Determination of carbonyl compounds in exhaled breath by on-sorbent derivatization coupled with thermal desorption and gas chromatography-tandem mass spectrometry

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Abstract

A reliable method for the determination of carbonyl compounds in exhaled breath based on on-sorbent derivatization coupled with thermal desorption and gas chromatography-tandem mass spectrometry is described. The analytical performances were optimized for a mixture of C2-C9 aldehydes and C3-C9 ketones, particularly interesting for clinical applications, by using an internal standard and applying a 2^3 full factorial design. A volume of sample (250 mL) was loaded at 50 mL/min into a Tenax GR sorbent tube containing 130 nmol of O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride. All compounds showed a limit of detection lower than 200 pptv. The yield of the derivatization procedure was normalised by adding to the sample a known amount of ^{6}D-acetone as internal standard. This allowed to halve the relative standard deviation to 10 and 15% for mono- and di-carbonyl compounds, respectively, thus improving reliability. The optimized method was applied to the determination of carbonyl compounds in twelve breath samples collected from four patients suffering from heart failure during the hospitalization.

Keywords:
Breath analysis, Carbonyl compounds, On-sorbent derivatization, Thermal desorption, GC-MS/MS.

1. Introduction

Over the last decades, exhaled breath analysis has become more and more attractive [1, 2] for its potential as an easy, painless and non-invasive tool to monitor in real time physiological and pathological conditions [3] as well as exposure to environmental contaminants [4]. A main advantage of the approach is that it can be used with people of all ages and conditions (e.g. newborns, infants [5] and mechanically ventilated patients [6]) to reduce problems associated with blood sampling (e.g. acceptance from patients, risk of infections, production of potentially infected waste and need of trained personnel working in dedicated environment). Despite potential, the development of diagnostic breath tests and their use in the clinical practice is hampered from the lack of standardized procedures for breath collection [7]. Several studies highlighted that real-time monitoring of carbon dioxide during breath sampling allows to selectively collect the end-tidal fraction, reduce the variability of the composition and improve the representativeness of samples [8, 9].

Off-line breath analysis is generally carried out in three steps: (i) collection of the sample in a canister [10] or a sampling bag [11, 12], (ii) pre-concentration of the compounds of interest using different techniques (e.g. solid phase extraction [13], solid phase micro-extraction [14] and needle trap micro-extraction [15]) and finally, (iii) analysis by thermal desorption coupled to gas chromatography-mass spectrometry (TD-GC-MS). Devices that allow combining breath sample collection and analyte extraction/pre-concentration in a single step [6, 14, 15] and prompt analysis by TD-GC-MS minimize the possible losses of compounds from bags [16, 17]. Instruments like proton-transfer-reaction mass spectrometer and selected-ion flow-tube mass spectrometer are commercially available for the real-time analysis of trace gases in breath with high sensitivity and wide dynamic range [18, 19], but the high cost and the uncertain identification of isobaric compounds make off-line methods still more common for breath analysis.

Various classes of volatile organic compounds (VOCs) can be detected in exhaled breath [20]. A total of 870 compounds have been found in exhaled breath, and many of them have been also identified in urine (10%), skin emanations (19%), blood (5%), saliva (13%) and faeces (13%) [21]. Among these compounds, there is a considerable interest for monitoring in breath oxygen-containing substances like aldehydes and ketones because they mainly result from oxidative stress [22]. Liver, heart, and brain represent tissues with a higher oxygen consumption rate and physiologically express higher antioxidant enzyme levels than those with lower oxygen consumption [23]. In fact, considerable evidences are now emerging that these carbonyls are also involved in the progression of chronic obstructive pulmonary disease [24], neurodegenerative disease [25], liver disease [26] and heart failure [27], due to their chemical reactivity that may cause further lesions to proteins and membranes [22]. These pathways may in turn exaggerate oxidative stress, forming a vicious cycle [28].

Unfortunately, the low concentration levels (ranged between tens and hundreds of pptv) of most carbonyls in exhaled breath and the instability due to their chemical reactivity make the analysis of these compounds quite challenging. Underestimation of concentrations due to carbonyls' reactivity can be reduced by direct sampling in sorbent tubes instead of bags, although several studies discussed the hypothesis of on-tube oxidation of aldehydes [29, 30]. Chemical derivatization coupled with pre-concentration techniques may overcome the reactivity issue and allow the analysis of carbonyl in breath. Several analytical approaches have been proposed for the determination of carbonyls in ambient air [31, 32]. Sorbent cartridges coated with a derivatization agent (e.g. 2,4-dinitrophenylhydrazine, DNPH) are typically used for the collection of aldehydes and ketones from gaseous samples [33-35]. The hydrazone derivatives are eluted from the cartridge with acetonitrile and then analysed by high-pressure liquid chromatography (HPLC) coupled with an UV-Vis [36] or a mass spectrometric detector [37]. This approach is currently the standard analytical method suggested by US EPA [38] for the determination of carbonyls in ambient air. However, the effect of humidity on the collection efficiency and the co-elution of larger carbonyl compounds (e.g. >C5) are the main drawbacks of the DNPH approach [31] that limit its application to breath analysis. Hence, various attempts have been made to find more reliable methods based on different derivatization reagents. In this perspective, pentafluorobenzylhydroxylamine (PFBHA) has shown better performance in GC-based analysis compared to the classical DNPH method [39-41]. Only few authors have proposed the use of PFBHA for the determination of carbonyls in breath [42-44]. In these procedures, reactive aldehydes and ketones were transformed into stable oximes by means of on-fiber derivatization (SPME-OFD). To the best of our knowledge, no study has been published on the determination of carbonyls in breath samples based on sorbent tubes derivatization with PFBHA coupled with TD-GC-MS/MS analysis. This approach should improve selectivity and sensitivity and also allow the analysis of a larger number of analytes than SPME.

2. Materials and methods

2.1. Chemicals and materials

A stock liquid mixture (L1) of 2-butanone, 2-pentanone, 3-pentanone, 2-hexanone, 4-heptanone, 3-heptanone, 2-heptanone, 3-octanone, 2-octanone, 5-nonanone, and 2-nonanone was purchased from AccuStandard, Inc. Chemical Reference Standard (USA). Acetone, 3-hydroxy-2-butanone, 2,3-butanedione, acetaldehyde, propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, benzaldehyde, glyoxal, methylglyoxal, methacrolein, acrolein and methanol were purchased from Sigma Aldrich (Italy). All compounds were GC grade standard with a purity higher than 99%.

O-2,3,4,5,6-pentafluorobenzylhydroxylamine hydrochloride powder (purity >99%) was purchased from Alfa Aesar (Germany).

Labelled ^{6}D-acetone with a purity of 99.8% was purchased from ARMAR Chemicals (Switzerland).

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%) and nitrogen 5.0 IP were purchased from Sol Group Spa (Italy). Each gas was further purified with a super clean filter from Agilent Technologies (USA) to remove water, oxygen and hydrocarbon contaminants.

Commercial stainless steel sorbent tubes for thermal desorption (O.D. 6.4 mm, I.D. 5 mm, 89 mm length) packed with 250 mg of 60/80 mesh Tenax GR phase (70% Tenax TA, 2,6-diphenyl-p-phenylene oxide and 30% graphite) were purchased by Markes International (UK).

Handmade Nalophan bags were fabricated at (film) surface-to-(sample) volume ratio (S/V) of 0.3 cm^{-1} from a roll of Nalophan tube (diameter 47 or 23.5 cm, film thickness 20 µm), supplied by Kalle (Germany), according to the procedure described elsewhere [17].

2.2. Preparation of standards

2.2.1. Liquid mixtures

A stock liquid mixture of aldehydes (L2) was prepared by mixing 50 µL each of these compounds, acetaldehyde, propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, benzaldehyde, glyoxal, methylglyoxal, methacrolein, acrolein, in a 1 mL glass vial equipped with a screw cap Mininert Valve (Supelco, USA). In the same way, another 1 mL glass vial was used to prepare a stock liquid mixture (L3) by mixing 50 µL of 3-hydroxy-2-butanone, 50 µL of 2,3-butanedione and 150 µL of acetone. These two stock solutions (L2 and L3) were prepared daily and stored at 4 °C.

Each stock liquid mixture (L1, L2 and L3) was diluted (100-fold) with methanol into a 1 mL vial equipped with a screw cap Mininert Valve. For the optimization of GC-MS parameters, the oximes were prepared in accordance to Cancho *et al* [45] by mixing 100 µL of each diluted solution with 1 mL of methanol containing 1 g/L of PFBHA and keeping the solution at 40 °C for two hours. Then, 1 µL of this solution was loaded into Tenax GR sorbent tubes using the calibration solution loading rig (CSLR) supplied by Markes International (UK). This device, specifically designed for loading sorbent tubes with gaseous or liquid standards, consists of an unheated injector port with a valve to flow the carrier gas sequentially through each tube. The methanol solution was introduced at room temperature (25 ± 2 °C) through the injector septum using a 1 µL syringe (Hamilton, USA) and then vaporized with a flow of 250 mL of dried medical air (50 mL/min) that allowed the oximes to reach the sorbent bed in the vapour phase. Under these conditions, methanol was completely purged out from the sorbent tube since its breakthrough volume at 20 °C for 1 g of Tenax GR is 0.2 L [46].

PFBHA solutions were prepared by dissolving the required amount of derivatizing agent in 50 mL of methanol. These solutions were prepared weekly and stored at 4 °C.

2.2.2. Gaseous mixtures

Three different stock gaseous mixtures of aldehydes and ketones were prepared by injecting 20 µL of L1, L2 and L3 solutions into three pre-evacuated 2 L glass flasks equipped with a screw cap Mininert Valve. Each glass flask was heated at 37 ± 1 °C until complete evaporation of the liquid and subsequently filled with dried medical air from a bag up to the ambient pressure. These gaseous mixtures were kept in a 1.1 m^3 thermostat at 37 ± 1 °C (RH 15 ± 3%) for 1 week. The concentrations of carbonyls in the glass flask are reported in table 1.

Table 1. Concentrations of analytes in the glass flask calculated at 37 °C and ambient pressure.

	Concentration in the glass flask (ppmv)
Acetaldehyde	130
Acetone	2550
Propanal	100
Acrolein	110
2-butanone	200
Methacrolein	140
Butanal	180
2-pentanone	170
3-pentanone	170
3-hydroxy-2-butanone	430
Pentanal	100
2-hexanone	150
4-heptanone	130
Hexanal	210
3-heptanone	130
2-heptanone	130
3-octanone	120
Heptanal	200
2-octanone	120
5-nonanone	110
Benzaldehyde	350
Octanal	230
2-nonanone	110
Nonanal	260
Glyoxal	320
Methylglyoxal	320
2,3-butanedione	350

These stock gaseous mixtures were further diluted with humidified medical air into Nalophan bags to simulate water vapour contained in breath samples. Humid working gaseous mixtures were prepared at room conditions (25 ± 2 °C and RH of 50 ± 5%) by flowing medical air (500 mL/min) through a gas bubbler filled with 5 mL of ultrapure water. In these conditions, the RH values in the gaseous mixtures ranged between 80% and 90%. After preparation, Nalophan bags were stored in the thermostat for half an hour to reduce the sample humidity down to 15 ± 3% of RH.

In the same way, a stock gaseous solution of ^{6}D-acetone was obtained by evaporating 5 µL of liquid labelled standard into a 2 L glass flask equipped with a screw cap Mininert Valve. This gaseous solution was kept in the thermostat at 37 ± 1 °C (RH 15 ± 3%) for 1 month. Under these conditions (37 °C and ambient pressure), the calculated concentration of ^{6}D-acetone resulted 870 ppmv.

2.3. Preparation of sampling tubes

Prior to first usage, Tenax GR sorbent tubes were conditioned at 320 °C for 2 hours and then at 335 °C for 30 min, under permanent He flow (70 mL/min) and a flow path temperature of 190 °C. After conditioning, tubes were capped at both ends with ¼" brass storage caps fitted with ¼" combined PTFE ferrules. Just before use, Tenax GR sorbent tubes were conditioned again for 15 min at 250 °C using a He flow of 70 mL/min.

Tenax GR tubes were spiked at room temperature (25 ± 2 °C and RH 50 ± 10%) by injecting 1 µL of

methanol solution containing 130 nmol of PFBHA, using the calibration solution loading rig. This aliquot was vaporized by a flow of 250 mL of dried medical air (50 mL/min) to let PFBHA reach the sorbent bed. Afterwards, tubes were capped at both ends using the same storage caps to avoid any contamination.

2.4. Sample collection and analysis

Mixed breath samples were collected by using Nalophan bags prepared according to the procedure described elsewhere [17]. All the components of the sampling system consisted of sterile inert materials. Each subject was asked to calmly fill the bags with multiple deep breaths. All breath samples were processed within 3 hr from sampling.

After collection, Nalophan bags were stored in the thermostat for half an hour to reduce the sample humidity down to 15 ± 3% of RH and prevent water condensation on the bag walls. Then, 250 mL of sample were flowed at 50 mL/min through a drying tube, filled with 9 g of anhydrous sodium sulphate (SKC, USA) for water removal, connected to a Tenax GR sorbent tube previously spiked with PFBHA (130 nmol). An aliquot (25 μL) of ^{6}D-acetone gaseous solution (870 ppmv) was injected during the sample loading using a long life non-stick septum (Agilent Technologies, USA). The sample flow through the tubes was controlled by a rotameter (0–150 mm) connected to the pump (KNF, Italy) (figure 1). The Tenax tube was capped as described before and stored in the thermostat for 24 h.

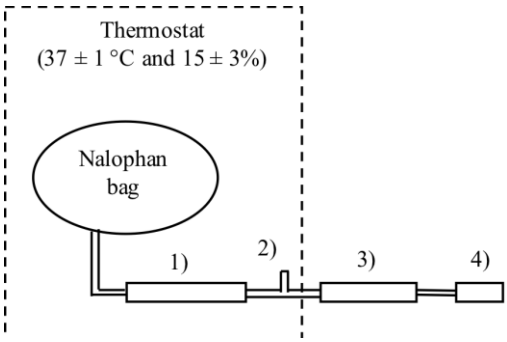

Figure 1. Sample transfer from Nalophan bags into Tenax GR sorbent tubes: 1) drying tube packed with 9 g of anhydrous sodium sulphate, 2) septum to inject ^{6}D-acetone, 3) Tenax GR sorbent tube and 4) rotameter and sampling pump.

After derivatization, adsorption tubes were thermally desorbed by a TD-100 multi-tube auto-sampler equipped with an automated re-collection system controlled by Maverick TD software (Markes International, UK). Tubes were desorbed in splitless mode at 250 °C for 8 min with a helium flow rate of 35 mL/min and compounds were cryogenically trapped at 5 °C into an internal focusing trap packed with 70 mg of Graphitized Carbon (Markes International, UK). Finally, the analytes were transferred to the chromatographic column in split mode (split ratio of 11) by heating the cold trap at 100°C/s up to 300 °C for 60 min. A flow path temperature of 140 °C was used during sample analysis. The thermal desorption unit was directly connected to the GC column via a fused silica transfer line (I.D. 0.25 mm) supplied by Markes International (UK). 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 GC-MS/MS system was controlled by the MassHunter Workstation software (Agilent Technologies, USA). A reference library (NIST/MS software v. 2.2) was used to support the identification of the compounds by comparing the obtained MS spectra with the spectra reported in the library. Chromatographic separation of oximes was carried out by a DB-5ms ultra inert capillary column (60 m × 0.25 mm, 1.0 μm film thickness) from Agilent Technologies (USA) at constant He flow of 1 mL/min. The oven temperature program was: 100 °C for 1 min and 4 °C/min to 250 °C (9.5 min hold time). After the elution of the last compound (i.e. 2,3-butanedione-PFBHA, retention time of 45.93 min), a post run step of 13 min with an oven temperature of 260 °C and a carrier gas flow of 1 mL/min was accomplished. The triple quadrupole mass spectrometer was operated in full scan (m/z 31–500), selected ion monitoring (SIM) and MS/MS mode (multiple reaction monitoring, MRM). Temperatures of transfer line, ion source and quadrupoles were set at 260, 250 and 150 °C, respectively. Helium was used as the quench gas at a flow of 4 mL/min and nitrogen as the collision gas at a flow of 1.5 mL/min. The retention times of the investigated oximes obtained in our conditions as well as ions or MRM transitions used for the quantification in real

breath samples are shown in table 2. A deviation of ±0.10 min of the expected retention time compared to stock carbonyl mixture and a qualifier/quantifier (q/Q) ratio within 10% of the ratio measured in stock mixtures were required for analyte identification [47].

Table 2. Retention times, molecular weights and quantifier ions (m/z values) or MRM transitions (Precursor ion->Product ion) of the investigated compounds. Collision energy (eV) is reported in round brackets.

Compound	Retention time (min)	MW	Quantifier ion	Quantifier MRM transition
^{6}D-acetone	4.72	64[a]	64	
PFBHA	16.63	213	181	
Acetaldehyde-PFBHA (E)	16.98	239		239->181 (1 eV)
Acetaldehyde-PFBHA (Z)	17.62			
^{6}D-acetone-PFBHA	18.94	259	181	
Acetone-PFBHA	19.11	253	181	
Propanal-PFBHA (E)	19.96	253		253->236 (2 eV)
Acrolein-PFBHA (E)	20.05	251		251->181 (2 eV)
Propanal-PFBHA (Z)	20.21	253		253->236 (2 eV)
Acrolein-PFBHA (Z)	20.58	251		251->181 (2 eV)
2-butanone-PFBHA (E)	21.82	267		267->250 (6 eV)
2-butanone-PFBHA (Z)	21.95			
Methacrolein-PFBHA (E)	22.57	265		265->181 (6 eV)
Methacrolein-PFBHA (Z)	22.86			
Butanal-PFBHA (E)	23.11	267	181	
Butanal-PFBHA (Z)	23.32			
2-pentanone-PFBHA (E)	23.47	281		281->181 (12 eV)
3-pentanone-PFBHA	24.26	281		281->264 (12 eV)
2-pentanone-PFBHA (Z)	24.66	281		281->181 (12 eV)
3-hydroxy-2-butanone (E)	24.93	283	181	
3-hydroxy-2-butanone (Z)	25.22			
Pentanal-PFBHA (E)	26.32	281	181	
Pentanal-PFBHA (Z)	26.45			
2-hexanone-PFBHA (E)	27.14	295	181	
2-hexanone-PFBHA (Z)	27.56			
4-heptanone-PFBHA	28.91	309		309->128 (8 eV)
Hexanal-PFBHA (E)	29.30	295	181	
Hexanal-PFBHA (Z)	29.56			
3-heptanone-PFBHA	29.53	309	181	
2-heptanone-PFBHA (E)	30.01	309	181	
2-heptanone-PFBHA (Z)	30.54			
3-octanone-PFBHA	30.79	323	181	
Heptanal-PFBHA	32.52	309	181	
2-octanone-PFBHA	33.43	323	181	
5-nonanone-PFBHA	33.91	337		337->253 (3 eV)
Octanal-PFBHA	35.44	323	181	
2-nonanone-PFBHA	35.64	337	181	
Benzaldehyde-PFBHA (E)	36.30	301		301->284 (12 eV)
Benzaldehyde-PFBHA (Z)	36.66			
2,3-butanedione-PFBHA	38.97	476	181	

(isomer 1)			
2,3-butanedione-PFBHA (isomer 2)	40.03	476	181
Nonanal-PFBHA	41.07	337	181
Methylglyoxal-PFBHA (isomer 1)	42.06	462	181
2,3-butanedione-PFBHA (isomer 3)	42.31	476	181
Glyoxal-PFBHA (isomer 1)	43.26	448	181
Glyoxal-PFBHA (isomer 2)	43.51	448	181
Methylglyoxal-PFBHA (isomer 2)	43.58	462	181
Glyoxal-PFBHA (isomer 3)	43.69	448	181
Methylglyoxal-PFBHA (isomer 3)	44.17	462	181
Methylglyoxal-PFBHA (isomer 4)	44.88	462	181
2,3-butanedione-PFBHA (isomer 4)	45.93	476	181

[a] molecular weight of non-derivative compound.

2.5. Optimization of the derivatization reaction

The influence of temperature (x_1, °C), time (x_2, min) and amount of PFBHA added into sorbent tubes (x_3, nmol) on the oxime signals was evaluated by a 2^3 full factorial design, which includes the eight possible combinations of three variables at two levels. Tests were carried out by inflating Nalophan bags with gaseous working mixtures obtained with a 40000-fold dilution of the stock mixtures with humidified medical air. After preparation, Nalophan bags were stored in the thermostat for half an hour to reduce sample humidity down to 15 ± 3%. Aliquots (250 mL) of these mixtures were transferred at 50 mL/min into sorbent tubes spiked with different amount of PFBHA and analysed according to the procedure described in section 2.4. In our experiments, temperature was evaluated at 25 and 100 °C, time at 240 and 2880 min and amount of PFBHA at 0.4 and 4 nmol. These amounts lead to PFBHA/total carbonyl molar ratios of 1 and 8, respectively. Three replicate experiments were performed at the centre of the experimental domain (60 °C, 1560 min and 2.2 nmol of PFBHA) to assess reproducibility and to validate the models, so eleven experiments were performed overall in a random order. Results were analysed by a dedicated software (MODDE 11.0, Umetrics, Sweden).

The yield of reaction (%) between ^{6}D-acetone and PFBHA was estimated. For this purpose, three aliquots (10, 25 and 50 µL) of ^{6}D-acetone (870 ppmv) were spiked in two sets of sorbent tubes during the transfer of 250 mL of medical air at 50 mL/min. The first set included five tubes without derivatizing agent, whereas the other five tubes were spiked with PFBHA (4 nmol). These latter tubes were capped as described before and stored in the thermostat (37 ± 1 °C and RH 15 ± 3%) for 24 h. Both sets were analysed according to the procedure described in section 2.4. The yield of reaction (%) was estimated from the ratio of ^{6}D-acetone peak areas obtained from sorbent tubes containing and not containing PFBHA.

2.6. Validation of the method

Detection limits were evaluated by analysing five times a gaseous humid standard mixture prepared in Nalophan bag (50 cm × 47 cm, S/V = 0.3 cm^{-1}) by diluting 320000-fold stock gaseous mixtures. Limits of detection were calculated in accordance with IUPAC guidelines as three times the standard deviation (s_b) of the lowest level spiked blank [48].

Calibration curves were determined from ratios between the sum of peak areas of isomers of a same carbonyl-PFBHA and ^{6}D-acetone-PFBHA (Y, A_{Oxime}/A_{IS}) versus the concentration ratio (X, C_{Oxime}/C_{IS}) of the five calibration standards, which were prepared by diluting (320000-, 100000-, 20000-, 5000- and 100-fold) stock gaseous mixtures in humidified medical air. The calibration curves (Y = mX) for all the carbonyls

were evaluated by the Deming regression analysis, which considers measurement errors for both dependent and independent variable [49].

According to the US EPA guidance (no. TO-15), the linearity of the 5-point calibration curves was calculated as percent relative standard deviation (RSD) of the relative response factor (RRF), which is calculated as follow $A_{Oxime}/A_{IS} \times C_{IS}/C_{Oxime}$ [50].

Five gaseous working mixtures were prepared by diluting (320000-, 100000-, 20000-, 5000- and 100-fold) stock gaseous mixtures with humidified medical air. Each mixture was analysed in triplicate within the same day and on three consecutive days to evaluate intra- and inter-day precision, respectively.

2.7. Influence of sample volume on the recovery of extracted carbonyl compounds and carry over

The effect of sample volume on the amount of extracted carbonyls was evaluated in triplicate at four volumes (50, 100, 250 and 500 mL) and a fixed flow rate (50 mL/min), using a diluted (10000-fold) humidified gaseous mixture of selected compounds prepared in a Nalophan bag. After preparation, the bag was stored in the thermostat for half an hour to reduce sample humidity down to 15 ± 3%. The corresponding PFBHA/carbonyl molar ratios were 43, 25, 11 and 6, respectively. After sample transfer, the Tenax GR tubes were analysed according to the procedure described in section 2.4.

Five sorbent tubes containing 130 nmol of PFBHA were loaded (flow rate 50 mL/min) with 250 mL of the 40000-fold diluted mixture to assess carry over. Each tube was analysed two times consecutively. Carry over of the internal focusing trap was evaluated by analysing an empty stainless steel tube after desorption of a sorbent tube containing 130 nmol of PFBHA and loaded with 500 mL of gaseous sample (40000-fold dilution from the stock gaseous mixtures).

2.8. Stability

A PFBHA solution in methanol (1.4 g/L) was split in two aliquots after preparation, one stored at room temperature (25 ± 2 °C) and the other at 4 °C. The stability of this solution over time was evaluated weekly up to four weeks after preparation. For this purpose, 1 µL of each solution was spiked every week into three Tenax GR sorbent tubes using the CSLR system, then tubes were analysed according to the procedure described in section 2.4.

Six Tenax GR sorbent tubes were loaded with 1 µL of a methanol solution containing 1.4 g/L of PFBHA using the CSLR system. After purging out the solvent, three tubes were analysed immediately (t_0), whereas the other three were capped and analysed after one-day storage (t_{0+24h}) at room conditions (T = 25 ± 2 °C, RH = 50 ± 10 %) according to the procedure described in section 2.4.

The stability of PFBHA adducts in Tenax GR sorbent tubes during a typical sequence of chromatographic analyses (storage at room temperature in the thermal desorption autosampler for about 24 h) was also evaluated. For this purpose, a diluted gaseous working mixture (40000-fold from the stock mixtures of selected compounds with humidified medical air) was prepared in a Nalophan bag and then stored in the thermostat for half an hour to reduce the sample humidity down to 15 ± 3%. This mixture was transferred into six Tenax GR tubes spiked with PFBHA (130 nmol). Three sorbent tubes were immediately analysed whereas the remaining three tubes were closed using autosampler caps, stored into the thermal desorption unit and then analysed after 24 h. All the analyses were carried out according to the procedure described in section 2.4.

3. Results and discussion

3.1. Optimization of thermal desorption, gas-chromatographic and mass-spectrometric conditions

The influence of split ratio during the primary (i.e. from sorbent tube to internal focusing trap) and secondary desorption (i.e. from internal focusing trap to GC column) was investigated. Data (not shown) highlighted that a good compromise between the sensitivity and peak shape was obtained by desorbing the sorbent tube in splitless mode and applying a split ratio of 11 in the secondary desorption. Flow path temperature is reported to be critical for labile and reactive species (e.g. terpenes and sulphur compounds) [51] but in our conditions a flow path temperature of 80 or 140 °C does not show any statistically significant influence on the oximes signals (p >0.05). Table S1 (supplementary information) reports the optimal conditions for the desorption of oximes, which were produced in the reaction between aldehydes and ketones with PFBHA, from Tenax GR sorbent tube.

Many analytes (table 2) showed two chromatographic peaks corresponding to (E) and (Z) isomers. Both isomers are formed for asymmetrical carbonyl compounds that are not sterically hindered due to the rigid nitrogen–carbon double bond. The first eluting peak has been assigned to isomer (E) and the second peak to (Z) [52], thus we followed this assumption. In our conditions the (E) and (Z) isomers of 3-heptanone, heptanal, 2-octanone, octanal and nonanal were not chromatographically resolved, although the GC column used in this work had a phase ratio (ß = 63) lower to those reported in other works (ß = 250) [53]. Moreover, compounds with two carbonyl groups have even more possible isomers. In our conditions, glyoxal showed three (ZE, EE and ZZ) isomers, whereas methylglyoxal and 2,3-butanedione showed four (ZE, EE, EZ and ZZ) isomers, confirming the results reported elsewhere [54].

The optimization of the mass spectrometric method was performed in three steps: i) acquisition of full scan spectra (m/z 31–500) to select a suitable precursor ion for each compound, ii) acquisition of the full product ion scan mass spectra to identify the most suitable collision energy value and iii) set up a reliable MRM method by considering at least two transitions for each compound. The most intense transition was identified as the quantifier (Q), whereas the other transitions were labelled as qualifier (q).

Figure S1 (supplementary information) shows the product ion mass spectrum (precursor ion m/z 265) of methacrolein-PFBHA oxime at three collision energies (2, 4 and 6 eV). Four transitions (m/z 265->84, m/z 265->56, m/z 265->248 and m/z 265->181) were selected for this oxime, setting the collision energy at 6 eV. Some compounds (e.g. 3-heptanone) were detected in SIM mode, by monitoring at least two ions per compound, since even at the lowest collision energy (1 eV) they did not show precursor ion. Also in this case, the most intense ion signal was used as quantifier whereas the other was used as qualifier. Table S2 (supplementary information) reports the qualifier (q) and quantifier (Q) ions or MRM transitions and their qualifier/quantifier ratio (q/Q) calculated for all analytes.

3.2. Optimization of the derivatization reaction parameters

Derivatization temperature (x_1, °C), derivatization time (x_2, min) and amount of PFBHA spiked into sorbent tubes (x_3, nmol) were selected as the parameters determining the oxime yield and consequently the 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 following model:

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

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

Figure 2 shows an overview of results obtained for two representative compounds (i.e. butanal and 2-butanone).

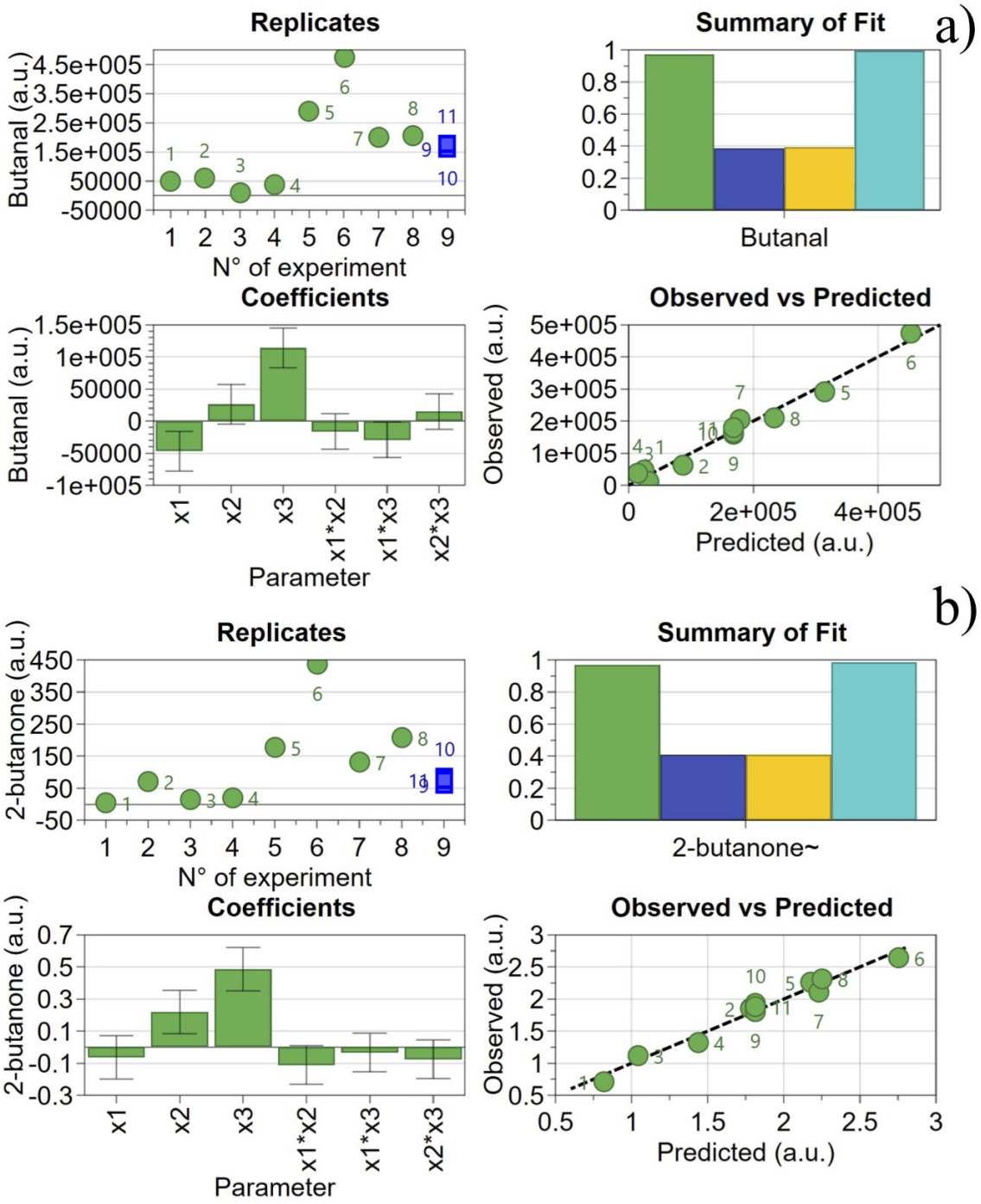

Figure 2. Analysis of results obtained for butanal (a) and 2-butanone (b): plot of replicates, summary of fit (R^2 (green), Q^2 (blue), model validity (yellow) and reproducibility (light blue)), plot of coefficients and model predictions (observed *vs.* predicted). The values of the coefficient of determination (R^2 = 0.99 for both analytes) prove that the regression models appropriately fit the raw data. The fraction of the total variation (Q^2) of X or Y variables that can be predicted by the model was 0.4 for both analytes, proving a satisfactory prediction power of the model. All these statistical indexes (R^2, Q^2, model validity and reproducibility are dimensionless. Confidence interval bars are included for each factor.

The plot of replicates shows the peak areas obtained in the different experiments, allowing a quick inspection of raw data. Measurements performed in repeated experiments (9-11) appear as blue points connected by a line. Model diagnostics are plotted in the summary of fit. In this plot, the coefficient of determination R^2 (green box) quantifies how well the model is able to reproduces the training set data (experiments 1-8) from which it was constructed, while the predictive power Q^2 (blue box) indicates how well the model predicts results of an independent set of validation observations (experiments 9-11).

Generally, a significant model should have R^2 and Q^2 values higher than of 0.5 and 0.1, respectively. The model validity (yellow bar) tests if the right type of model has been used to fit the data (e.g. a linear or a quadratic model). A value lower than 0.25 indicates the existence of statistically significant deviations of the data from the model, as it may happen in the presence of outliers. The light blue bar indicates reproducibility, and typically repeated experiments are performed at the centre of the experimental domain (in our case it was 60 °C, 1560 min and 2.2 nmol of PFBHA). Reproducibility is close to 1 in case of low variability, but a value greater than 0.5 is sufficient to validate the experimental model. For all the investigated analytes, all these statistical indexes (R^2, Q^2, model validity and reproducibility) were always higher than recommended values, highlighting that the experimental model appropriately fitted the raw data and was validated. The plot of coefficients shows values of model coefficients, if positive (e.g. time and amount of PFBHA) an increase of the corresponding parameter increases the response, whereas the contrary is true for parameters having negative coefficients (e.g. derivatization temperature). The error bars reflect the uncertainty on the estimate of a coefficient and the significance of the related parameter. Coefficients that have a confidence interval overlapping zero (e.g. temperature) are non-significant term. Experimental values and model predictions are compared in the observed *vs* predicted plot.

The amount of PFBHA spiked into sorbent tubes and the derivatization time (in the case of 2-butanone) were the only significant coefficients, and this means that increasing the amount of derivatizing agent or letting the reaction to proceed longer results in higher signals, as expected. The effect of both parameters on the oxime signals can be discussed considering the reaction mechanism between carbonyl compounds and PFBHA [55, 56]. In fact, nucleophilic addition of the active electron pair on the nitrogen atom in PFBHA to the carbonyl group is greatly determined by the partial positive charge on this carbon atom. This charge is caused by the strong negative inductive effect of the oxygen atom. Substitution with alkyl groups on the other hand, will render the carbon atom less positive due to a weak positive inductive effect. Additionally, because of the large size of the pentafluorobenzyl group, steric hindrance can exert a large effect on the reaction progress. Therefore, compounds with a carbonyl group that is substituted with the weaker electron-releasing and less voluminous groups, such as aldehydes, will react fast with PFBHA and consequently they can be more easily analysed. In fact, the ratio between the peak area observed in the experiment 5 (x_1 = 25 °C, x_2 = 240 min and x_3 = 4 nmol) and experiment 6 (x_1 = 25 °C, x_2 = 2880 min and x_3 = 4 nmol) resulted 0.4 and 1.0 for 2-butanone and butanal, respectively. The influence of the PFBHA amount on the oxime peak area can be discussed considering the reaction mechanism between PFBHA and carbonyl compounds. In fact, being an equilibrium reaction, the concentration of PFBHA-oxime at equilibrium (assuming the activity coefficient equal to 1) is expressed as follow:

$$[\text{PFBHA-oxime}] = K_n \times [\text{PFBHA}]^n \times [\text{carbonyl}_n]$$

where n = 1 and 2 for mono-carbonyl and di-carbonyl compounds, respectively. Therefore, according to this equation, a higher amount of PFBHA reagent will produce more oxime, which forces the equilibrium of reaction heavily in favour of the oxime formation. In our case, a quantitative reaction of aldehydes and ketones was assured by adding PFBHA in excess (at least 3-fold based on molar ratios). Thus, a PFBHA amount of 130 nmol guarantees the detection of carbonyls in a typical breath sample collected from healthy [57] and patients suffering from oxidative stress-related diseases (e.g. lung cancer [43]).

Although we achieved the maximum oxime areas at the maximum derivatization time tested (2880 min), subsequent experiments were carried out using a reaction time of 1440 min to reduce the time of analysis required to process a sample. Generally, in this condition the peak area of each oxime was 20% lower to those observed at 2880 min, confirming the results discussed by other authors [58, 59].

Using the final experimental conditions, the yield of reaction (%) was estimated considering the reaction between ^{6}D-acetone and PFBHA. Results obtained from the first set of tubes (n = 5), highlighted that the GC signal (m/z 64, SIM mode) of ^{6}D-acetone increased linearly (R^2 >0.970) with the sampling volume without saturation of the sorbent material. This calibration curve was used to determine the concentration of unreacted ^{6}D-acetone with the excess of PFBHA (second set of tubes, n = 5). The ratio (mean ± standard deviation) between the concentration of unreacted ^{6}D-acetone and the concentration of ^{6}D-acetone spiked into sorbent tube was 0.10 ± 0.01, thus the reaction between ^{6}D-acetone and PFBHA can be considered almost quantitative.

3.3. Analytical figures of merits

3.3.1. Limits of detection, calibration curves and linearity

Table 3 reports the linear regression parameters for the calibration curves, calibration ranges and limits of detection for the investigated carbonyl compounds.

Table 3. Summary of linear regression parameters for the calibration curves, calibration range and limits of detection of carbonyl compounds.

Carbonyl	Range (ppbv)	Slope[a] (RSD)	R^2	LOD (pptv)
Acetaldehyde	6.5-130.3	0.03 (7%)	0.996	1100
Acetone	8.0-2550.0	11.3 (6%)	0.991	180
Propanal	0.3-100.0	0.002 (13%)	0.998	80
Acrolein	0.3-110.0	0.006 (15%)	0.997	110
2-butanone	0.6-200.0	0.005 (7%)	0.993	140
Methacrolein	0.4-140.0	0.001 (12%)	0.998	20
Butanal	0.6-180.0	0.8 (15%)	0.986	150
2-pentanone	0.5-170.0	0.001 (7%)	0.998	60
3-pentanone	0.5-170.0	0.001 (15%)	0.998	5
3-hydroxy-2-butanone	1.3-430.0	1.1 (13%)	0.998	80
Pentanal	0.3-100.0	0.7 (12%)	0.992	160
2-hexanone	0.5-150.0	0.4 (7%)	0.997	90
4-heptanone	0.4-130.0	0.004 (12%)	0.996	10
Hexanal	0.7-210.0	1.0 (14%)	0.998	10
3-heptanone	0.4-130.0	0.4 (10%)	0.991	90
2-heptanone	0.4-130.0	0.4 (10%)	0.998	70
3-octanone	0.4-120.0	0.4 (13%)	0.981	190
Heptanal	0.6-200.0	1.2 (12%)	0.997	80
2-octanone	0.4-120.0	0.001 (10%)	0.982	180
5-nonanone	0.3-110.0	0.001 (13%)	0.994	5
Octanal	1.1-350.0	0.7 (13%)	0.999	90
2-nonanone	0.7-230.0	0.04 (14%)	0.991	160
Benzaldehyde	0.3-110.0	0.5 (13%)	0.998	130
Nonanal	0.8-260.0	2.6 (11%)	0.997	130
Glyoxal	1.0-320.0	0.2 (13%)	0.997	360
Methylglyoxal	1.0-320.0	0.2 (14%)	0.991	350
2,3-butanedione	1.1-350.0	2.6 (11%)	0.999	100

[a] Calculated from three replicates at five concentration levels (320000-, 100000-, 20000-, 5000- and 100-fold dilution from stock gaseous mixtures) according to the following formula: $A_{Oxime}/A_{IS} \times C_{IS}/C_{Oxime}$

As reported in table 3, we observed detection limits in the range 10-350 pptv and 5-200 pptv for aldehydes and ketones, respectively. Only acetaldehyde showed a detection limit close to 1 ppbv as a consequence of the higher standard deviation of spiked blank samples due to sample contamination from ambient air.

Mochalski *et al* determined very low concentration levels of most carbonyl compounds in breath of healthy subjects [60]. For example, 2-butanone, 2-pentanone, hexanal, 2-hexanone and heptanone isomers were detected at concentrations ranging between 10 and 600 pptv. Therefore, considering that the rate of carbonyls production is enhanced in oxidative stress-related diseases, such as chronic obstructive pulmonary disease [24], neurodegenerative disease [25] and heart failure [27], these limits are very satisfactory and allow a reliable monitoring of carbonyl compounds in breath samples.

For all the investigated carbonyls, the RSD values of the RRFs were below 20%, indicating a high degree of linearity over the tested concentration range.

3.3.2. Effect of sample volume on the recovery of extracted carbonyl compounds

A reliable quantification of VOCs using sorbent tubes can be performed when the amount of retained analyte linearly increases with sample volume until breakthrough occurs [61].

Acetone and 3-hydroxy-2-butanone showed a linear behaviour up to 250 mL, whereas all the other carbonyls had a linear behaviour within the investigated volumes range (50-500 mL), showing a coefficient of determination (R^2) higher than 0.995.

A desorption efficiency close to 100% was observed for all the investigated carbonyls, highlighting that Tenax GR material can be used without fear of any carry over effect. Moreover, no peaks of target compounds were observed when an empty stainless steel tube was analysed after the desorption of a sorbent tube, containing 130 nmol of PFBHA and loaded with 500 mL of sample (40000-fold dilution from the stock gaseous mixtures).

3.3.3. Method precision

Most carbonyls showed RSDs between 15 and 20%, whereas RSDs close to 30% were observed for di-carbonyl compounds such as 2,3-butandione. In a derivatization reaction, the use of an internal standard generally allows to improve the analytical performance. For example, Poli *et al* used 2-methylpentanal as an internal standard and obtained a method variability (RSD) close to 10% [42], but the possible presence of this branched aldehyde in breath samples [20] makes its use as internal standard critical for the quantification of analytes. For this reason, we tested ^{6}D-acetone, surely absent in the breath samples, to normalize the yield of the derivatization reaction. Results highlighted that this approach halved the relative standard deviation to 10% and 15% for mono- and di-carbonyl compounds, respectively.

The reproducibility of derivatization procedure was controlled by monitoring the ^{6}D-acetone-PFBHA signal over time. A control chart was drawn with the daily average ^{6}D-acetone-PFBHA peak areas, the relevant average area during the whole experimental period, the warning limits (average ± 1 standard deviation) and the control limits (average ± 2 standard deviations). The overall variability during six months was close to 10%, confirming that our derivatization procedure was highly reproducible and allowed a reliable determination of carbonyl compounds.

3.3.4. Stability

The stability of PFBHA was evaluated at 1, 2, 3 and 4 weeks after the preparation by analysis of variance (ANOVA) at a confidence level of 95%. We observed a significant decrease of concentration values (about 10% for week) over this time span, probably for the reaction of PFBHA with volatile contaminants during storage. In fact, a progressive increase of the formaldehyde-PFBHA adduct signal was observed after 2, 3 and 4 weeks in the solution stored at room temperature and 4 °C. Based on this, we suggest to prepare weekly a fresh PFBHA solution.

The stability of PFBHA in Tenax GR sorbent tubes at room conditions (25 ± 2 °C and RH 50 ± 10 %) was determined by spiking 1 µL of a PFBHA solution in methanol (1.4 g/L) into six tubes. Three of them were analysed immediately (t_0), whereas the others were closed with Swagelok cap, stored at room conditions and analysed after 24 h (t_{0+24h}). The mean area values measured at t_0 and t_{0+24h} were not statistically different and there was no increase of the signal produced from the formaldehyde-PFBHA adduct, thus meaning that PFBHA was stable for one day in Tenax GR. Similar results for PFBHA adducts were obtained with sorbent tubes sealed with the autosampler caps and stored into the thermal desorption unit. The different stability of PFBHA in solution and adsorbed in Tenax GR may be related to the more efficient protection from ambient contaminants of the tubes.

3.3.5. Interferences

The ratio of the peak areas of each isomer pair (Z/E) was monitored for quality control to avoid possible biases in the results caused by interfering compounds. In fact, comparable ratios of the (Z/E) isomer signals produced by each analyte should be calculated when analysing standards and real samples with the same procedure. According to US EPA guidance (no. 556), the (Z/E) isomer ratio should be within 50% of the

ratio observed in standards [62]. In our experimental conditions, the variability of (Z/E) ratio was below 20% and close to 30% for mono- and di-carbonyl compounds, respectively.

3.4. Application to real samples

The optimized on-sorbent derivatization procedure was tested by analysing 12 mixed breath samples collected from 4 patients (2 males and 2 females) suffering from chronic heart failure (HF) and hospitalized at the Cardio-Thoracic-Vascular Department of the Azienda Ospedaliera-Universitaria Pisana (AOUP) in the occasion of an acute exacerbation. The study was approved from the Ethics Committee of the Area Vasta Nord-Ovest (CEAVNO- Tuscany Region). Breath samples were collected in the morning (10.00 to 12.00 AM) at the time of hospital admission (t_0), after 48 h and at the discharge (t_d). The possible interference from fasting was partially reduced by collecting breath samples always in the morning, 2 h after breakfast.

Table 4 reports the carbonyl concentrations measured in the patients' breath samples during hospitalization.

Table 4. Breath carbonyl concentrations (pptv) measured in heart failure patients during hospitalization: at the admission (t_0), after 48-hour (t_{0+48h}) and at the discharge (t_d).

	P1			P2			P3			P4		
	t_0	t_{0+48h}	t_d	t_0	t_{0+48h}	t_d	t_0	t_{0+48h}	t_d	t_0	t_{0+48h}	t_d
Acetaldehyde[a]	13	36	8	3	21	7	5	9	9	16	6	4
Acetone[a]	2060	260	200	720	210	260	630	210	470	1190	430	140
Propanal[a]	6	1	3	4	5	5	180	110	260	4	4	2
Acrolein	4990	1350	1550	1800	2930	2510	3170	1760	2780	3430	2910	2140
2-butanone	2260	1080	1010	540	1000	1180	640	530	440	1150	800	370
Methacrolein	2820	780	1010	1600	1900	1660	2050	1570	1160	2670	3280	3100
Butanal	2280	790	1090	1940	1220	3000	1500	1210	1300	3500	3150	2800
2-pentanone	1070	390	260	230	190	190	230	200	§	480	260	§
3-pentanone	1290	530	660	730	1170	1290	1280	720	1480	1170	1440	1210
3-hydroxy-2-butanone[a]	36	5	13	10	7	5	23	7	9	200	50	20
Pentanal	870	710	640	690	1220	1370	*	*	*	1130	1030	900
2-hexanone	*	*	*	*	*	*	*	*	*	*	*	*
4-heptanone	60	§	§	§	§	§	§	*	§	40	60	50
Hexanal	270	190	140	310	100	140	290	130	180	180	60	40
3-heptanone	340	§	§	§	280	390	290	390	410	400	490	420
2-heptanone	*	*	*	*	*	*	*	*	*	*	*	*
3-octanone	*	*	*	*	*	*	*	*	*	*	*	*
Heptanal	2670	5600	760	810	1410	1390	*	*	*	2040	1970	1100
2-octanone	*	*	*	*	*	*	*	*	*	*	*	*
5-nonanone	*	*	*	*	*	*	*	*	*	*	*	*
Benzaldehyde	*	*	*	*	*	*	*	*	*	*	*	*
Octanal	4950	1400	1230	1230	2340	1920	*	*	§	2820	3100	2900
2-nonanone	*	*	*	*	*	*	*	*	*	*	*	*
Nonanal	1160	1120	890	1000	1410	1460	*	*	§	2450	2350	400
Glyoxal[a]	15	15	17	22	18	17	34	21	47	46	37	30
Methylglyoxal[a]	9	5	5	4	4	5	10	6	10	17	23	18
2,3-butanedione	900	1080	930	870	420	460	2050	900	460	2950	1410	350

[a] concentration expressed in ppbv
* concentration lower than detection limit
§ concentration lower than quantification limit

As reported in table 4, at the first collection time, patients showed high breath levels of acetone, 2-pentanone, 3-hydroxy-2-butanone, 2,3-butandione and hexanal, whereas the remaining breath components did not change their levels during hospitalization. Acute exacerbations in chronic heart failure patients are characterized by signs and symptoms resulting from fluid overload. The excessive fluid volume starts building in the intravascular compartment and then in the interstitial spaces leading to an increase in body weight [63]. When water seeps into the air spaces of the lungs (pulmonary edema), gas exchange is reduced and eventually the patient experiences shortness of breath (dyspnea), thus requiring hospitalization. At this stage, he/she shows a reduced cardiac output, i.e. amount of blood pumped from the heart per minute. The heart tries to compensate its reduced efficiency by increasing heart rate, while peripheral vasoconstriction helps sustaining blood pressure. The heart gets exhausted in the effort to provide organs with the necessary amount of blood, and metabolism shifts to anaerobic conditions. At this stage of the disease, an imbalanced redox state as well as metabolic and neuroendocrine responses are observed, leading the release of many chemicals (e.g. ketone bodies and aldehydes) in blood-stream [64, 65]. During hospitalization, all patients were treated with diuretics and β-blockers to manage HF and improved their clinical conditions, as highlighted by the reduction of BNP plasma levels (by at least three times). We speculate that decreasing trends of breath acetone, 2-pentanone, 3-hydroxy-2-butanone, 2,3-butandione and hexanal resulted from the improved clinical conditions, as discussed elsewhere [15, 64-67].

The limited number of patients does not allow us to produce firm conclusions, however the longitudinal approach allows to cope with the inter-individual variability of breath composition [1, 2]. In fact, monitoring a patient over time permits to him/her to act as his/her own control and to correlate more easily variations of breath composition to the evolution of the disease. If these data will be confirmed in a larger number of patients, it will be possible to demonstrate the usefulness of breath carbonyls as a source of clinical information regarding the pathogenesis of HF or the identification of subjects at risk.

4. Conclusions

This paper reported an analytical protocol for the determination of carbonyl compounds in exhaled breath samples by on-sorbent derivatization coupled with thermal desorption and gas chromatography-tandem mass spectrometry. The procedure entails the transfer of an aliquot of sample (250 mL) at a flow rate of 50 mL/min onto Tenax GR sorbent tubes spiked with 130 nmol of O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride.

The method has a broad applicability in medicine since alterations of the aldehyde and ketone content in blood, and therefore in exhaled breath, are directly related to unsaturated lipid peroxidation. Diseases causing oxidative stress, and therefore alterations of the carbonyl compound pattern, are extremely numerous and important for human health. As an example, we can mention diabetes, obesity, cardiovascular disease, heart failure, neurodegenerative diseases, liver disease, etc. In many cases, the appearance of the disease corresponds to an alteration of the normal pattern of carbonyl compounds, rather than the appearance of new compounds. This requires the availability of analytical procedures that, in addition to adequate accuracy and detection limits, offer above all a high precision in measuring the concentration of a given compound.

The analytical performance of the proposed method has confirmed that the PFBHA on-sorbent approach provides a sensitive and reliable procedure for the determination of carbonyls in human breath samples and, more importantly, ensures the required precision by using an internal standard.

As a clinical example, the present method was applied to monitor the altered oxidation of fatty acids and imbalanced redox state in patients suffering from heart failure. Acetone, 2-pentanone, 3-hydroxy-2-butanone, 2,3-butandione, and hexanal showed a clear decreasing trend that may correlate with the improved clinical conditions. These preliminary results are very encouraging for future applications of the method to monitor patients suffering from heart failure and other diseases.

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