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Determination of aldehydes in human breath by on-fibre derivatization, solid-phase microextraction and GC–MS

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Abstract

A GC–MS method for the simultaneous determination of hexanal, heptanal, octanal, nonanal and decanal in exhaled breath was established and validated. The aldehydes were derivatized on PDMS/DVB fibres using *O*-2,2,4,5,6-(pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA) as the headspace derivatization reagent. The resultant oximes were quantified by GC–MS in selected ion monitoring (SIM) mode. The method provides detection limits of 0.01–0.03 nM for the aldehydes, with a linear response in the concentration range 0.002–20 nM. Within-day precision values for the five aldehydes at 0.02–0.04 nM and 0.2–0.4 nM were in the ranges: 3–9% and 3–8%, respectively; the corresponding between-day precision values were 11–22% and 10–24%. Exhaled breath samples could be stored at −20 °C for 48 h.

Keywords: Breath analysis; Aldehydes; Lipid peroxidation; GC–MS; Solid-phase microextraction

1. Introduction

The detection and monitoring of biomarkers for oxidative stress has received increasing attention in the medical literature. Single biomarkers as well as biomarker patterns have been used to distinguish groups of patients, or monitor disease progression in order to manage medical treatment [1,2]. Among other substances, the aldehyde end-products of lipid peroxidation have been proposed as biomarkers of tissue damage caused by oxidative stress [3,4].

Elevated levels of reactive oxygen species (ROS) originate from inflammatory cells such as eosinophils and neutrophils. These species have e.g. been associated with chronic airway inflammation [5,6] by a mechanism involving the initiation of peroxidation in polyunsaturated fatty acids [7]. Decomposition of the resultant lipid peroxides proceeds by radical chain reactions, yielding either alkoxyl radicals (and ultimately, alkanes and aldehydes), or peroxyl radicals (leading to isoprostanes) [8,9].

Measurement of endogenous compounds in exhaled breath has potential use as a clinical tool, although the origin, distribution and exhalation kinetics of these species are not yet completely understood [2,10]. Using qualitative gas chromatography (GC), Pauling et al. detected about 250 volatile organic compounds (VOCs) in exhaled breath from healthy subjects [11]. In another study, Phillips developed a qualitative method to examine the entire range of exhaled VOCs, including the aldehydes nonanal and hexanal [12]. Lin et al. have described a method for the determination of aldehydes in breath, which involved derivatization on dinitrophenylhydrazine-impregnated silica cartridges, followed by HPLC/UV detection [13]. More recently, selected ion flow tube mass spectrometry has been used to quantify acetaldehyde in the breath of healthy volunteers [14].

Aldehyde compounds in the exhaled breath condensate (EBC) of asthmatics have also been determined by HPLC with fluorescence detection [5,15], and in the EBC of chronic obstructive pulmonary disease patients and healthy volunteers by LC–MS/MS [16,17]. However, EBC has several drawbacks as a sampling technique, including dilution of the sample with condensed water vapour [18], low concentrations of biomarkers [19] and possible contamination by saliva [20]. Clearly, there is

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still a requirement for improved, sensitive analytical techniques for exhaled aldehyde biomarkers.

Typical concentrations of potential biomarkers in exhalation samples are in the nanomolar range, therefore pre-concentration techniques, such as automatic thermal desorption with sorbents and cryofocusing, are required [12]. Another recently developed technique with considerable potential for pre-concentrating volatile analytes is 'solid-phase microextraction' (SPME) [21–24]. SPME, coupled to GC–MS, has been used to quantify levels of VOCs [22], acetone [21,25] and isoprene [21,26] in exhaled breath. An important feature of the study [25] was on-fibre derivatization of the volatile components using the reagent pentafluorobenzyl hydroxylamine hydrochloride (PFBHA), which has also been used to determine aldehyde concentrations in head-space of blood samples [27,28].

We have adopted several of the techniques mentioned above to produce a non-invasive, rapid and sensitive method for the quantification of aldehydes at low levels in exhaled air. Our method, which employs SPME and on-fibre derivatization, coupled to GC–MS in SIM mode, has been validated using nanomolar concentrations of five homologous, aldehyde biomarkers (hexanal, heptanal, octanal, nonanal and decanal). The use of the described technique for the determination of aldehydes in exhaled air has not previously been reported, to the best of our knowledge.

2. Experimental

2.1. Chemicals

A certified reference solution containing 1.0 mg/ml of each of 12 aldehydes dissolved in acetonitrile was obtained from Accustandard (New Heaven, CT, USA). Hexanal (98%), heptanal (95%), octanal (99%), nonanal (95%), decanal (99%), methanol (LC–MS quality: 99.9%) and *O*-2,2,4,5,6-(pentafluorobenzyl) hydroxylamine hydrochloride, PFBHA (98%) were obtained from Sigma–Aldrich (Steinheim, Germany). A 0.01 M aldehyde stock solution was prepared weekly in a volumetric flask and an aldehyde working solution was prepared daily in a volumetric flask to concentration levels of 10 μM dissolved in methanol. All aldehyde solutions were stored at 6 °C before use. Synthetic air of highest purity (scientific 5.5) was provided by AGA, Enköping, Sweden.

2.2. Sample collection

Subjects spent at least 10 min in an examination room prior to sampling. Single exhalations were collected at a flow of 50 ml/s, the first 750 ml being discarded. The flow rate was checked by the pneumotachometer of a CLD 77 AM NO analyzer from Eco-Physics (Dürnten, Switzerland). Breath aliquots (20 ml) were collected using a mouth-piece equipped with a nipple attached to 1 cm of polypropylene tubing, and a silanized gas-tight syringe was used to transfer them to evacuated 20 ml vials equipped with a silicone/PTFE septum (purchased from Supelco (Bellafonte, PA, USA)).

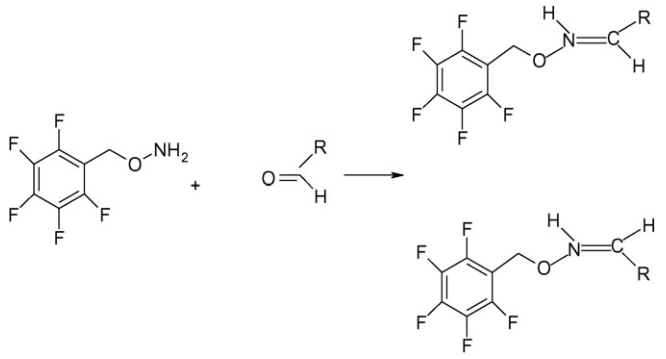

Fig. 1. Reaction scheme for the derivatization of an aldehyde with PFBHA to give *cis*- and *trans*-oxime isomers.

2.3. On-fibre derivatization

The on-fibre pre-derivatization of aldehydes was performed using 65 μM StableFlex polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fibre of 24 gauge needle size, purchased from Supelco (Bellafonte, PA, USA). Before its first use the fibre was prepared by desorbing possible contaminants in the injection port of the GC–MS equipment used in the subsequent analyses (see below), in split mode, for 30 min at 250 °C. Prior to each analysis the fibre was desorbed for 10 min at 250 °C to avoid carry-over. The fibre was then exposed to a headspace concentration of PFBHA (1 mg in a 20 ml vial filled with synthetic air) for 10 min at 50 °C by incubating the vial containing the dry reagent in a thermostatically-controlled water bath. Following this, the fibre was exposed for 8 min at 65 °C to a 20 ml vial containing either a vapourized calibration solution (5 μl) in synthetic air, or an authentic breath sample. (this derivatization period has been optimized previously [27]). The derivatization temperature (65 °C) was held constant by means of an ETS-D4 Fuzzy Electronic Contact Thermometer purchased from IKA (Staufen, Germany). The fibre was then transferred immediately to the injection port of the GC–MS, and desorbed for 10 min at 250 °C.

Fig. 1 shows a reaction scheme for the derivatization of aldehydes with PFBHA reagent to give oxime products.

2.4. Calibration

Determination of aldehyde levels in breath samples was performed with external standards (contained in 20 ml vials identical to those used for the authentic samples). To obtain calibration curves, triplicate blanks were used, together with measurements at three higher concentrations of 2 nM [29], with synthetic air as the matrix. The vials were flushed with synthetic air and evacuated, then 20 ml of synthetic air was added by gas-tight syringe. A 5 μl aliquot of methanol (blank sample) or calibration solution was then added to the vial. The calibration curves were adjusted according to the content of aldehydes in the methanol solvent.

Exhalation samples for the standard addition curve were collected directly in a 3 L Tedlar bag®, SKC Inc. (Eighty Four, PA, USA) which was flushed with nitrogen five times before sampling.

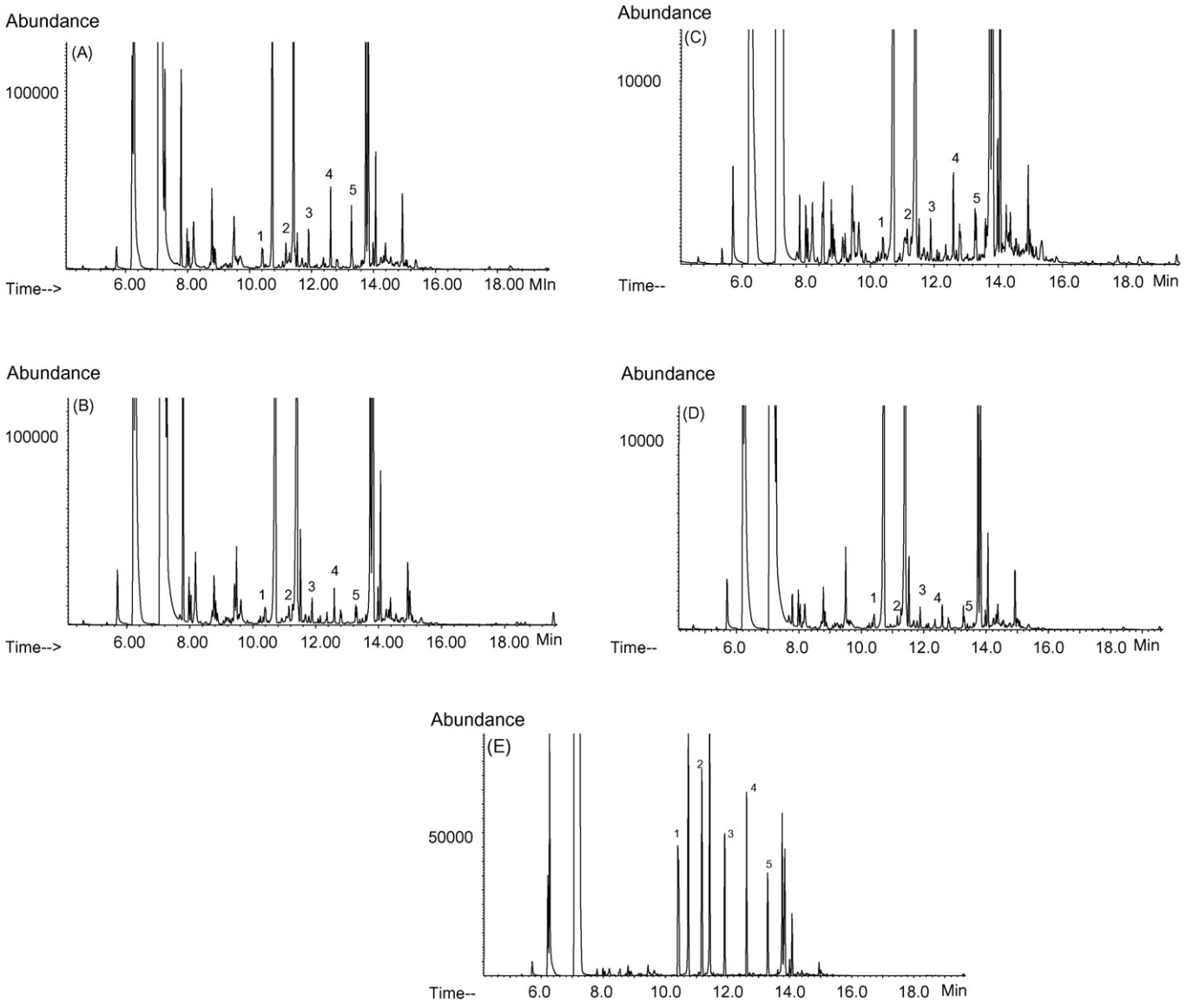

Fig. 2. The GC–MS SIM (*m/z* 181) chromatograms of aldehydes following on-fibre derivatization SPME. 1 = hexanal, 2 = heptanal, 3 = octanal, 4 = nonanal, 5 = decanal. (A) Asthmatic patient. Hexanal 0.04 nM, heptanal 0.02 nM, octanal 0.05 nM, nonanal 0.10 nM, decanal 0.07 nM. (B) Healthy volunteer. Hexanal 0.01 nM, heptanal 0.01 nM, octanal 0.01 nM, nonanal 0.01 nM, decanal 0.01 nM. (C) Blank. (D) Room air. (E) Calibration sample, 2 nM.

2.5. Gas chromatography–mass spectrometry

The analyses were performed using a HP 6890 gas chromatograph from Agilent (Palo Alto, CA, USA) coupled to a HP5973 mass detector. The gas chromatograph was run in splitless mode using a silanized injection liner with 0.75 mm I.D. purchased from Supelco (Bellafonte, PA, USA) and a HP-5 capillary column measuring 30 m × 0.25 mm I.D. with a film thickness of 0.25 μM (Agilent, Folsom, California, USA). The carrier gas (helium) flow rate was 5.18 ml/min, and the initial temperature of 50 °C was ramped at 15 °C/min to 270 °C. The transfer line and ionization source were maintained at 280 °C. The ionization energy was 71 eV. Full scan data were recorded from 28 to 550 *m/z*. Mass fragment *m/z* 181 was monitored in SIM mode (dwell time 0.5 s) and was used to quantify the aldehydes (Fig. 2). The mass spectrometer was used in the full scan mode as well as in single ion monitoring mode in each run, and Chemstation D.02 software (Agilent) was used for data acquisition and instrument control. This software was also equipped with a Mass Spectral Search Program 2.0 from NIST (Gaithersburg, MD, USA).

2.6. Subjects

Exhaled breath samples from four subjects were used for validation of the method. Two of the subjects were healthy volunteers (females, age 27 and 37). The other subjects were asthmatic volunteers (females, age 30 and 38), receiving treatment with corticosteroids and β-receptor agonists. None of the subjects were smokers. To test the applicability of the method, it was applied to one asthmatic and one healthy subject, see Section 3.3.6. The study was approved by the medical ethics committee of Göteborg University.

3. Results and discussion

3.1. Optimization of derivatization conditions

The recovery of aldehydes from the calibration solution was tested using three different fibre types: polydimethylsiloxane/divinylbenzene (PDMS/DVB), carboxen/divinylbenzene (CAR/PDMS) and DVB/CAR/PDMS. The maximum response was obtained with the PDMS/DVB coated fibre, which had previously been reported as being suitable for aldehyde analysis [24,27].

Impurities from the derivatization reagent were minimized by using dry reagent in the headspace (as opposed to aqueous solutions reported by others [25,30]). The dual purpose of the derivatization was to stabilise the aldehydes as their oximes and improve the quantification levels achievable by SIM. The effects of varying the loading temperature over the range 25–65 °C were studied, and the response (indicated by the areas under the curve) was strongest at 50 °C. Therefore, this loading temperature was used in all subsequent analyses. The influence of derivatization temperature was assessed by a calibration sample in the range 20–70 °C; the optimum being 65 °C.

In a recent study of the influence of derivatization time on the formation of unbranched aldehyde adducts in blood headspace, the adduct concentration reached a plateau after 8 min [27]. In the present study, we employed a reaction time of 8 min throughout to maximize the formation of oxime derivatives. Carry-over effects were avoided by employing desorption times of 10 min.

3.2. Optimization of GC–MS parameters

With a run-time of less than 20 min, the peaks for *cis*- and *trans*-isomers of the oxime derivatives were not fully resolved, but were combined to quantify each of the aldehydes examined in the study. The mass spectra were run in both full scan and SIM modes and the purity of the eluted oximes was checked by comparing the spectra acquired to spectra in the NIST 2.0 databases.

For quantification by SIM analysis, various dwell times in the range 200–500 μs were investigated; the maximum response was obtained with a dwell time of 500 μs.

3.2.1. Background levels

Indoor air is an unsuitable sample matrix due to the presence of relatively high and fluctuating background levels of aldehydes. This was confirmed by our finding that background levels of the various aldehydes in our laboratory varied between 0.01 and 0.05 nM over 24 h. These ambient aldehyde levels may originate from sources such as wall paint and laboratory personnel, and are likely to lead to large errors when aldehyde concentrations in exhalation samples are of the same order of magnitude [10]. For this reason, calibrations in the present study were performed with synthetic air. Other ways of compensating for background levels of aldehydes include supplying the patient with pure air in order to purge the lungs, and subtracting background levels from the breath signal [31]. However, optimum purge times are unknown, and there is a risk that purified air might irritate the airways due to its relatively low humidity.

3.3. Validation

3.3.1. Selectivity

Despite the high resolution offered by GC–MS, co-elution of other carbonyl compounds present in exhaled breath is always a potential problem. The selectivity was investigated by running a 50-min temperature program on authentic samples from one asthmatic and one healthy volunteer (the selectivity was not improved by longer temperature programs). Mass spectra were acquired in both full scan and SIM modes, and the purity of the eluted oximes was checked by comparing the spectra acquired to spectra in the NIST 2.0 databases.

3.3.2. Linearity and detection limits

Linearity was investigated by measuring aldehyde standards with concentrations in the range of 0.002–20 nM. The limit of detection (LOD) was calculated from the equation LOD = intercept + 3SD, taking a value for the intercept from the calibration equation. Standard deviations, SD, were calculated from eight replicates of determinations of synthetic air and the limit of quantification (LOQ) was calculated as LOQ = intercept + 10SD (Table 1).

3.3.3. Influence of the matrix

To determine whether water vapour or other compounds present in exhaled breath influenced the recovery of analytes, standards were added and calibration curves were constructed. The air used to prepare the standard addition curve was from an asthmatic subject, and was stored in a Tedlar sample bag. Synthetic air was used for the calibration curve. The exhalation sample was analyzed and aldehyde concentrations were calculated using both calibration techniques. Since the results acquired for all measured aldehydes using the two techniques were within 95% confidence intervals we concluded that the

Table 1
Calibration equations and parameters: range, R^2, LOD and LOQ

	Range (nM)	Equation	R^2	LOD (nM)	LOQ (nM)
Hexanal	0.002–20	$y = 5E+06x + 121941$	0.99	0.01	0.05
Heptanal	0.002–20	$y = 6E+06x + 83405$	0.99	0.01	0.02
Octanal	0.002–20	$y = 5E+06x + 149512$	0.99	0.01	0.05
Nonanal	0.002–20	$y = 4E+06x + 208603$	0.99	0.03	0.10
Decanal	0.002–20	$y = 4E+06x + 284789$	0.99	0.03	0.09

Table 2
Within-day and between-day precision

	Within-day precision (CV)		Between-day precision (CV)	
	0.02–0.04 (nM)	0.2–0.4 (nM)	0.02–0.04 (nM)	0.2–0.4 (nM)
Hexanal	7	3	22	24
Heptanal	3	8	20	10
Octanal	5	5	18	11
Nonanal	9	6	11	19
Decanal	7	6	31	12

composition of the matrix did not influence aldehyde determinations.

3.3.4. Precision

Within-day precision was estimated by performing triplicate determinations of standard solutions on the same day. The concentrations used equating to aldehyde levels of 0.02 and 0.2 nM. The average within-day precision, calculated as the coefficient of variation (CV) for the concentration levels, ranged from 3 to 9% for aldehyde concentrations of 0.02–0.04 nM, and 3–8% for aldehyde concentrations of 0.2–0.4 nM. Between-day precision was investigated on three different days using the procedure described above for within-day precision. For practical reasons, standard solutions of 5 μl were added to the vials; this resulted in differences in the working aldehyde standards, and a consequent contribution to between-day variation in the range 2–8%. The resulting CV values were found to be 11–22% for concentrations corresponding to 0.02–0.04 nM and 10–24% for concentrations corresponding to 0.2–0.4 nM for the various aldehydes, as shown in Table 2. These data are in agreement with, or higher than literature values. For example, Deng and Zhang investigated the within-day variation of acetone and reported variations of 3% in blood headspace using on-fibre derivatization with GC–MS detection [27]. Isoprene in breath has been studied using SPME-GC/MS. Hyspler et al. found a coefficient of variation of 15% [26] whereas Grote and Pawliszyn reported 2–6% [21].

3.3.5. Sample storage

An exhalation from a healthy volunteer was collected in a Tedlar bag. Aliquots of 20 ml were transferred to evacuated vials and spiked with 5 μl of a standard solution equating to an aldehyde level of 10 nM. The breath samples were analyzed immediately or stored at −20 °C or at ambient conditions (approximately +20 °C), respectively. Triplicates of the stored samples were analyzed after 6 h, 24 h and 2 weeks of storage. The recovery data are presented in Table 3. Storage of samples at −20 °C is possible for a maximum of 48 h prior to analysis, whereas storage at ambient conditions cannot be recommended due to the dramatic decrease in the recovery. These data are in accordance with other studies; Deng et al. reported that acetone stored in Tedlar bags was stable for 4 h, and had to be analyzed within 6 h [25], and Grote and Pawliszyn reported that storage of a fibre exposed to acetone, ethanol and isoprene for 8 h in dry ice increased acetone levels by 10% [21].

Table 3
Sample storage stability

	−20 °C (%)				Room temperature (%)
	6 h	24 h	48 h	2 weeks	6 h
Hexanal	113	134	117	2	41
Heptanal	99	111	104	0	27
Octanal	86	99	99	1	25
Nonanal	73	91	95	1	30
Decanal	65	146	94	2	33

3.3.6. Authentic samples from healthy and asthmatic subjects

Our optimized method was used to measure low levels of aldehydes from an asthmatic and a healthy volunteer (Fig. 2). Hexanal, heptanal, octanal, nonanal and decanal were detected in nanomolar concentrations in the breath samples (i.e. these aldehydes were present at levels higher than the limits of detection and quantification). Levels in the asthmatic and healthy subjects were 0.02–0.07 nM, and 0.01 nM, respectively.

4. Conclusions

We have optimized and validated a gas chromatographic method with mass spectrometric detection for the determination of five aldehydes in exhaled breath. Solid-phase on-fibre microextraction with GC–MS detection combines the advantages of pre-concentration, high selectivity, and SIM. Sampling is non-invasive, sample preparation and analysis time is less than 30 min, and LODs are below levels found in typical samples. Storage of exhaled breath samples at −20 °C is possible for up to 48 h prior to analysis.

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