum (Agilent Technologies, USA) was used.

In the same way, an internal standard gaseous stock solution containing labeled ^{8}D-toluene was prepared by evaporating 5 μl of liquid compound into a 2 l glass flask equipped with a screw-cap mininert valve. This gaseous mixture was stored in the thermostatic chamber and used for 1 month or prepared again when the amount of subtracted volume exceeded 10% of the glass flask volume. The concentrations of ^{8}D-toluene, calculated at 37 °C and ambient pressure, was 600 ppmv.

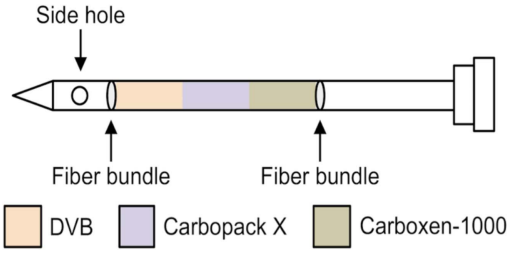

Figure 1. Needle trap design and sorbent materials used. (DVB = divinylbenzene).

A stock gaseous mixture with 5% CO_2 was diluted with humidified medical air at 1%, 2%, 3% and 4% CO_2 to calibrate the CO_2 sensor. These mixtures were prepared in 2 l hand-made Nalophan bags (S/V 0.3 cm^{-1}) and kept in the thermostatic chamber for 1 d.

2.3. Needle trap device

Commercial three-bedside-hole NTDs (23-gauge stainless-steel needle, length 60 mm), packed with 1 cm each of Divinylbenzene (80/100 mesh), Carbopack X (60/80 mesh) and Carboxen 1000 (60/80 mesh) were purchased from PAS-Technology (Germany) (figure 1).

Prior to first use, NTDs were conditioned in a custom-made heating device (PAS-Technology, Germany) at 250 °C under a permanent nitrogen flow (1 bar front pressure) for at least 20 h to remove impurities from the sorbent materials. NTDs were then sealed with Teflon caps and stored at room conditions (25 °C ± 2 °C and RH of 50% ± 5%) until use. Right before use, the NTDs were conditioned again for 30 min using the same conditions.

A plot showing the relationship between the applied pressure different (mBar) and sampling flow rate (ml min^{-1}) was obtained for each NTD by drawing ambient air (25 °C ± 2 °C and RH of 50% ± 5%). The same measurements were repeated after 20 conditioning/analysis cycles to exclude possible changes of the pressure drop through the NTD due to a rearrangement of sorbents or needle occlusion.

2.4. Study population

The study was carried out in the framework of HEARTEN project ('A co-operative mHEALTH environment targeting adherence and management of patients suffering from HF', protocol number: 643694) and was approved by the Ethics Committee of the Area Vasta Nord-Ovest (CEAVNO- Tuscany Region). Forty patients (30 males, 10 females), hospitalized at the Cardio-Thoracic-Vascular Department of the Azienda Ospedaliera-Universitaria Pisana

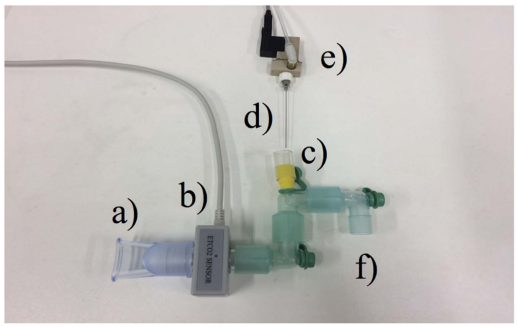

Figure 2. Breath sampler prototype composed by (a) sterile and disposable mouthpiece, (b) CO_2 sensor, (c) septum connector, (d) needle trap device, (e) electronic valve and (f) polyethylene T-piece connectors.

(AOUP), were enrolled in the study after receiving appropriate information on the whole protocol and signing the informed consent.

2.5. Sample collection

Twenty-five milliliters of end-tidal breath samples were collected at 15 ml min^{-1} from the enrolled patients at the time of hospital admission (t_0) and every 48 h until discharge (t_d) using a sampling device (figure 2), made by a mouthpiece (Spectra 2000, Italy), a Capnostat 5 CO_2 sensor (Respironics, Philips), an electronic valve and three polyethylene T-piece connectors. Generally, three samples were collected from each patient during hospitalization. An aliquot (25 ml) of ambient air was also collected at 15 ml min^{-1} and analyzed to exclude contamination risks, which is a critical problem in clinical environments [30].

Before sample collection, each subject was asked to breathe normally through the breath sampler for 1 min to familiarize with the sampler and measure end-tidal CO_2. After setting an appropriate CO_2 threshold (80% of the end-tidal CO_2), a NTD was connected to the automatic system via the electronic valve and inserted into the yellow adapter connected to the series of polyethylene T-pieces. Based on the real-time CO_2 values, the automated NTD sampler device (PAS-Technology, Germany) opened the electronic valve (delay < 50 ms) to sample end-tidal breath fraction and accurately controlled the sampling flow rate, pressure and sample volume [1]. The electronic mass-flow meter was calibrated using a mini-BUCK M-5 primary gas flow calibrator (A. P. Buck Inc., USA) operating in the range 1–6000 ml min^{-1}.

The clinical data of the patients were provided from the Cardio-Thoracic-Vascular Department of the AOUP.

The CO_2 sensor was calibrated at room conditions (25 °C ± 2 °C and RH of 50% ± 5%) by flowing standard working mixtures containing 1%, 2%, 3%, 4% and 5% of CO_2 at 1 l min^{-1}. A linear relationship between the voltage output (V) and CO_2 content (%) was observed ($r > 0.998$, slope of the calibration curve 1.2 V/%).

2.6. Sample analysis

Analyses were performed by a 7890B GC (Agilent Technologies, USA) coupled to a 7010 triple quadrupole GC/MS (Agilent Technologies, USA) with an electron ionization source operating at 70 eV.

The NTDs were thermally desorbed at 250 °C for 30 s in the multimode inlet, which was equipped with a long life non-stick septum (Agilent Technologies, USA) and an ultra-inert SPME liner (inner diameter 0.75 mm, internal volume 35 μl) (Agilent Technologies, USA). The automatic desorption was performed using a Concept GC-Autosampler (PAS-Technology, Germany) optimized for the fast-expansive flow technique.

Chromatographic separation of VOCs was carried out by a DB-5 ms ultra-inert capillary column (60 m × 0.25 mm, 1.0 μm film thickness) supplied by Agilent Technologies (USA). The oven temperature program was: 30 °C for 13 min, 4 °C min^{-1} to 130 °C (3 min hold time) and 10 °C min^{-1} to 220 °C (1 min hold time). Post run was 15 min with an oven temperature of 300 °C. Helium 5.6 IP was used as the carrier gas at 22.8 cm s^{-1} constant average linear velocity and 4 ml min^{-1} split flow.

Triple quadrupole MS detector was operated in full scan (range set from m/z 31 to m/z 200) and MS/MS mode (multiple reaction monitoring, MRM). ^{8}D-toluene (m/z of 98) was monitored using SIM mode with a dwell time of 0.4 s. The temperature of transfer line, ion source and quadrupoles was set at 280 °C, 280 °C and 150 °C, respectively. Helium was used as quench gas at a flow rate of 4 ml min^{-1}, and nitrogen as collision gas at 1.5 ml min^{-1}. A solvent delay of 6 min was set to protect the filament from water vapor. The retention times of the investigated compounds for the applied chromatographic parameters as well as the quantifier ions or MRM transitions used for the quantification are shown in table 2.

A deviation of ±0.1 min of the expected retention time compared to stock MIX22 and a qualifier/quantifier (q/Q) ratio within 20% of the ratio measured in stock MIX22 were required for analyte identification.

The analyte concentrations were calculated in real breath samples according to the following equation (equation (1)):

$$\mathrm{RRF} = \frac{R_a}{R_{\mathrm{IS}}} = \frac{A_a}{A_{\mathrm{IS}}} \times \frac{m_{\mathrm{IS}}}{m_a}, \qquad (1)$$

where subscripts a indicates the analyte and IS the internal standard, m the amount (ng) loaded into the NTD, relative response factor (RRF), and V (ml) the volume of breath sample collected into the NTD. The RRFs of compounds were calculated according to the EPA TO-15 method [31]. To determine the response factors, an aliquot (10 μl) of ^{8}D-toluene stock gaseous solution was dispersed in a volume (10 ml) of dry medical air flowing through each NTD at 5 ml min^{-1}.

Table 2. Retention times and quantifier ions (m/z values) or transitions (Precursor ion −> Product ion) of the investigated compounds. Collision energies (eV) are reported in brackets.

Compound	Retention time (min)	Quantifier ion	Quantifier transition
Ethanol	7.61	45	
Acetone	9.13	58	
2-propanol	9.45	45	
Pentane	9.45	43	
Isoprene	10.02		67 −> 41 (18 eV)
Dimethyl sulfide	10.75	62	
Carbon disulfide	12.08	76	
1-propanol	13.71	31	
Butanal	16.71	44	
2-butanone	16.92		72 −> 43 (5 eV)
Hexane	17.18	57	
1-butanol	22.67	56	
2-pentanone	24.39		86 −> 58 (5 eV)
Pentanal	25.28	44	
Dimethyl disulfide	28.80		94 −> 79 (26 eV)
^{8}D-toluene	30.02	98	
Toluene	30.33		91 −> 39 (34 eV)
2-hexanone	31.38		100 −> 85 (1 eV)
Hexanal	32.14	56	
4-heptanone	36.23		114 −> 71 (9 eV)
2-heptanone	37.13	43	
Heptanal	37.85	70	
Benzaldehyde	41.80		120 −> 105 (1 eV)

In these conditions, each NTD was spiked with 24 ng of ^{8}D-toluene. Then, three working gaseous mixtures were prepared in double-walled Nalophan bags (S/V 0.3 cm^{-1}) by diluting (1000-, 10 000- and 100 000-fold) the MIX22 with humidified medical air. Aliquots (25 ml) of each standard working mixture were transferred at 15 ml min^{-1} into three NTDs, whose content was determined according to the aforementioned procedure.

The instrumental detection limits (IDLs), i.e. the analyte concentrations producing signals with a signal-to-noise ratio equal to 3, were estimated by extracting the quantifier ions and quantifier MRM transitions.

The data quality assurance was assessed by systematically monitoring the GC signal of ^{8}D-toluene spiked into NTD. A control chart was drawn reporting the daily average ratios between the ^{8}D-toluene peak areas, the relevant average ratio during the whole experimental period, the warning limits (average ±1 standard deviation) and the control limits (average ±2 standard deviations). In about 12 months of experiments, control limits were exceeded five out of 35 times, indicating very good control of experimental conditions. The overall variability of the internal standard signal was close to 20%.

2.7. Optimization of adsorption and desorption parameters

The influence of desorption temperature (T_{des}), desorption time (t_{des}) and sampling flow rate (Flow) on the NTD performances was evaluated in our experimental conditions using a 2^3 full factorial design. Table 3 reports the selected experimental levels for each factor. Five replicate experiments were performed at the center of the experimental domain (250 °C, 30 s and 15 ml min^{-1}), so 13 runs were randomly performed overall. For this purpose, a gaseous working mixture was prepared by diluting 1000-fold the MIX22 with humidified medical air in a double-walled Nalophan bag. Results were analyzed using the MODDE 11.0 Software (Umetrics, Sweden).

Table 3. Experimental levels for the investigated factors.

Factor	Low level (–)	High level (+)
Desorption temperature (°C)	220	280
Desorption time (s)	15	45
Sampling flow rate (ml min^{-1})	5	25

The effect of sample volume on the amount of extracted VOCs was evaluated in triplicate at five levels (15, 25, 50, 75 and 100 ml) and a fixed flow rate (15 ml min^{-1}), using a gaseous working mixture prepared by a 1000-fold dilution of the MIX22 with humidified medical air in a double-walled Nalophan bag.

In addition, two NTDs were connected in series and two aliquots (25 and 50 ml) of diluted MIX22 (1000-fold) were transferred into the NTDs to verify that the breakthrough volume of any the analyte was not exceeded. Due to the doubled pressure drop of two NTDs in series, also pressure was doubled to obtain the usual flow rate (15 ml min^{-1}).

Furthermore, five NTDs were loaded (flow rate 15 ml min^{-1}) with 25 ml of the previous 1000-fold

diluted MIX22 to assess carry over, and then each NTD was analyzed two times consecutively. The desorption efficiency was calculated for all the VOCs as the ratio between the area measured in the first desorption (A_{I°) and the sum of the areas obtained in the first and the second desorption step ($A_{I^\circ} + A_{II^\circ}$).

2.8. Stability studies

The stability over time of the MIX22 components was evaluated weekly up to four weeks after the preparation of the mixture in the glass flask. For this purpose, an aliquot (50 μl) of stock gaseous MIX22 was manually injected ($n = 3$) in the GC system to be analyzed according to the analytical method described in section 2.6.

The stability of MIX22 components when loaded in the NTD was evaluated by comparing measurements performed immediately after collection, after 6 and 24 h. Nine NTDs were loaded (25 ml at 15 ml min^{-1}) with the 1000-fold diluted MIX22 and analyzed in triplicate at each observation time according to the method described in section 2.6. During the experiments, NTDs were sealed with a Teflon cap and stored at room conditions (25 °C ± 2 °C and RH of 50% ± 5%). To simulate the conditions occurring during a typical GC sequence, the luer-lock end of the NTD was closed with the autosampler stainless-steel cap, which was equipped with a GC septum.

2.9. Statistical analysis

Demographic and clinical data are reported as mean ± standard deviation and ranges. The distribution of variables was tested for normality by a Shapiro–Wilk test, whereas the gender difference was evaluated by a Mann–Whitney test. Deming regression and analysis of variance (ANOVA) were used to evaluate the statistical significance of the analytical method parameters. A two-tailed p value lower than 0.05 was considered statistically significant. Statistical analysis and principal component analysis were performed using GraphPad Prism v.6.0 (GraphPad, La Jolla, USA) and XLSTAT v.2015.4.01 (Addinsoft, New York, USA), respectively.

A software (G* Power, version 3.1) was used to calculate the sample size assuming a level of significance (α) of 5%, power = 95% and effect size = 0.51 [32]. With these values of parameters, the calculated sample size was 39.

3. Results and discussion

3.1. Optimization of MS conditions

Optimization of the tandem MS conditions was done in a multistep process. First, 50 μl of stock gaseous MIX22 were directly injected into the GC and a total ion chromatogram ($31 < m/z < 200$) was acquired. Then, analysis of the full product scan MS at different collision energies allowed to select at least two product ions and set up MRM transitions for each compound. The most abundant mass transition was selected as the quantifier transition (Q) whereas the other was selected as the qualifier transition (q).

Table S1 is available online at stacks.iop.org/JBR/11/047110/mmedia in the supplementary information reports the qualifier and quantifier transitions with the corresponding collision energies, and the q/Q ratio.

3.2. Optimization of the NTD loading and desorption procedure

The effect of the injection mode, e.g. split (10:1 and 5:1) and splitless, on the shape of GC signals was investigated (figure S1). The splitless mode caused a peak broadening that was more pronounced for more volatile compounds such as isoprene compared to less volatile compounds such as 2-hexanone. In fact, the splitless mode increases the time needed to molecules to exit the needle and reach the column and favors diffusion. This is particularly true for more volatile compounds, which are retained from the stronger sorbent material (Carboxen 1000) positioned at a higher distance from the side hole. These results led to choose the injection split mode with a split ratio of 5:1 for VOCs desorption from the NTD and their transfer to the chromatographic column.

Desorption temperature (x_1), desorption time (x_2) and sampling flow rate (x_3) were selected as the parameters determining the analyte peak area (y) to be included in the 2^3 full factorial design. For each analyte, peak areas resulting from the experiments were used to fit the model:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{1,2}x_1x_2 + b_{1,3}x_1x_3 + b_{2,3}x_2x_3 + b_{1,2,3}x_1x_2x_3, \quad (2)$$

where y represents the predicted response (analyte peak area), and x_i and b_i the model parameters and coefficients, respectively.

For the majority of the compounds, desorption temperature and sampling flow rate were the most significant coefficients, whereas desorption time was not important. For each compound, a refined model was determined including only the significant terms. Figure 3 shows an overview of the results obtained for a representative compound (2-propanol). The plot of replicates (figure 3(a)) shows the peak areas obtained in the different experiments, whereas the histogram in figure 3(b) reports the model diagnostics such as the degree of fit (R^2, explained variation) that quantifies how well the model is able to reproduce the data of the training set (experiments 1–8), and predictive power (Q^2, predicted variation) that quantifies how well the model predicts the results of an independent set of validation observations (experiments 9–13). Figure 3(c) shows the values of the main model coefficients: an increased value of a parameter with a positive coefficient (e.g. time and temperature) rises the

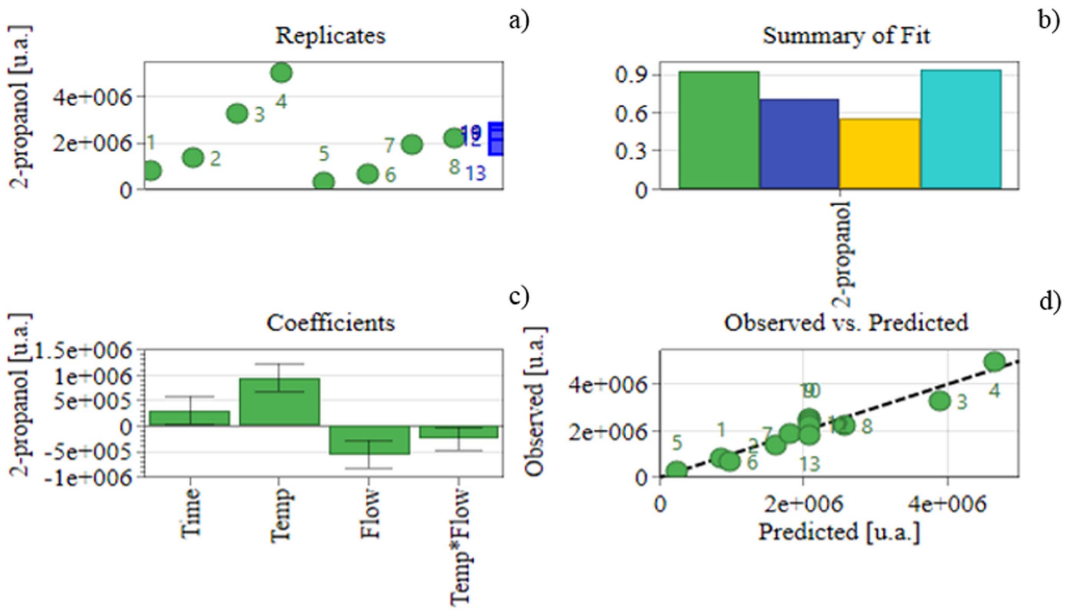

Figure 3. Overview of results obtained for 2-propanol: (a) plot of replicates, (b) summary of fit (R^2 (green), Q^2 (blue), model validity (yellow) and reproducibility (light blue)), (c) plot of coefficients and (d) model predictions. In this last plot, the correct prediction of results from experiments 9–13, not included in the model development, demonstrate the validity of the model.

response, whereas the contrary is true for parameters having negative coefficients (e.g. flow). The error bars reflect the uncertainty on the estimate of a coefficient and significance of the related parameter. Experimental values and model predictions are compared in figure 3(d).

Very VOCs showed an increased peak area (from 60% to 80%) at the higher desorption temperature, whereas a decrease (from 10% to 30%) was observed in less VOCs, probably due to their catalytic degradation on the surface of carbon sorbents at temperatures close to 300 °C [33, 34].

The optimal experimental conditions for 18 out of 22 compounds (from ethanol to hexanal), calculated from the models, were desorption temperature 280 °C, sampling flow rate 5 ml min^{-1} and desorption time 30 s, whereas for the others resulted 260 °C, 5 ml min^{-1} and 30 s. However, the following aspects were further considered: (a) stability of the NTD stationary phases and device reusability, (b) sampling invasiveness and patient discomfort. Among the three-stationary phase, DVB is the less thermally stable: at 290 °C, its reusability is very limited, and this increases the cost per analysis since NTDs are quite expensive [35]. Thus, the desorption temperature was set at 250 °C to preserve DVB from degradation and extend the use of NTDs up to 60 thermal cycles. Breath sampling is considered non-invasive, but patient discomfort increases very quickly when sampling is longer than 2–3 min, especially when samples are taken at the bedside. This sampling time represents also a limit to avoid hyperventilation. If these sampling times are to be respected, then a NTD loading flow rate of at least 15 ml min^{-1} is required. Further tests and analyses of real breath samples were then carried out using a desorption temperature of 250 °C, a sampling flow rate of 15 ml min^{-1} and desorption time 30 s. An acceptable decrease of peak areas from 30% to 50% was observed when compared to the optimal conditions. Using these conditions the desorption efficiency was higher than 95% for all compounds except carbon disulfide, whose efficiency was close to 90%.

3.3. Effect of sample volume on NTD efficiency

Five sample volumes (15, 25, 50, 75 and 100 ml) of diluted MIX22 (1000-fold from stock gaseous solution) were transferred at 15 ml min^{-1} into each NTD for evaluating breakthrough volume. In these conditions, the total amounts of VOCs loaded into NTD were: 200 ng (15 ml), 340 ng (25 ml), 680 ng (50 ml), 1100 ng (75 ml) and 1440 ng (100 ml), respectively.

Ethanol, acetone and carbon disulfide showed a linear behavior up to 50 ml, whereas a deviation from linearity was evident for dimethyl sulfide even at 25 ml. All the other compounds had a linear behavior within the investigated range (15–100 ml), with a coefficient of determination (R^2) higher than 0.995. These results were confirmed by connecting two NTDs in series with a modified yellow adapter, so that compounds not been retained in the first NTD were adsorbed in the second one. Two volumes (25 and 50 ml) of diluted MIX22 (1000-fold from the stock gaseous mixture) were transferred at 15 ml min^{-1} into the series NTDs by applying a two-fold pressure, due to the increased resistance of the system. The results confirmed that all the compounds, except for dimethyl sulfide, were quantitatively collected in the first NTD since no VOCs were found in the second one.

3.4. Release of contaminants from sampler device and materials

Five NTDs were analyzed immediately after the conditioning step (30 min at 250 °C with a nitrogen flow at 1 bar) to verify the effectiveness of the conditioning procedure. Only toluene and benzaldehyde, probably produced by the thermal degradation of DVB at 250 °C, were found in all chromatograms.

The possible contamination of a breath sample due to the contact with the sampling device (figure 2) was excluded by analyzing humid air samples flown through the device (results not shown).

A Nalophan bag (S/V ratio of 0.3 cm^{-1}) was filled with 10 l of humidified medical air and kept at room conditions (25 °C ± 2 °C and RH of 50% ± 5%) to evaluate the release of contaminants from Nalophan material and the effectiveness of cleaning procedures (the stopcock and polypropylene valve were washed 5-times with 2 ml of methanol). After 3 h, three aliquots (25 ml) were transferred from the bag to NTDs at 15 ml min^{-1}. Besides toluene and benzaldehyde, only 2-methyl-1,3-dioxolane, a compound emitted by recycled polyethylene terephthalate bags due to polymeric impurities [36], was found in these blank samples, confirming the results reported elsewhere [29].

3.5. Stability studies

The ANOVA evidenced that most compound recoveries from the glass flask measured right after the preparation and after a 6 d storage were not statistically significant different ($p < 0.05$). Only aldehydes (i.e. butanal, pentanal, hexanal, heptanal and benzaldehyde) showed a marked decrease (about 20%–50%) after 6 d, probably due to spontaneous reactions with oxygen to produce carboxylic acids [37]. The occurrence of such reactions was confirmed by the presence in the extracted ion chromatogram of the signal corresponding to an m/z ratio of 60, resulting from the McLafferty rearrangement typical of carboxylic acids. A good correlation ($r = 0.80$, $p < 0.05$) was found between the peak areas of aldehydes and the corresponding carboxylic acids. After 1 month, alcohols and ketones showed recoveries ranging between 70% and 90%, whereas dimethyl sulfide decreased at about 50%.

In the case of the double-walled Nalophan bag ($S/V = 0.3\ cm^{-1}$), no significant variations ($p < 0.05$) were observed within 3 h in the content of water and for the majority of the investigated compounds (1000-fold dilution in humid conditions), confirming the results reported elsewhere [29]. In fact, only carbon disulfide and aldehydes showed a loss of 15% and 20% within 3 h, respectively. For this reason, we suggest to transfer samples into NTDs as soon as possible.

All analytes were stable in NTDs for 24 h, except for ethanol, 2-propanol and acetone, which showed a decrease of about 20%. The recovery of dimethyl sulfide and carbon disulfide was lower than 60%. No improvement was observed by sealing the luer-lock end of NTDs with autosampler stainless-steel caps instead of Teflon caps.

3.6. IDLs and RRFs

The IDLs, i.e. the minimum analyte concentrations producing a signal distinguishable from noise within a statistical confidence limit (i.e. a signal-to-noise ratio equal to 3), were estimated by extracting the quantifier ions from the TIC chromatogram and/or using quantifier MRM transitions.

Table S2 in the supplementary information report the RRFs and IDLs for all the investigated compounds. The IDLs resulting from the extraction analyses were lower than 500 pptv for all compounds, except for ethanol (3 ppbv), C4-C7 aldehydes (1 ppbv) and C3-C4 alcohols (1 ppbv), whereas the IDLs from MRM analyses were always below 200 pptv, and was close to 20 pptv for dimethyl sulfide. The pre-concentration factors, calculated from the ratios of peak areas obtained by the manual injection of the MIX22 (50 μl) and NTD desorption, ranged between 500 and 2000. Inter- and intra-day relative standard deviations of the RRFs were always below 15% for all analytes except for carbon disulfide (25%), confirming NTME as a reliable method for the quantification of VOCs in human breath.

3.7. Application to real samples: HF patients

The optimized NTME procedure was applied to monitor the chemical composition of breath samples collected from forty patients suffering from HF. During the collection time, the self-paced respiratory rate was constant and the real-time end-tidal CO_2 partial pressures fluctuated randomly within a 10%. The simplicity (and very low pressure drop) of the sampling device and the short sampling time (i.e. <3 min) avoided the hyperventilation observed by other authors [37]. The constant respiratory rate also allowed to exclude modifications of breath composition due to changes of this parameter [38, 39].

Table 4 reports the demographic and clinical data of patients at the first observation time (hospital admission).

The Mann–Whitney test did not reveal statistically significant gender differences for any of the above cardiorespiratory variables as well as for blood sample values, whereas weight and body mass index were significantly different ($p < 0.05$). Moreover, glycaemia and glycated hemoglobin were not statistically different between diabetic and non-diabetic patients ($p > 0.05$).

All exhaled breath samples contained hydrocarbons (e.g. pentane and isoprene), carbonyl compounds (e.g. acetone, 2-butanone and 2-pentanone), alcohols (e.g. ethanol, 2-propanol and 1-butanol) as well as other compounds (e.g. 1-propanol). Aldehydes

Table 4. Characteristics of enrolled patients ($n = 40$) at the first observation time. Data are shown as mean ± standard deviation (range).

Characteristics	HF patients ($n = 40$)
Age (years)	70 ± 10 (42–85)
Gender, male:female	30:10
Weight (g)	male: 80 ± 14 (60–123) female: 63 ± 8 (51–75)
Body mass index (Kg m^{-2})	male: 27 ± 4 (20–41) female: 23 ± 3 (20–29)
NYHA, *n*	
1	4
2	12
3	17
4	7
Systolic blood pressure (mmHg)	122 ± 20 (90–180)
Diastolic blood pressure (mmHg)	74 ± 14 (50–110)
Heart rate (bpm)	75 ± 18 (53–150)
Respiratory rate (breaths per minute)	19 ± 7 (8–39)
Left ventricular ejection fraction (%)	33 ± 12 (15–65)
Low-density lipoprotein (mg dl^{-1})	98 ± 38 (42–182)
High-density lipoprotein (mg dl^{-1})	43 ± 12 (26–77)
Triglyceride (mg dl^{-1})	105 ± 43 (43–274)
Calcium (mg dl^{-1})	9 ± 1 (7–10)
Sodium (mg dl^{-1})	140 ± 7 (105–146)
Potassium (mg dl^{-1})	4 ± 1 (1–5)
Blood creatinine (mg dl^{-1})	1.3 ± 0.5 (0.8–3.3)
Estimated glomerular filtration rate (ml min^{-1})	55 ± 20 (19–104)
Blood brain natriuretic peptide (pg ml^{-1})	1230 ± 970 (140–3500)
Oxygen saturation (%)	95 ± 4 (79–99)
Glycaemia (mg dl^{-1})	diabetics, 145 ± 90 (80–410) non-diabetics, 105 ± 36 (40–183)
Glycated hemoglobin (mmol mol^{-1})	diabetics, 49 ± 14 (28–74) non-diabetics, 60 ± 27 (40–146)

(e.g. hexanal and heptanal) and dimethyl sulfide were detected only occasionally and therefore were not included in the data preliminary evaluation. The ethanol and 2-propanol concentrations in breath ranged between 40–2200 ppbv and 3–800 ppbv, respectively. These values were comparable with those measured in the ambient air, confirming the exogenous origin of these compounds, which are generally contained in the disinfectants used in hospitals [30].

Over 90% of patients had underlying comorbidities, including diabetes mellitus ($n = 15$), hypertension ($n = 29$), dyslipidemia ($n = 22$), chronic obstructive pulmonary disease ($n = 8$), chronic kidney disease ($n = 12$), chronic liver disease ($n = 2$), coronary artery disease ($n = 18$), atrial fibrillation ($n = 13$), and oncological diseases ($n = 6$).

Principal component analysis was used to obtain an overall view of the internal structure of the dataset (figure 4), including the data from clinical analyses and the chemical characterization of exhaled breath at the first observation time (t_0).

The loading plot (figure 4(a)) shows that the concentration of BNP, a commonly accepted biomarker for the diagnosis and monitoring of HF [16], was positively correlated with acetone ($r = 0.51$, $p < 0.05$) and creatinine ($r = 0.62$, $p < 0.05$) and negatively correlated with oxygen saturation ($r = -0.48$, $p < 0.05$), isoprene ($r = -0.36$, $p < 0.05$) and estimated glomerular filtration rate ($r = -0.36$, $p < 0.05$). In addition, low levels of left ventricular ejection fraction (LVEF) were related to high levels of BNP ($r = -0.44$, $p < 0.05$), acetone ($r = -0.38$, $p < 0.05$) and 2-pentanone ($r = -0.37$, $p < 0.05$). These data are consistent with the acute conditions and the disease severity degree of most patients, whose majority (60%) belonged to NYHA class III ($n = 17$) and IV ($n = 7$). The low LVEF and oxygen saturation document that patients' hearts were unable to perform their function correctly and that respiration was hampered. In some patients, disease manifestations, such as the reduction of cardiac output and the presence of different amounts of water in the lungs (up to pulmonary edema), induce a progressive impaired perfusion of other organs and apparatus. This condition, sometimes associated to comorbidities, produces a failure of other functions like renal and/or liver. As an example, the reduced estimated glomerular filtration rate and the high creatinine levels suggest a poor kidney function in some patients of this study. In the HF syndrome, there are changes of metabolic and neuroendocrine responses with a cardiac release of natriuretic peptides like BNP and an increase of heart rate.

The possible enhancement of lipolysis could trigger the increase of circulating free fatty acids [40] and induce an overproduction of ketone bodies (i.e. acetoacetate, beta-hydroxybutyrate and acetone). Ketone bodies are the most efficient substrate to produce adenosine triphosphate and therefore could be a particularly useful energy source when the oxygen delivery to tissues is reduced, such as in acute HF [41]. The impaired metabolism was discussed by Lommi *et al* [42], who hypothesized that free fatty acids could be an alternative source of energy for the heart during acute HF. On the other hand, ketone bodies are also produced in different conditions, e.g. unbalanced diabetes mellitus or fasting [43], making the relationship of breath acetone with HF less clear. In our study, the possible interference from fasting was partially reduced by collecting breath samples always in the morning at the same time, 2 h after breakfast. The non-statistically different average values for blood glycaemia and glycated hemoglobin between diabetic and non-diabetic patients suggest that diabetes was under control for most patients.

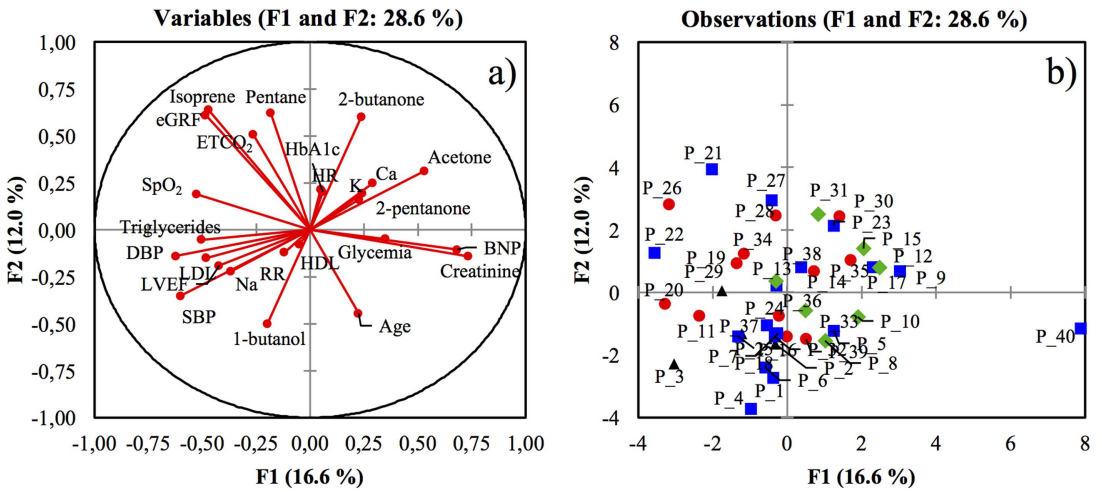

Figure 4. Principal component analysis of the patients' dataset ($n = 40$) at t_0: loading plot (a) and score plot (b). Legend: high-density lipoprotein (HDL), low-density lipoprotein (LDL), left ventricular ejection fraction (LVEF), oxygen saturation (SpO_2), heart rate (HR), respiratory rate (RR), estimated glomerular filtration rate (eGFR), end-tidal CO_2 ($ETCO_2$), brain natriuretic peptide (BNP), calcium (Ca), potassium (K), sodium (Na), glycated hemoglobin (HbA1c), systolic blood pressure (SBP) and diastolic blood pressure (DBP). Black up-pointing triangle (NYHA I), red dots (NYHA II), blue circle (NYHA III) and green diamond (NYHA IV).

Furthermore, median (interquartile range) breath acetone concentrations at the admission calculated for diabetic ($n = 15$) and non-diabetic subgroup ($n = 25$) [1200 ppbv (800–1700 ppbv) and 1160 ppbv (700–2600 ppbv), respectively] were not significantly different ($p > 0.05$). We hypothesized that breath acetone levels in our HF patients could be related to cardiac decompensation more than to other factors (i.e. diabetes and fasting). Moreover, in the literature a relationship between BNP and acetone has been previously described [44, 45]. A good correlation ($r = 0.77$, $p < 0.001$) between these two analytes was found in 59 HF patients in acute conditions [44], and high acetone levels ($\geqslant 3.7\ \mu g\,l^{-1}$, 50th percentile) in exhaled breath were associated to poor prognosis in HF patients showing a reduced ejection fraction [45].

In our case, only BNP values in blood samples, LVEF measurements and 2-pentanone in exhaled breath were significantly different among patients belonging to different NYHA functional classes. In fact, the median (interquartile range) concentration of BNP was 350 pg ml^{-1} (300–760 pg ml^{-1}) among patients in class I ($n = 4$), 560 pg ml^{-1} (250–1100 pg ml^{-1}) in class II ($n = 12$), 1250 pg ml^{-1} (700–2100 pg ml^{-1}) in class III ($n = 17$) and 2100 pg ml^{-1} (800–3100 pg ml^{-1}) in class IV ($n = 7$). The LVEF was 45% (35%–55%) for patients in class I, 35% (30%–40%) in class II, 30% (20%–40%) in class III and 25% (20%–30%) in class IV. The concentration of 2-pentanone in exhaled breath was 110 pptv (80–250 pptv) among patients in class I, 360 pptv (190–580 pptv) in class II, 370 pptv (200–1000 pptv) in class III and 850 pptv (500–1100 pptv) in class IV. Slightly higher breath acetone concentrations, even if not significant ($p > 0.05$), were also observed in higher NYHA classes: NYHA I had a median of 900 ppbv and an interquartile range 700–1000 ppbv, whereas NYHA IV had a median of 1600 ppbv and an interquartile range 1200–2600 ppbv. On the contrary, the corresponding values of exhaled breath isoprene slightly decreased from 450 ppbv (280–570 ppbv) to 160 ppbv (100–400 ppbv). The score plot (figure 4(b)) shows that patients belonging to different NYHA functional classes are not separated, as the large inter-subject variability of clinical and breath data could hide other possible differences, as discussed for acetone from other authors [46]. For these reasons, we postulate that patient's monitoring by breath analysis would be more successful if performed in single subjects over time, as we already reported for breath measurements in general terms [10]. In this way, each patient would act as his/her own control and variations of breath composition could be correlated more easily to the disease evolution [47].

Figure 5 shows the variations over time of end-tidal CO_2 partial pressure and breath concentration of acetone, isoprene, 2-pentanone and blood BNP levels normalized to values measured at the admission (t_0) for two representative patients (P_9 and P_13).

In our population, isoprene showed different patterns. In fact, about 40% of patients showed a 40% decrease of breath isoprene concentration between admission and discharge from the hospital, whereas 30% showed an isoprene increase of about 50%. No difference in isoprene concentration was observed between the correspondent breath samples collected from the remaining patients (30%). Due to the low affinity with blood and the fast diffusion of isoprene through alveolar capillaries, the increase may be related to an improved hemodynamic condition, as recently pointed out by others [48, 49]. In addition, the combination of a reduced sterol synthesis and a reduced pool of squalene peroxidation by free radicals could also be the reason of the isoprene lower

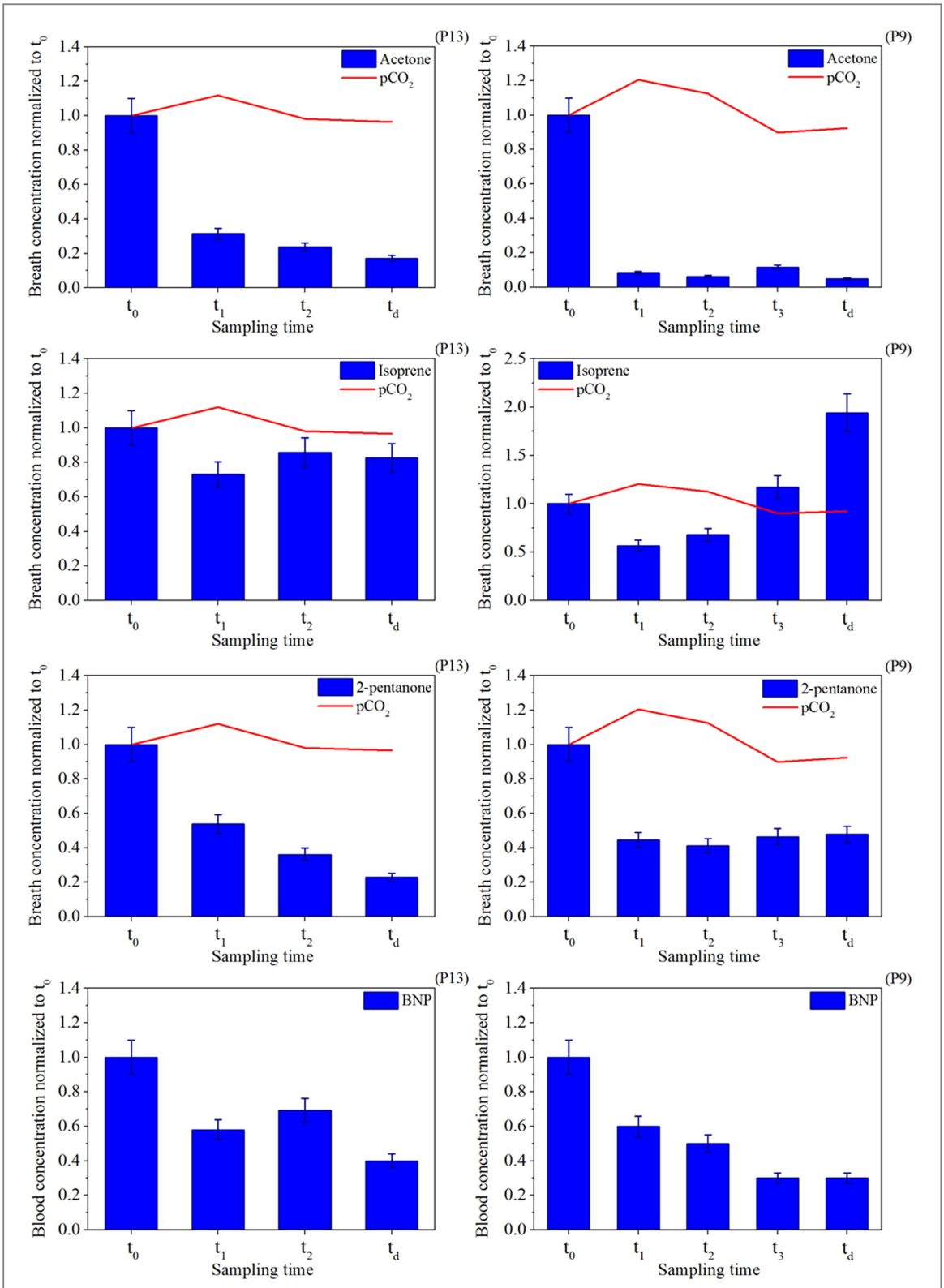

Figure 5. Acetone, isoprene and 2-pentanone breath concentrations, end-tidal CO_2 partial pressure and BNP blood concentrations measured over time. Data were normalized to the values measured at the first sample collected at the admission from patients P_9 and P_13. Error bars indicate the standard deviation.

concentration observed in the acute phase [50]. Also for pentane and 1-butanol, no similar trends in different patients were found. These data confirmed that one or more confounding factors (e.g. diet and drug therapy) could increase the complexity with a real difficulty for the meaning of these breath compounds in HF [51].

Relatively to breath concentrations of 2-butanone and 2-pentanone, in about 75% of patients, there is a decrease of approximately 40% and 60% from the admission to the discharge, respectively. These

Table 5. Breath acetone concentration (ppbv) measured for all the enrolled patients ($n = 40$) during the hospitalization: at the admission (t_0), after 48 h time spans (t_1, t_2 and t_3) and at the discharge (t_d).

	Collection points				
ID patient	t_0	t_1	t_2	t_3	t_d
P_1	1200	430			500
P_2	930	620			240
P_3	880	500			260
P_4	1580	750			710
P_5	1140	580			500
P_6	1840	340			300
P_7	1050	600			320
P_8	1750	820			570
P_9	3650	310	230	420	170
P_10	1560	490			500
P_11	300	110			260
P_12	2620	1770			1670
P_13	710	220	170		120
P_14	1000	310			350
P_15	1610	1140	680		1240
P_16	1000	740			760
P_17	3420	1640	1070	620	400
P_18	400	610			390
P_19	2250	1010			810
P_20	700	660	540		380
P_21	4530	4200	2680		1290
P_22	470	310			330
P_23	27520	5920	4620	880	380
P_24	1030	1060	470		360
P_25	330	330	380	420	330
P_26	790	510	630	770	820
P_27	1710	360	590	460	240
P_28	3720	330			240
P_29	590	1540			420
P_30	6440	1020			600
P_31	4360	770	540	370	320
P_32	600	240	520		250
P_33	1220	520	140		340
P_34	3560	2720			350
P_35	1160	1490	3210		1080
P_36	600	400			430
P_37	260	110			140
P_38	1710	270			230
P_39	210	200			230
P_40	6040	15890			300

substances could derive by fatty acids degradation [52], which is presumably related to the increased lipolysis occurring during acute HF [42].

Table 5 reports the breath acetone concentrations measured in all the enrolled patients ($n = 40$) at the admission, after 48 h time spans (when available) and at the discharge.

The majority of HF patients ($n = 31$) showed a remarkable decrease of breath acetone concentration over time (a factor 3 at least) as their health conditions improved. Among these, the subgroup of nine patients characterized by the lowest LVEF values (<20%) and highest BNP blood levels (>1000 pg ml^{-1}) at the admission exhibited the most pronounced decreases (a factor of 6 or higher, up to a factor of 80 in the case of patient P_23). As mentioned above, this behavior could be related to an altered oxidation of fatty acids during the acute phase leading to an increased production of ketone bodies such as acetone [41, 42]. Only nine HF patients, namely P_11, P_15, P_18, P_22, P_25, P_26, P_29, P_35 and P_39, did not display any decrease of the breath acetone levels between admission and discharge. These nine patients, belonging to NYHA class I and II, showed the classical symptoms of HF (e.g. dyspnea) at the admission and maintained a low level of BNP (<500 pg ml^{-1}) during all the hospitalization time.

Interestingly, patients suffering from both diabetes and HF displayed the same decreasing trend of breath acetone and, from this point of view, they were indistinguishable from the other patients. Even if breath acetone can change for a variety of reasons, our findings suggest that the evolution of the HF condition was the leading factor explaining the observed variations in the enrolled population.

4. Conclusions

This paper reports a validated analytical procedure for the determination of VOCs (e.g. ketones, aldehydes, alcohols, hydrocarbons, sulfur and aromatic compounds) in exhaled breath samples by NTME followed by GC-MS/MS analysis. The analytical protocol was optimized using humidified gaseous standard mixtures to accurately simulate the performance of the technique with real breath samples. The use of experimental design allowed us to clarify the role of the sampling flow rate on NTD efficiency in extracting VOCs from breath samples.

An aliquot (25 ml) of sample was loaded into the NTD at 15 ml min^{-1}, and the analytes were thermally desorbed at 250 °C for 30 s using a split ratio of 5:1. These conditions, that guarantee a quantitative desorption from the three-bed NTD without the occurrence of carry over effects, represent in our opinion the best compromise between optimum performance, cost of measurement (extended NTD life time) and reduced discomfort to patients.

The method was successfully applied to monitor the chemical composition of breath samples collected from forty patients suffering from HF. Our results highlighted the potential role of breath acetone for monitoring the health conditions of these patients, as a remarkable decrease (at least to a factor of 3) of acetone concentration was observed for the majority patients at the discharge compared to admission time.

The results obtained during this study strongly support the hypothesis that the acetone concentration in breath increases when the patient is at home and health conditions worsen for a HF relapse. Thus, the use of point of care devices equipped with a reliable sensor system for monitoring acetone in breath may be useful to reveal a possible HF exacerbation event, as

they might provide an early advice to the patients and the family doctors.

Acknowledgments

This work was financially supported by the HEARTEN project, which has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 643694. The authors would like to thank PAS-technologies (Magdala, Germany) for the technical support, and the ROMBAT group at the University of Rostock for their friendly and helpful collaboration.

ORCID iDs

T Lomonaco https://orcid.org/0000-0002-1822-7399
V Barletta https://orcid.org/0000-0002-4231-7781

References

[1] Trefz P, Rösner L, Hein D, Schubert J K and Miekisch W 2013 *Anal. Bioanal. Chem.* **405** 3105–15
[2] Qin T, Xu X, Polak T, Pacakova V, Stulik K and Jech L 1997 *Talanta* **44** 1683–90
[3] Jurdáková H, Kubinec R, Jurcisinová M, Krkosová Z, Blasko J, Ostrovskỳ I, SojáK L and Berezkin V G 2008 *J. Chromatogr.* A **1194** 161–4
[4] Alonso M, Cerdan L, Godayol A, Anticó E and Sanchez J M 2011 *J. Chromatogr.* A **1218** 8131–9
[5] Mesarchaki E, Yassaa N, Hein D, Lutterbeck H E, Zindler C and Williams J 2014 *Mar. Chem.* **159** 1–8
[6] Filipiak W, Filipiak A, Ager C, Wiesenhofer H and Amann A 2012 *J. Breath Res.* **6** 1–19
[7] Warren J M and Pawliszyn J 2011 *J. Chromatogr.* A **1218** 8982–8
[8] Sanchez J M 2014 *J. Chromatogr.* A **1369** 18–25
[9] Helming D and Vierling L 1995 *Anal. Chem.* **67** 4380–6
[10] Ceccarini A, Di Francesco F, Fuoco R, Ghimenti S, Onor M, Tabucchi S and Trivella M G 2012 *Analytical Techniques for Clinical Chemistry: Methods and Applications* ed S Caroli and G Zaray (New York: Wiley) pp 651–77
[11] Lourenco C and Turner C 2014 *Metabolites* **4** 465–98
[12] Yancy C W *et al* 2016 *Circulation* **128** 240–327
[13] Mozzafarian D *et al* 2015 *Circulation* **131** e29–322
[14] Pang P S, Xue Y, Defilippi C, Silver M, Januzzi J and Maisel A 2012 *Congest. Heart Fail.* **18** S5–8
[15] de Boer R A, Daniels L B, Maisel A S and Januzzi J L 2015 *Eur. J. Heart Failure* **17** 559–69
[16] Emdin M, Vittorini S, Passino C and Clerico A 2009 *Eur. J. Heart Failure* **11** 331–5
[17] Cao W and Duan Y 2006 *Clin. Chem.* **52** 800–11
[18] Di Francesco F, Fuoco R, Trivella M G and Ceccarini A 2005 *Microchem. J.* **79** 405–10
[19] Beauchamp J, Herbig J, Gutmann R and Hansel A 2008 *J. Breath Res.* **2** 1–7
[20] Ghimenti S, Tabucchi S, Lomonaco T, Di Francesco F, Fuoco R, Onor M, Lenzi S and Trivella M G 2013 *J. Breath Res.* **7** 1–7
[21] Ghimenti S, Di Francesco F, Onor M, Stiegel M A, Trivella M G, Comite C, Catania N, Fuoco R and Pleil J D 2013 *J. Breath Res.* **7** 1–10
[22] Grote C and Pawliszyn J 1997 *Anal. Chem.* **69** 587–96
[23] Di Francesco F, Loccioni C, Fioravanti M, Russo A, Pioggia G, Ferro M, Roehrer I, Tabucchi S and Onor M 2008 *J. Breath Res.* **2** 1–10
[24] Salvo P, Ferrari C, Persia R, Ghimenti S, Lomonaco T, Bellagambi F G and Di Francesco F 2015 *Med. Eng. Phys.* **37** 539–44
[25] Herbig J, Muller M, Schallhart S, Titzmann T, Graus M and Hansel A 2009 *J. Breath Res.* **3** 1–10
[26] Spanel P and Smith D 2011 *Mass Spectrom. Rev.* **30** 236–67
[27] de Lacy Costello B, Amann A, Al-Kateb H, Flynn C, Filipiak W, Khalid T, Osborne D and Ratcliffe N M 2014 *J. Breath Res.* **8** 1–29
[28] Amann A, de Lacy Costello B, Miekisch W, Schubert J, Buszewski B, Pleil J, Ratcliffe N and Risby T 2014 *J. Breath Res.* **8** 1–17
[29] Ghimenti S, Lomonaco T, Bellagambi F G, Tabucchi S, Onor M, Trivella M G, Ceccarini A, Fuoco R and Di Francesco F 2015 *J. Breath Res.* **9** 1–13
[30] Ghimenti S, Tabucchi S, Bellagambi F G, Lomonaco T, Onor M, Trivella M G, Fuoco R and Di Francesco F 2015 *J. Pharm. Biomed. Anal.* **106** 218–23
[31] McClenney W A and Holdren M W 1999 *Air Method, Toxic Organics-15 (TO-15): Compendium of Methods for the Determination of Toxic Organic Compounds in Ambient Air, 2nd Edn: Determination of Volatile Organic Compounds (VOCs) in Air Collected in Specially-Prepared Canisters and Analyzed by Gas Chromatography/Mass Spectrometry (GC/MS)* EPA 625/R-96/010b Environmental Protection Agency (EPA)
[32] McCrum-Gardner E 2010 *Int. J. Ther. Rehabil.* **17** 10–4
[33] Dettmer K and Engewald W 2002 *Anal. Bioanal. Chem.* **373** 490–500
[34] Sanchez J M and Sacks R D 2005 *J. Sep. Sci.* **28** 22–30
[35] http://vici.com/hayesep/polyspec.php (Accessed: 2 December 2016)
[36] Ohkado Y, Kawamura Y, Mutsuga M, Tamura H O and Tanamoto K 2005 *Food Hyg. Safe Sci.* **46** 13–20
[37] Vanoye L, Favre-Requillion A, Aloui A, Philippe R and de Bellefon C 2013 *RSC Adv.* **3** 18931–7
[38] Cope K A, Watson M T, Foster W M, Sehnert S S and Risby T H 2003 *J. Appl. Physiol.* **96** 1371–9
[39] Lomonaco T, Salvo P, Ghimenti S, Biagini D, Bellagambi F G, Fuoco R and Di Francesco F 2015 *Conf. Proc. IEEE Engineering Medicine Biology Society* vol 2015, pp 7618–21
[40] Wang Z V, Li D L and Hill J A 2014 *J. Cardiovasc. Pharmacol.* **63** 302–13
[41] Kodde I F, van der Stok J, Smolenski R T and de Jong J W 2007 *Comp. Biochem. Physiol.* A **146** 26–39
[42] Lommi J, Kupari M, Koskinen P, Näveri H, Leinonen H, Pulkki K and Härkönen M 1996 *J. Am. Coll. Cardiol.* **28** 665–72
[43] Laffel L 1999 *Diabetes Metab. Res. Rev.* **15** 412–26
[44] Marcondes-Braga F G, Gutz I G R, Batista G L, Saldiva P H N, Ayub-Ferreira S M, Issa V S, Mangini S, Bocchi E A and Bacal F 2012 *Chest* **142** 457–66
[45] Marcondes-Braga F, Batista G L, Gutz I G R, Saldiva P H N, Mangini S, Issa V S, Ayub-Ferreira S M, Bocchi E A, Pereira A C and Bacal F 2016 *PLoS One* **11** 1–10
[46] Spanel P, Dryahina K, Rejskova A, Chippendale T W and Smith D 2011 *Physiol. Meas.* **32** 23–31
[47] Beauchamp J and Pleil J 2013 *J. Breath Res.* **7** 1–3
[48] Sukul P, Trefz P, Schubert J K and Miekisch W 2014 *J. Breath Res.* **8** 1–10
[49] Pabst F, Miekisch W, Fuchs P, Kischkel S and Schubert J K 2007 *J. Cardiothoracic Surg.* **18** 2–37
[50] McGrath L T, Patrick R and Silke B 2011 *Eur. J. Heart Failure* **3** 423–7
[51] Stone B G, Besse T J, Duane W C, Evans C D and DeMaster E G 1993 *Lipids* **28** 705–8
[52] van den Velde S, Quirynen M, van Hee P and van Steenberghe D 2007 *J. Chromatogr.* B **853** 54–61