Journal of Chromatography B, 877 (2009) 1856–1866

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Improved pre-concentration and detection methods for volatile sulphur breath constituents

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ARTICLE INFO

Article history:
Received 1 December 2008
Accepted 8 May 2009
Available online 15 May 2009

Keywords:
Breath analysis
Volatile sulphur compounds
SPME
Sorbent trapping
H_2S
MeSH
EtSH
DMS
COS
CS_2

ABSTRACT

Suitability of different types of pre-concentration (solid phase microextraction and sorbent trapping) and detection (flame photometric detector (FPD) and mass selective detector (MSD)) for gas chromatographic determination of sulphur-containing compounds (H_2S, MeSH, EtSH, DMS, COS and CS_2) in breath-gas was assessed in this study. Several factors like influence of humidity, influence of oxygen, or stability of target compounds in extraction vessels (SPME vials and sorbent tubes) were investigated. Despite poor stability of VSCs in SPME vials and matrix effects (unfavorable influence of humidity), SPME was found to be a fast and reliable enrichment method, which coupled with mass selective detector provided satisfactory LODs of target compounds at the ppt level (from 0.15 ppb for CS_2 to 2.3 ppb for H_2S). Application of sorbent trapping with two-bed sorbent tubes containing Tenax TA and Carboxen 1000 gave excellent LODs (0.03–0.3 ppb for 200 ml sample and MSD). Stability of investigated VSCs in sorbents was found to be very poor (30–40% losses after 2 h). FPD showed satisfactory sensitivity only when it was coupled with sorbent trapping. Breath samples were collected into Tedlar bags in a CO_2-controlled manner. Humidity was removed during sampling (permeation dryer – Nafion) to avoid unfavorable water dependent effects during analysis.

1. Introduction

Exhaled breath analysis is a highly promising non-invasive diagnostic technique of great medical potential [1–4]. Over the last years a great number of volatile organic compounds (VOCs) have been found in exhaled breath and for some of them correlations between the concentrations and numerous diseases have been established. Currently, an effort is made to find new biomarkers in human breath and develop reliable and sensitive methods for their determination.

Volatile sulphur compounds (VSCs) are promising biomarkers present in human breath-gas [3]. Increased concentrations of VSCs were found in breath of individuals with impaired liver functions (carbonyl sulphide (COS), dimethyl sulphide (DMS), methanethiol (MeSH), ethanethiol (EtSH), dimethyl disulphide (DMDS), carbon disulphide (CS_2) [5–7], halitosis (H_2S, MeSH, DMS, CS_2) [8–11], organ rejection after lung transplantation (COS) [12], lung cancer (DMS) [13], or schizophrenia (CS_2) [14].

Trace analysis of VSCs in human breath is challenging due to their highly reactive nature and trace concentrations [15]. Currently, breath VSCs are analysed using gas chromatographic techniques (GC) [7,12,16] frequently coupled with mass spectrometry (GC–MS) [9,17,18], or sensor-based techniques employing semiconductor gas sensors [8,10,13]. However, the last technique cannot separate and distinguish between different VSCs.

Gas chromatography is a gold standard in breath research [1,2,19]. In case of analysis of sulphur-containing species it offers wide range of sulphur-selective detectors, namely flame photometric detector (FPD), pulsed flame photometric detector (PFPD), sulphur chemoluminescence detector (SCD) and atomic emission detector (AED) [20–23]. Unfortunately, they are in most cases impractical due to some technical problems (PFPD, SCD), or high cost (AED). FPD is a stable, relatively inexpensive sulphur-selective detector, which has been employed in trace analyses of VSCs in different matrices [6,7,12,21,24–26]. This detector together with a universal mass selective detector (MSD) found a wide application in VSCs analysis in human breath.

Currently, breath analysis of VOCs relies on two pre-concentration methods, namely solid phase microextraction (SPME) and solid phase extraction with subsequent thermal desorption (SPE/TD) [1,2,4,19].

SPME has been successfully applied in VSCs analyses in gaseous [22–24,27] and liquid [21,24,26] matrices. The advantages of this

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doi:10.1016/j.jchromb.2009.05.010

technique are its ease of operation, good limits of detection (ppb–ppt) and small amounts of sample required to perform extraction. SPME can be also easily automated, which significantly reduces laboratory effort and increases samples' throughput.

Solid phase extraction with subsequent thermal desorption (SPE/TD) is the most popular VSCs pre-concentration method in breath analysis [7,9,12,13,25]. It provides excellent limits of detection and possibility of targeting specific compounds by a proper choice of sorbents [28]. This method, however, is much more time- and effort-consuming.

Sampling is a crucial stage in breath analysis. Dilutions, contaminations and losses, which very frequently occur during this analytical step irreversibly modify sample composition and distort the analysis results. Consequently, special precautions have to be taken during the breath sampling. Currently, two sampling methods are commonly used in breath studies: mixed expiratory sampling and alveolar sampling [19,29]. The alveolar sampling is much more profitable as it reduces the risk of contamination and ensures higher concentrations of breath volatile species [29,30]. The main problem of breath sampling is, however, the lack of a commonly accepted sampling protocol. Recently Miekisch et al. [29] have developed a simple CO_2-controlled sampling method targeting alveolar air. This method was shown to be reliable and reproducible, and seems to be a significant progress toward the standardized sampling protocol.

In this paper suitability of different types of pre-concentration (solid phase microextraction and sorbent trapping) and detection (flame photometric detector (FPD) and mass selective detector (MSD)) for gas chromatographic determination of sulphur-containing compounds (H_2S, MeSH, EtSH, DMS, COS and CS_2) in breath-gas were assessed. To select the optimal configuration several factors like sensitivity, matrix effects, choice of optimal sorbent, or stability of target compounds in extraction vessels were investigated. In addition to this a CO_2-controlled breath sampling method targeting VSCs was presented.

2. Experimental

2.1. Calibration mixtures and test gases

Two certified cylinder-based primary standards of VSCs were used within this study (Linde gas, Germany). Standard no. 1 had a certified tolerance of 5% and contained MeSH (10 ppm), EtSH (10.7 ppm) and DMS (10.7 ppm) balanced with dry helium. Standard no. 2 was also balanced with dry helium and contained H_2S (12.5 ppm), COS (10.2 ppm) and CS_2 (12.5 ppm). The certified tolerance was 5% for H_2S and CS_2 and 10% for COS. These primary standards were statically diluted to prepare test gases with VSCs concentrations ranging from 0.2 to 200 ppb. This was achieved by transferring 0.015–15 ml of primary standards with gas-tight silanized syringes (Hamilton, USA) into previously filled with 800 ml of nitrogen/air 1 L Tedlar bags (SKC Inc., USA). Tedlar bags were chosen as the secondary standards containers due to their inertness and suitability for short-time storage of VSCs [31]. Prepared in such a way calibration mixtures were analysed after 30 min.

2.2. SPME materials and method

SPME fibers coated with 85 μm carboxen/polydimethylsiloxane (CAR-PDMS) were obtained from Supelco (Canada). This type of coating is commonly recommended for pre-concentration of VSCs [21–24,26,32,33]. Prior to the use all fibers were preconditioned in GC injector at 290 °C for 2 h. SPME was performed in two types of vials: 21 ml in volume (PerkinElmer, USA) and 20 ml in volume (Gerstel, Germany). To prevent adsorption all vials were silanized with hexamethyldisilazane (Sigma–Aldrich, USA). Five types of sealing septa were used: silicone/PTFE (3 mm, Perkin Elmer, USA), silicone/PTFE (1.3 mm, Gerstel Germany), silicone/TEF (1.5 mm, Markus Bruckner Analysentechnik, Austria), butyl/PTFE (1.3 mm, Gerstel, Germany) and natural rubber/TEF (1.3 mm, Gerstel, Germany).

Prior to the extraction the vial was evacuated with a membrane pump and 19 ml of sample were introduced into it using a 20 ml glass gas-tight syringe (Roth, Germany). Subsequently, the pressure in vial was balanced with pure nitrogen (6.0–99.9999%). Extraction was performed manually by inserting a fiber into a vial and exposing it to a sample for 10 min at 30 °C. Afterward, the fiber was immediately introduced into the injector of gas chromatograph. The fibers were desorbed at 260 °C in a splitless mode (30 s).

Table 1
Characteristics of sorbents. CMS – carbon molecular sieve, GCB – graphitized carbon black.

Adsorbent	Manufacturer	Type	Particle size (mesh)
Carbopack X	Supelco	GCB	40/60
Carbotrap X	Supelco	GCB	20/40
Carboxen 569	Supelco	CMS	20/45
Carboxen 1000	Supelco	CMS	60/80
Carboxen 1003	Supelco	CMS	40/60
Unicarb	Markes International	CMS	60/80
Tenax TA	Markes International	Organic polymer	60/80
Chromosorb 106	Supelco	Organic polymer	60/80

2.3. SPE materials and methods

Eight sorbent materials were used within this study: two organic polymers (Tenax TA and Chromosorb 106), two graphitized carbon blacks (Carbotrap X and Carbopack X) and four carbon molecular sieves (Unicarb, Carboxen 569, Carboxen 1000 and Carboxen 1003). The characteristics of the sorbents are presented in Table 1.

Stainless steel thermal desorption tubes (1/4 in. O.D., 3½ in. long) pre-packed with Tenax TA and Unicarb were purchased from Markes Int. (UK). All other sorbent tubes were prepared in-house. Sorbents were placed in silanised glass thermal desorption tubes (1/4 in. O.D. × 3½ in. long, Markes International, UK) and protected with silanised glass wool. Sorbent tubes containing carbon adsorbents were preconditioned using the following temperature program: 0.5 h at 100 °C, 1 h at 200 °C, 1 h at 300 °C and 0.5 h at 350 °C under the dry helium flow of 50 ml/min. In case of Tenax TA sorbent tubes preconditioning temperature program was as follows: 0.5 h at 300 °C and 2 h at 325 °C. Chromosorb 106 was conditioned for 0.5 h at 100 °C and 1 h at 200 °C.

Sorbent trapping of VSCs was accomplished at room temperature by manual drawing of appropriate amount of sample through a thermal desorption tube using a 100 ml glass syringe (ROTH, Germany). Prior to the extraction on the sampling end of a sorbent tube an inert stainless steel needle (Sulphinert treatment) was installed. The other end was connected to the syringe. Next, the needle was inserted through the septum into the sampling bag and sample was sucked with a steady flow rate of 40 ml/min. After extraction both ends of the sorbent tubes were sealed with PFTE ferrules and Swagelok caps.

A two-stage thermal desorption of target compounds was performed in commercial thermal desorber (UNITY, Markes International Limited, UK). Over the course of the primary desorption sorbent tubes were heated to 280 °C for 10 min and purged with pure helium (6.0–99.9999%) at the flow rate of 20 ml/min. Released during this step, analytes were immediately focused in the designed for sulphur compounds cold trap (Markes International Limited, UK) packed with graphitized carbon black and maintained at

Table 2
Retention times, mass/charge ratios (m/z) and dwell times used in the SIM mode.

Compound	Retention time (min)	m/z	Dwell time (us)
H_2S	4.94	33	150
		34	250
COS	5.39	60	300
		62	100
MeSH	7.57	46	150
		47	150
		48	150
EtSH	10.15	29	200
		47	200
		62	200
DMS	10.7	46	200
		47	200
		62	250
CS_2	11.7	76	250
		78	200

−10 °C. Analysed sulphur species were introduced into the capillary column in the splitless mode by the rapid heating of the cold trap to 280 °C (secondary desorption). The 1 m-long transfer line (silica tube) connecting thermal desorber with the column was maintained during the analysis at the lowest possible temperature of 120 °C.

2.4. Gas chromatography

Slightly modified GC system presented in our recent paper [31] was used during analyses. Analytes of interest were separated using the DB1 column (60 m × 0.32 mm, film thickness 5 μm, Agilent, USA) working in a constant pressure mode (19.28 psi). The column temperature program was as follows: 60 °C for 7 min, increase to 230 °C at a rate of 15 °C/min and 230 °C for 5 min. The three-way spl

itter (type G3183B, Agilent, USA) was used to divide the effluent from the column among three different detectors: mass selective detector (MSD) (type 5975, Agilent, USA), flame photometric detector (FPD) (type G2333B, Agilent USA) and electron capture detector (ECD). The split ratio determined by the length and diameter of tubing connecting the splitter to the detectors was set to 2:2:1 for the MSD, FPD and ECD, respectively. This means that both MSD and FPD detectors received equal amounts of the effluent, namely 40%. The ECD detector, despite being a part of the system, was not used within this work. The splitter pressure (the pressure at the column outlet) was set to 3.8 psig. The application of the splitter allowed on the simultaneous detection of investigated VSCs by the MSD and FPD detectors and, consequently, significantly shortened the time of the experiments. The whole system was adapted to analyses of reactive compounds (Sulphinert treatment).

Mass spectrometer worked in a combined SCAN and selected ion monitoring mode (SIM). SCAN monitoring mode was used for compounds identification, whereas SIM mode was utilized for quantification. The scan range was set from m/z 33 to 100. The retention times, mass/charge ratios (m/z) and dwell times used in the SIM mode are presented in Table 2.

2.5. Effect of matrix on efficiency of SPME

To evaluate possible matrix effects in the SPME of VSCs two factors were investigated, namely presence of oxygen and high humidity. For this purpose three test gases containing concentrations of approximately 20 ppb of the VSCs of interest were prepared and analysed using the same SPME procedure and MSD detector. The first one was balanced with neutral gas (nitrogen 6.0), second one with synthetic air (80% N_2, 20% O_2) and the last one with humidified synthetic air (RH = 100% at 37 °C). To avoid water condensation and related to it possible losses of hydrophilic compounds humid mixtures were produced only in SPME vials. First, SPME vials were evacuated with a membrane pump and heated to 40 °C for 2 min. Next, 0.82 μl of distilled water – amount which corresponds to the water content in 19 ml of human breath (100% RH at 37 °C) – was injected into the vials. After approximately 2 min (time necessary for the complete water evaporation), appropriate amounts of dry mixtures were added to obtain target concentrations. All vials were maintained at 40 °C in the course of the whole experiment.

2.5.1. Background tests of SPME vials

During these tests background of vials sealed with different types of septa was investigated to estimate its influence on sample integrity. Crimped with tested septa vials were filled with high-purity nitrogen and stored at room temperature. For each type of septa two vials were prepared. The first one was analysed immediately after filling the second one after 6 h of storage.

2.5.2. Recovery tests from SPME vials

Stability of target compounds in extraction vessels (in our case in SPME vials) is a crucial factor in SPME of breath samples. This is particularly important when long sequences of breath samples are analysed with the help modern autosamplers. Septum closing the extraction vial is in this case the most vulnerable element. To select the optimal septum material five different types of septa were tested with respect to their influence on the VSCs stabilities. Recoveries of VSCs were examined using a test gas containing approximately 10 ppb of each VSC. For each type of septum a set of 8 vials was prepared. These vials were evacuated and filled with the test gas at the same time. Next, the content of these vials was analysed with SPME–GC–MS method after certain periods of time to monitor the time evolution of VSCs concentrations. The first analysis was done immediately after the vials' filling, next ones after approximately 0.5, 1, 2, 3, 4, 6 and 24 h of storage.

2.6. Selection of optimal sorbent

The main goal of this test was to select the optimal sorbent (or set of sorbents) for trapping and retaining the investigated VSCs. All the eight aforementioned sorbents were investigated. In case of the carbon based sorbents tested tubes contained mass of 200 mg. For organic polymers the corresponding mass amounted to 100 mg. This difference was caused by a limited volume of glass tubes and relatively low mass to volume ratio in case of polymer sorbents. During the test 200 ml of the test gas (20 ppb of VSCs) were allowed to pass through each sorbent tube using the earlier described extraction procedure. The tubes were desorbed and analysed with GC–MS method immediately after extraction.

2.6.1. Recovery of VSCs from sorbent tubes

Suitability of sorbent tubes for storage of breath VSCs was checked in a similar way like for SPME vials. Prior to the experiment four two-bed sorbent tubes containing 100 mg of Tenax TA and 150 mg of Carboxen 1000 were prepared and preconditioned. As it will be shown in the Section 3, this combination of sorbents was found to be optimal for trapping of target compounds. Next, 200 ml of a test gas containing about 20 ppb of investigated VSCs were transferred into each tube using the earlier described procedure. The first tube was analysed (GC–MS) immediately after extraction, next ones after 1, 2.5 and 24 h of storage. Two storage temperatures were investigated to check the influence of this factor on the VSCs stability: room temperature (23 °C) and 4 °C. During the storage time all the tubes were sealed with PFTE ferrules and Swagelok caps.

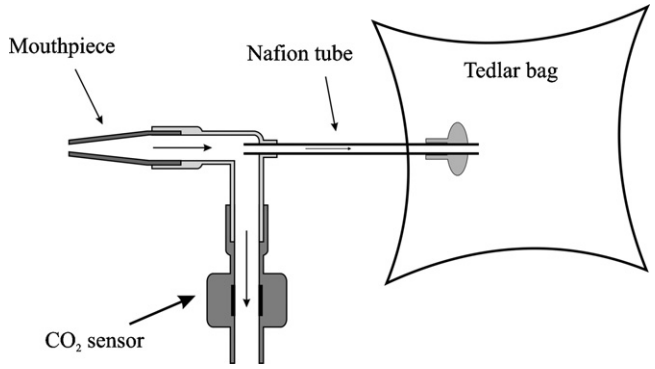

Fig. 1. Scheme of breath sampling device.

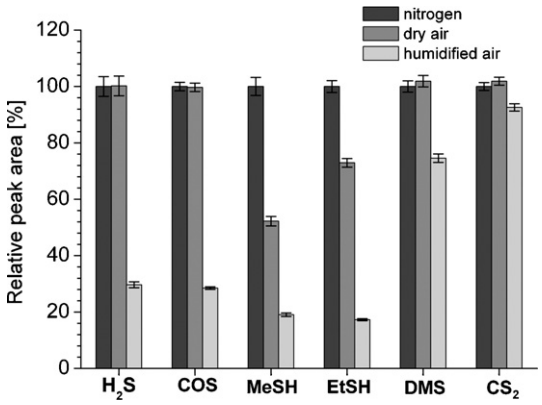

Fig. 2. Influence of the different matrices (nitrogen, dry air, humidified air) on the efficiency of SPME of H_2S, COS, MeSH, EtSH, DMS and CS_2 (GC–MSD). Peaks areas are normalized to signals obtained for mixtures balanced with N_2.

2.7. Breath sampling

The applied sampling procedure was based on the breath sampling method developed by Miekisch et al. [29]. Our modifications of this system faced two problems: losses related to the high reactivity of VSCs, and humidity dependant effects in VSCs analysis. To circumvent the first one an effort was made to delimit the contact of breath samples with sampling system elements. This was done by shortening of the transfer line. Humidity of breath samples strongly affects the GC analyses of VSCs. The most undesirable effects are: decrease of the sorbents adsorption capacity, plugging of cold traps, retention time variations and decrease of the extraction efficiency in SPME [15,23,34,35]. Several methods of water removal have been proposed to avoid these effects. The most popular ones are: usage of drying agents (e.g. $CaCl_2$, K_2CO_3, $MgSO_4$) and usage of permeation membranes (e.g. Nafion – perfluorosulphonic acid) [15,27]. Drying agents, despite their popularity, strongly affects the recoveries of numerous VSCs and their application is limited [15]. Much more useful seems to be the permeation removal of water engaging membrane tubes. This drying method has been proved to be an efficient one in VSCs analyses [15].

The sampling device used in this study consisted of a disposable mouthpiece installed on a L-shaped plastic tube (Polyvinyl chloride – PVC), CO_2 sensor cell (Capnogard, Novametrix, USA) attached to the other side of the L-tube, and 68 cm-long Nafion tube in a polypropylene casing (Perma Pure LLC, USA) (see Fig. 1). The one end of the Nafion tube was connected through an additional luer-lock aperture to the L-shaped tube, whereas, the other end was connected to the valve of a sampling bag. In our investigations samples were collected into 1 L Tedlar or Flexfoil bags (SKC Inc., USA), which were found to be the most suitable materials for storage of investigated species [31]. During the first phase of sampling exhaled breath flowed solely through the L-tube and the sensor cell, where the CO_2 concentration was monitored. The alveolar phase of the exhalation air was recognized with the help of the P_{CO2} monitor (Capnogard, Novametrix, USA). Breath-gas was introduced into a sampling bag only during the alveolar phase by closing the outlet of the sensor cell. Consequently, only mouthpiece, part of the L-shaped tube, Nafion tube and valve were in contact with a sample during its flow to the polymer bag. To improve the efficiency of drying the Nafion tube was additionally purged from outside with ambient air.

To investigate losses of the VSCs during the passage through the sampling device, a 1 L in volume Tedlar bag was filled with the test gas containing approximately 20 ppb of sulphur compound. The content of this bag was analysed using the SPME–GC–MS. Next, the bag was connected to the mouthpiece of the sampling device and its content was transferred to another Tedlar bag connected to the distant end of the Nafion tube. Prior to the experiment the sampling device was purged with approximately 1 L of the test gas to remove air and avoid dilution. Finally, gas collected in the second Tedlar bag was analysed with the same method as the first one.

3. Results

3.1. Effect of matrix on efficiency of SPME

The comparison of VSCs peak areas obtained for different matrices is presented in Fig. 2. For H_2S, COS, DMS and CS_2 no significant differences were found between test gases prepared in nitrogen and air. There was, however, a notable difference in the peak areas obtained for mercaptans. The peak area of methanethiol was 50% smaller when this compound was extracted from dry air. For ethanethiol the analogous loss amounted to 30%. This phenomenon was also described elsewhere [23] and can be attributed to the oxidation of MeSH and EtSH in the GC injector at high temperature during desorption. An effort was made to reduce this effect by decreasing the injector temperature. However, this did not significantly improve signals. Furthermore, for the temperatures below 250 °C significant peak tailings were noticed. Consequently, the injector temperature of 260 °C was recognized as the optimal one.

For the test gas balanced with humidified air the peak areas of H_2S, COS, MeSH and EtSH were about 80% smaller than the ones obtained for the test gas prepared in dry nitrogen. For DMS and CS_2 the drop was slightly smaller and amounted to 25% and 8%, respectively. Two possible reasons of such a decrease can be indicated: loss of hydrophilic compounds related to water condensation on the vials' walls and decrease of SPME efficiency. Condensed water vapor attracts well soluble compounds and affects, thereby, the original concentrations. Since the test gas was humidified only in vials, which were maintained within the course of the experiment at the temperature guaranteeing that no water condensation occurred, the second option seems to be much more probable. Particularly that similar humidity effect in SPME of VSCs was described also elsewhere [23]. These findings led us to a conclusion that the water removal from the sample prior to the SPME could significantly improve the VSCs extraction from breath.

3.2. Background tests of SPME vial

All the three types of silicone septa exhibited a good background. They did not emit any sulphur species as well as other contaminants, which could interfere with the investigated VSCs. For the case of butyl septa and natural rubber septa significant emissions

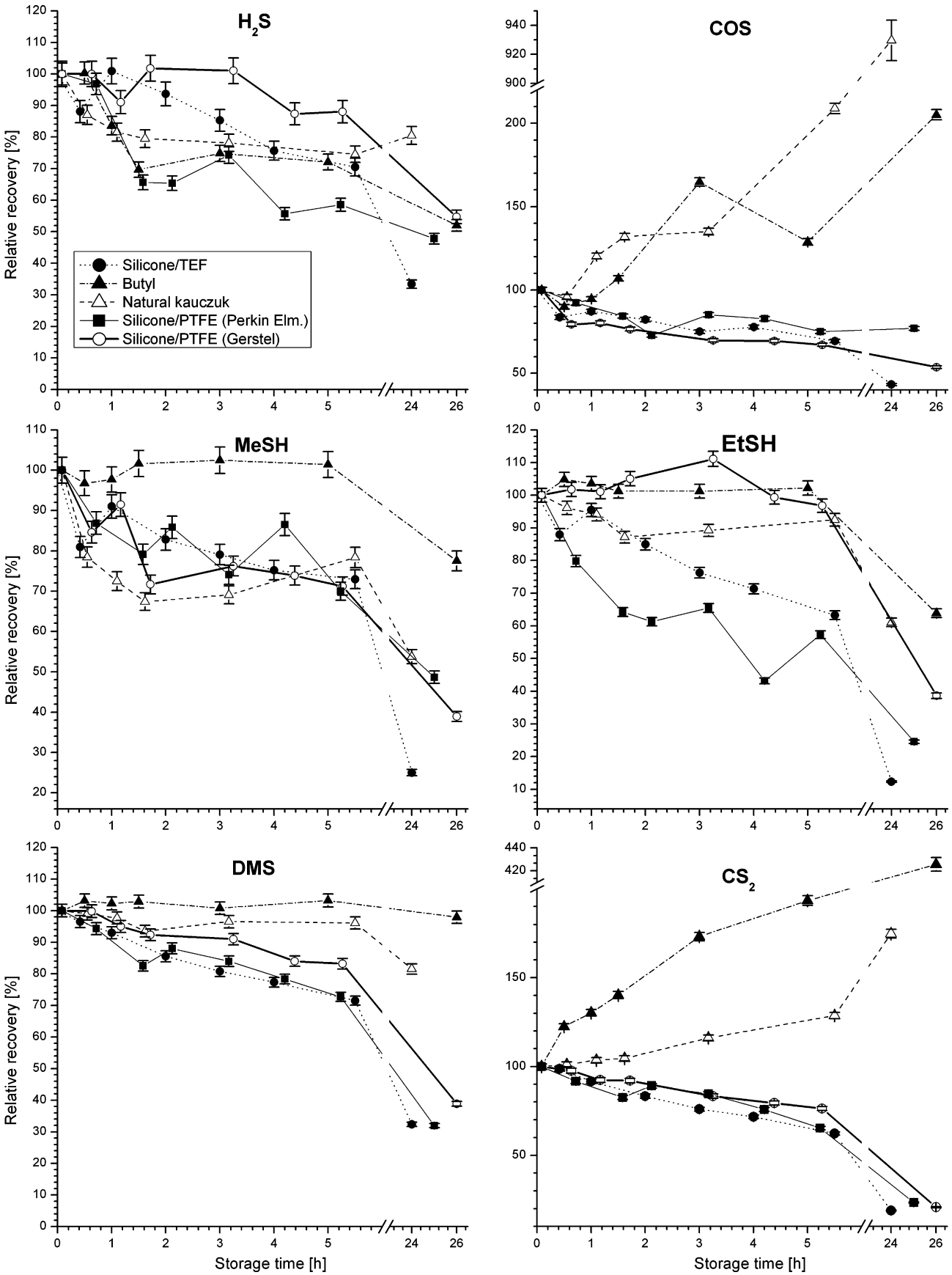

Fig. 3. Effect of storage time on recoveries of investigated VSCs from vials sealed with different types of septa (SPME–GC–MSD). Peak areas normalized to signals obtained for samples analysed immediately after filling.

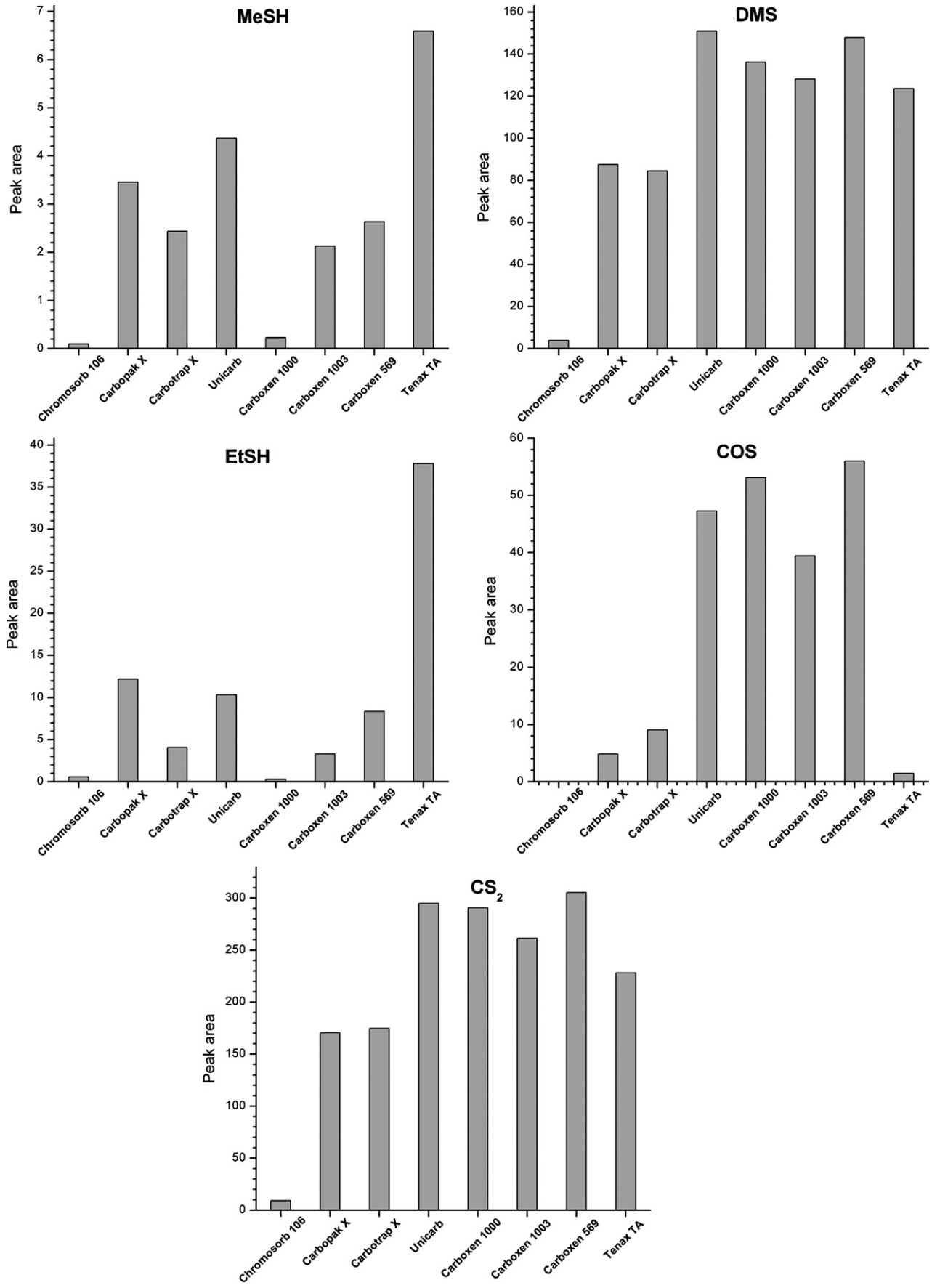

Fig. 4. Extraction efficiency of investigated sorbents for COS, MeSH, EtSH, DMS and CS_2 (SPE–GC–MSD).

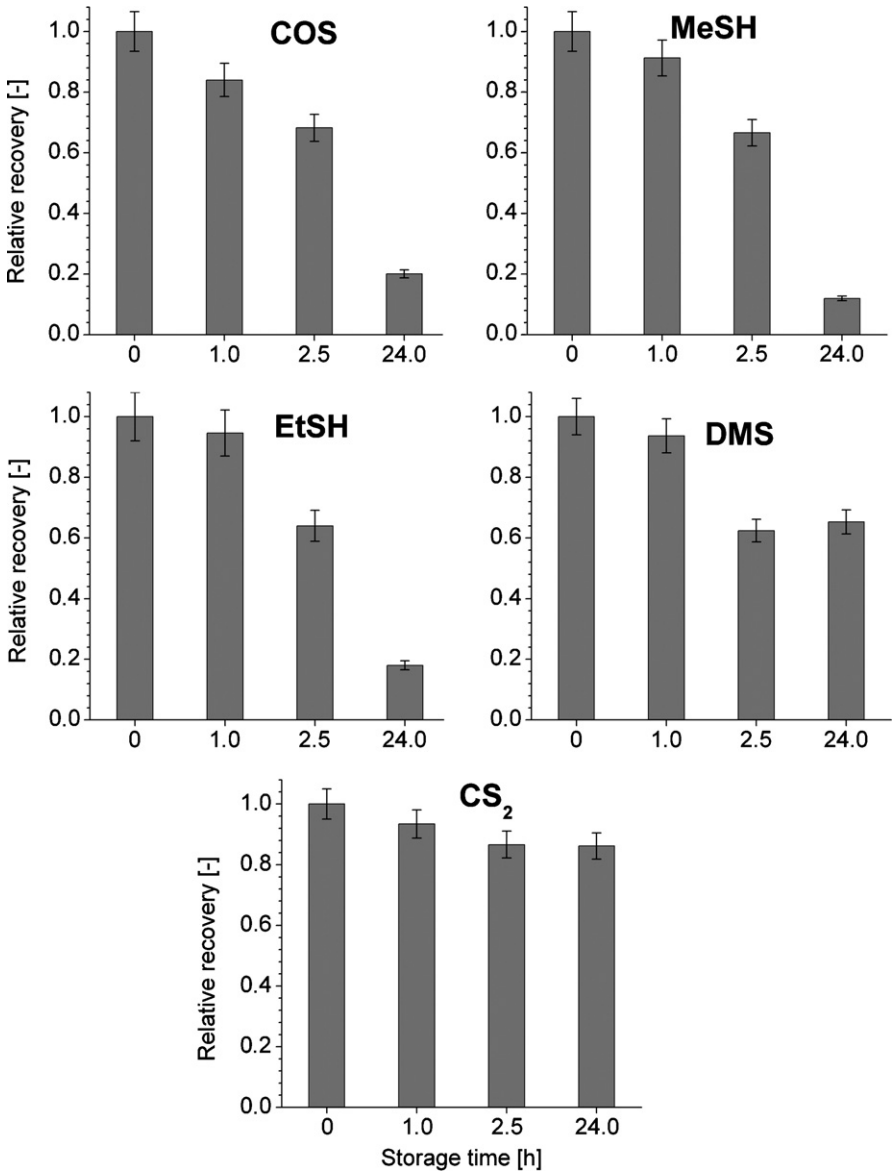

Fig. 5. Effect of storage time on recoveries of COS, MeSH, EtSH, DMS and CS_2 from Tenax TA/Carboxen 1000 sorbent tubes.

of COS and CS_2 were noted. In vials crimped with natural rubber septa the COS and CS_2 concentrations increased to 10 and 3 ppb, respectively after 24 h of storage, whereas, for butyl septa these concentrations amounted to 3 and 9 ppb.

3.3. Recovery tests from SPME vials

Silicone septa from different manufacturers exhibited similar time evolution of VSCs concentrations (see Fig. 3). In case of COS, MeSH, DMS and CS_2 recoveries ranged from 70% to 75% after 6 h to drop to 20–40% after 24 h. More perceptible differences between different silicone septa were noted for H_2S and EtSH. The best recovery after 6 h was observed for septa manufactured by Gerstel (88% for H_2S and 95% for EtSH). For the two remaining silicone septa losses of H_2S and EtSH amounted to 30–40%.

For the butyl septa, recoveries of the organic VSCs were excellent and did not change significantly during the first 6 h of storage. After 24 h they dropped to 80% for MeSH, 63% for EtSH and 95% for DMS. The H_2S stability was poorer. There was a considerable loss of 28% in its concentration after 6 h, which reached 50% after one day of storage. The concentrations of COS and CS_2 increased to 20 and 40 ppb at the end of the investigated period, which is consistent with the results of the background test.

Recoveries of organic VSCs were also relatively good in vials sealed with natural rubber septa. DMS and EtSH concentrations remained stable for 6 h after the vial filling (losses smaller than 10%) to drop by 20% and 40%, respectively after one day. For H_2S and MeSH decrease was more evident and amounted to 25% after the first 6 h. Similarly like for butyl septa, notable gains in COS and CS_2 concentration were observed. It is worth mentioning that the emission of COS from natural rubber septa was particularly intensive. The concentration of this compound reached 93 ppb after 24 h of storage.

None of the examined septa was perfect material for SPME of investigated VSCs. Despite good background silicone septa do not provide a good recovery of sulphur compounds. Rubber septa (particularly butyl septa) seem to be a good material for SPME of organic VSCs. Due to the excellent stability of EtSH, MeSH and DMS, vials sealed with these types of septa can be recommended as storage containers.

In view of these results, silicone septa with the best general background (PerkinElmer) were selected for experiments within

Table 3
Correlation coefficients, slopes (a), intercepts (b), LODs, LOQs and RSDs obtained for investigated configurations The concentrations used for RSDs determinations were as follows: 6 ppb for SPME/SPE–GC–MSD, 20 ppb for SPE–GC–FPD and 50 ppb (200 ppb for H_2S) for SPME–GC–FPD. The R^2 have been estimated for the range from LOD to 100 pbb (MSD) and 200 ppb (FPD).

	SPME (CAR/PDMS)											
	MSD (SIM)						FPD					
	LOD (ppb)	LOQ (ppb)	RSD (%)	a	b	R^2	LOD (ppb)	LOQ (ppb)	RSD (%)	a	b	R^2
H_2S	2.3	6.9	3.5	11.02 ± 0.19	11.43 ± 6.80	0.9992	120	250	8.0	0.14 ± 0.01	12 ± 2	0.9821
COS	0.32	0.96	1.7	57.92 ± 0.37	−1.68 ± 7.21	0.9997	43	76	5.0	1.04 ± 0.18	2.54 ± 5.80	0.9727
MeSH	0.65	1.95	2.6	80.36 ± 2.04	−41.04 ± 25.22	0.9980	43	74	3.5	1.07 ± 0.07	0.88 ± 1.98	0.9968
EtSH	0.31	0.93	1.5	171.82 ± 6.01	−115 ± 102	0.9973	20	35	2.3	2.27 ± 0.15	0.39 ± 4.27	0.9923
DMS	0.18	0.54	1.9	476.1 ± 10.2	243.7 ± 181.3	0.9991	9	15	1.6	5.19 ± 0.61	0.62 ± 2.91	0.9988
CS_2	0.15	0.45	1.6	641.1 ± 12.6	167.4 ± 155.1	0.9992	6	10	1.6	7.59 ± 0.19	−9.08 ± 6.16	0.9981
	SPE/TD (Tenax/Carboxen 1000)											
	MSD (SIM)						FPD					
	LOD (ppb)	LOQ (ppb)	RSD (%)	a	b	R^2	LOD (ppb)	LOQ (ppb)	RSD (%)	a	b	R^2
H_2S	–	–	–	–	–	–	–	–	–	–	–	–
COS	0.06	0.18	6.5	232.7 ± 31.2	145.1 ± 256.2	0.9901	5	12	6.8	5.95 ± 0.32	9.56 ± 8.09	0.9918
MeSH	0.31	0.93	6.8	241.1 ± 9.4	−165.0 ± 199.1	0.9958	18	32	9	2.26 ± 0.07	−0.87 ± 1.96	0.9993
EtSH	0.13	0.39	7.5	692.6 ± 41.4	−600 ± 540	0.9965	5	8	8	8.80 ± 0.29	−10.37 ± 8.57	0.9979
DMS	0.04	0.12	6.2	2992 ± 111	−1254 ± 1675	0.9958	1	2.1	5	32.83 ± 1.15	−50.56 ± 30.98	0.9964
CS_2	0.03	0.09	5.8	4811 ± 206	−776 ± 2241	0.9952	0.8	1.3	6	55.51 ± 2.44	−93.47 ± 76.99	0.9943

this study. However, to avoid losses of VSCs SPME was performed immediately after the vials filling.

3.4. Selection of optimal sorbent

The comparison of the extraction efficiencies of tested sorbents is presented in Fig. 4. With the exception of Chromosorb 106 DMS and CS_2 were satisfactorily trapped by all tested sorbents. The highest peak areas for DMS and CS_2 were obtained for carbon molecular sieves (CMSs). They outstripped by 40% the ones obtained for graphitized carbon blacks (GCBs) and by 25% the one obtained for Tenax TA. COS was trapped satisfactorily only by CMSs. Within this group Carboxen 569 and Carboxen 1000 produced the largest peaks. Tenax TA was found to be the most efficient sorbent for trapping of mercaptans. In case of this sorbent signal for EtSH outstripped 4–10 times the EtSH signals obtained for other sorbents. Similar pattern was observed for methanethiol. Poor peak areas of MeSH and EtSH for carbon sorbents can be ascribed to mass losses due to the reactions induced by active centers. However, we did not detect any potential products of such reactions.

H_2S turned out to be too volatile for sorbent trapping with applied materials. A signal for this compound was not obtained for any of the tested sorbents. It was either not retained by sorbents or purged out during the thermal desorption. This problem could be eliminated by the usage of special materials designed for trapping of H_2S (e.g. cold trap filled with silica gel). Since we were interested in the most universal sorbents this compound was excluded from further investigations.

Amongst the tested sorbents Unicarb was the most universal one. It successfully trapped COS, DMS and CS_2 and provided relatively good signals for mercaptans. On the other hand, Tenax TA was found to be an excellent sorbent for retaining of MeSH and EtSH. Consequently, the combination of Tenax TA and one of CMSs was recognized as optimal for sorbent trapping of investigated VSCs. The two-bed tubes used in further experiments contained 100 mg of Tenax TA (front end) and 150 mg of Carboxen 1000 (far end).

To estimate the efficiency of the extraction with Tenax TA/Carboxen 1000 200 ml of test gas (40 ppb of VSCs) were sucked through two sorbent tubes connected in a row and analysed. The estimated extraction efficiency amounted to 98.8% for COS, 96.7% for MeSH, 98.7% for EtSH, 98.2% for DMS and 99.8% for CS_2. These values were recognized as satisfactory for analysis of all components of interest.

3.4.1. Recovery of VSCs from sorbent tubes

VSCs stabilities on the investigated sorbents were poor (see Fig. 5). For tubes stored at 23 °C recoveries amounted to approximately 90% after 1 h of storage. 1.5 h later they dropped to 65% for COS, MeSH, EtSH and DMS and 85% for CS_2. After 24 h there were considerable losses (80–85%) observed for COS, MeSH and EtSH, whereas, the concentrations of DMS and CS_2 remained stable. CS_2 with the concentration drop of 15% after 24 h was the only compound whose recovery was satisfactory over the investigated period. Very similar recoveries were obtained for tubes stored at 4 °C in the refrigerator. These findings led us to a conclusion that sorbent tubes should not be considered as storage containers for breath VSCs. Furthermore, the retained sulphur compounds should be analysed within 1 h after extraction.

3.5. Validation parameters

The validation parameters were established for both tested detectors (FPD and MS) and both pre-concentration techniques (SPME and SPE). The linearity of the calibration graphs was checked using nine gas mixtures with concentrations of 0, 0.2, 0.5, 1, 5, 10, 25, 50, 100 and 200 ppb. The last concentration was used only for

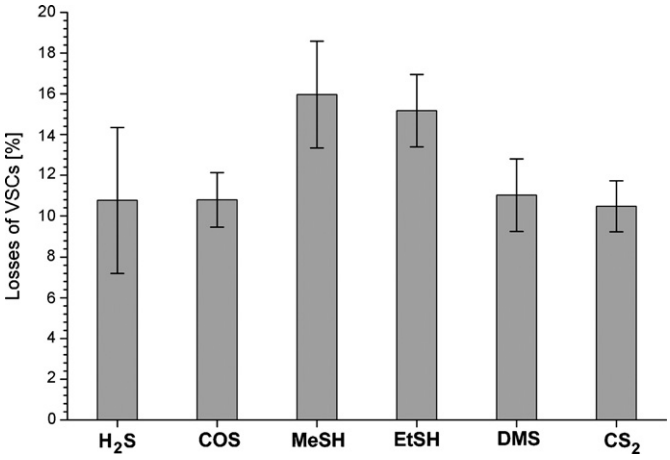

Fig. 6. Losses of H_2S, COS, MeSH, EtSH, DMS and CS_2 after the passage through the sampling device.

the configurations employing the FPD, which showed higher limits of detection than the MSD. For the FPD the sulphur response is quadratic [20]. To compensate it, the square roots of the peak areas were used for calculations concerning this detector. The calibration graphs were also used to estimate limits of detections and limits of quantifications. The limit of detection (LOD) and limit of quantification (LOQ) were defined as signal-to-noise ratios of 3 and 9, respectively.

The estimations of the relative standard deviations (RSDs) were based on five consecutive analyses of appropriate test gases. For experiments employing mass selective detector the test gas with 6 ppb of each VSC was used. In case of the FPD configurations the test gases contained slightly higher concentrations of VSCs 20 ppb (SPE) and 50 ppb (SPME).

The correlation coefficients, slopes, intercepts, LODs, LOQs and RSDs obtained for both investigated detectors and both preconcentration methods are presented in Table 3. It must be stressed that the LODs presented in Table 3 were obtained for only 40% of the column effluent.

For all configurations the responses were found to be linear over the investigated ranges of concentrations. The configurations with mass selective detector (MSD) produced 60–100 times better LODs than the ones employing flame photometric detector (FPD). For the SPME–GC–MSD they ranged from 0.15 ppb for CS_2 to 2.3 ppb for H_2S, whereas, for SPE–GC–MSD they were between 0.03 ppb for CS_2 and 0.31 ppb for MeSH. These values can be considered as satisfactory for breath analysis. For the case of FPD the limits of detection fell in the range of 6–120 ppb when SPME was used as the preconcentration method and in the range of 1–18 ppb when SPE was employed for sample enrichment.

The sensitivity of the SPME–GC–FPD system was too poor for quantitative analyses of investigated compounds in breath-gas. Consequently, the application of the FPD detector in breath anal-

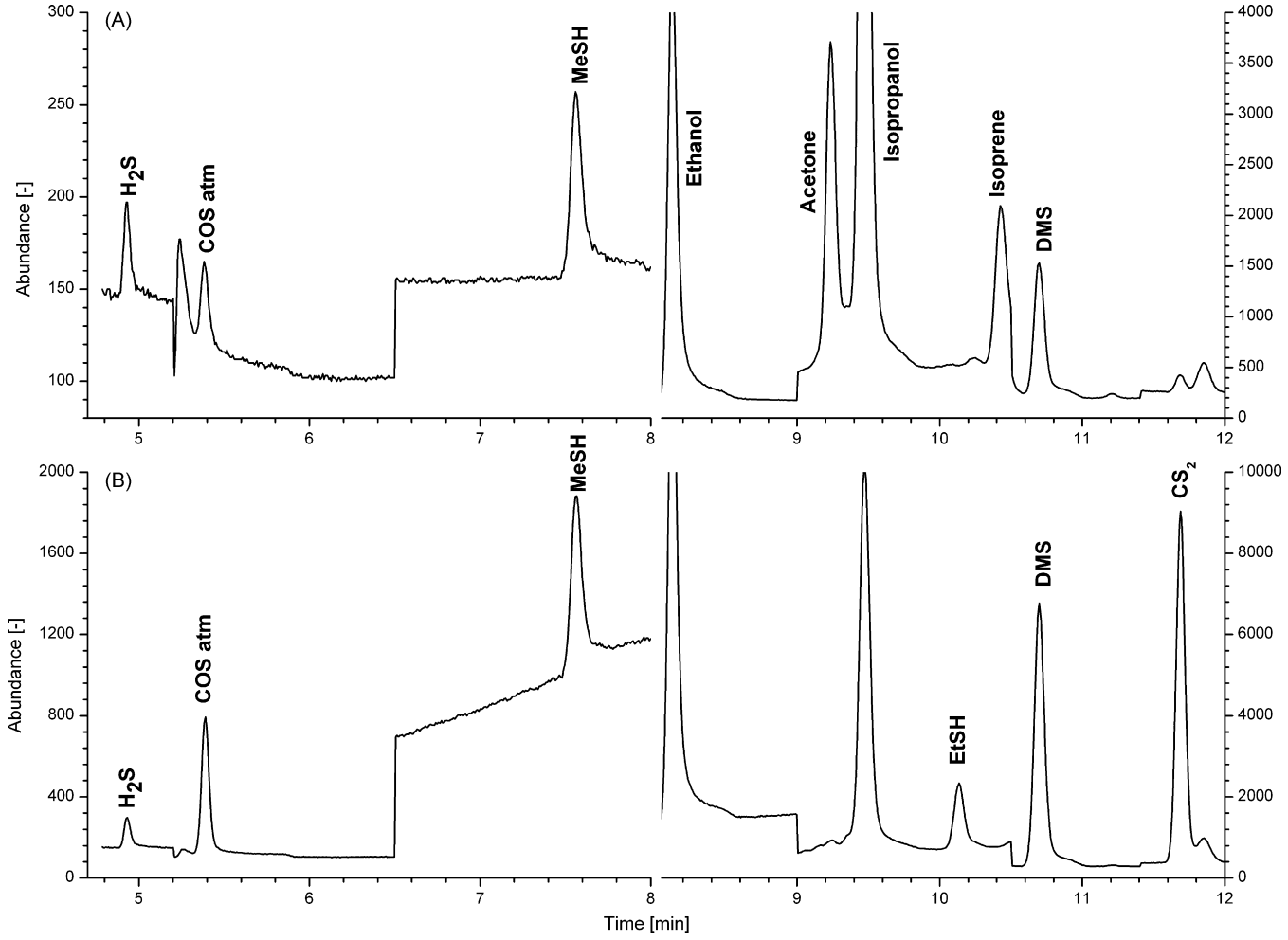

Fig. 7. (A) Exemplary SPME–GC–MS chromatogram of a breath sample (volunteer no. 8). COS atm. denotes atmospheric COS (about 0.5 ppb). (B) Reference chromatogram of a standard mixture containing 50 ppb of VSCs.

Table 4
VSCs compounds detected in breath of healthy volunteers.

No.	Gender	Age	Smoking status	H_2S (ppb)	COS (ppb)	MeSH (ppb)	DMS (ppb)	CS_2 (ppb)
1	F	40	non-smoker	7.3 ± 0.5	<0.26	<0.56	10.5 ± 0.9	<0.1
2	F	28	non-smoker	7.1 ± 0.5	<0.26	<0.56	5.8 ± 0.7	<0.1
3	M	30	smoker	6.6 ± 0.5	<0.26	<0.56	5.7 ± 0.7	<0.1
4	F	28	non-smoker	14.4 ± 0.6	0.7 ± 0.2	2.1 ± 0.3	16.0 ± 1.0	<0.1
5	M	35	non-smoker	6.5 ± 0.5	<0.26	1.6 ± 0.3	4.4 ± 0.7	<0.1
6	F	45	non-smoker	3.0 ± 0.5	<0.26	<0.56	6.8 ± 0.8	<0.1
7	F	24	non-smoker	<2	<0.26	<0.56	14.0 ± 1.0	<0.1
8	F	31	non-smoker	11.9 ± 0.6	<0.26	2.3 ± 0.3	15.8 ± 1.1	<0.1
9	M	24	non-smoker	<2	<0.26	<0.56	4.1 ± 0.7	<0.1
10	M	55	non-smoker	29.5 ± 0.7	<0.26	1.6 ± 0.3	6.0 ± 0.8	0.6 ± 0.2

ysis is limited to sorbent trapping. The great advantage of sorbent trapping is the improvement of LODs by the increase of exploited sample volume. For the SPE–GC–FPD system 500 ml of breath-gas seem to be the minimum volume to obtain satisfactory sensitivity. The mass selective detector working in SIM mode ensured satisfactory LODs both for SPME and SPE. However, the SPME–GC–MS configuration is more practical with respect to laboratory effort, sample stability and automation.

3.6. Breath sampling

Losses of approximately 10% were observed for H_2S, COS, DMS and CS_2 after the test gas passage through the breath sampler (see Fig. 6). In case of mercaptans they were higher and amounted to 15%. These losses were slightly higher than the ones reported for Nafion driers by other authors [15]. However, in our experiment they cannot be ascribed exclusively to the influence of Nafion tube, but also to the adsorption on other elements such as the mouthpiece or bag's valve. Considering profits resulting from the water removal the recorded losses were recognized as acceptable for the developed method.

3.7. Examples of breath analysis

Breath samples of 10 healthy volunteers were analysed using the SPME–GC–MS. This method was chosen due to its simplicity, good sensitivities and possibility of H_2S detection. Two samples were taken per subject: alveolar air and room air. The latter was used to make the background corrections of the VSCs concentrations in exhaled air. Samples were collected in sitting stance after 5 min rest. Since all subjects were recruited from the laboratory staff longer equilibration with environmental air was not necessary. Under normal conditions this time should be increased to at least 30 min. Samples were collected into 1 L in volume Tedlar bags and analysed within 2 h after sampling. An exemplary chromatogram of breath sample analysis is presented in Fig. 7.

Five compounds of interest were found to be present in the expired breath of volunteers: H_2S, COS, MeSH, DMS and CS_2 (see Table 4). H_2S was detected in breath of eight persons. Its concentrations ranged from 3 to 29.5 ppb (mean: 10.8 ppb). DMS was present in all breath samples. The concentrations of this compound were between 4 and 16 ppb (mean: 8.9 ppb). Breath of only four people contained detectable amounts of methanethiol (1.5–2.3 ppb). COS like CS_2 was detected only in breath of one volunteer. Interestingly volunteers with elevated concentrations of COS and CS_2 exhibited higher concentrations of other sulphur species.

4. Discussion

Low breath concentrations and reactive features of VSCs impose severe precautions during all steps of VSCs analysis. To ensure reliable analysis all materials and elements of the system being in contact with the sample have to be carefully selected. This means application of inert materials showing no emission of target compounds. The last problem particularly afflicts determination of COS and CS_2. As it was demonstrated above and in our recent paper [31] COS and CS_2 are frequently emitted from rubber parts of the system (e.g. O-rings, septa, gaskets) that distorts the analysis results. Consequently, rubber should be eliminated from all elements being in contact with the sample particularly during the sample storage.

Breath sampling is the most vulnerable stage of breath analysis. In case of VSCs we recommend CO_2-controlled breath sampling method developed by Miekisch et al. [29]. However, to reduce VSCs losses transfer line should be as short as possible and heated. It is also advisable to reduce sample humidity during sampling. Such an approach eliminates numerous unfavorable effects during the VSCs analysis. In the sampling system used in this study humidity problem was solved by the application of the permeation dryer (Nafion). The main disadvantage of this approach is a slightly increased resistance during the breath flow into the bag, induced by a small internal diameter of the Nafion tube. However, it can be eliminated by the use of commercial multitube Nafion driers adapted to high flow rates.

The choice of sample vessel is a crucial issue for sample storage. The perfect one should be inert for samples, inexpensive and reusable. High reactivity and very low concentrations of breath VSCs put severe demands on potential sample vessels. Unfortunately, the most inert containers are impractical due to the high costs (stainless steel with Sulphinert treatment) or fragility (silanised glass bulbs). In our recent paper [31] we investigated suitability of several polymer bags for storage of sulphur-containing compounds relevant in breath analysis. Flexfoil and transparent Tedlar were found to be the optimal materials for VSCs storage up to 24 h. In this paper we additionally tested alternative sample containers: sorbent tubes and SPME vials. Unfortunately, due to the poor stability of VSCs (vials and sorbent tubes) or emission of some sulphur species (some types of septa) they do not meet all requirements. Only vials equipped with butyl septa could be used for short-time (6–8 h) storage of organic sulphur species (e.g. DMS, EtSH, MeSH). Consequently, Tedlar or Flexfoil bags can be recommended for storage of breath samples in VSCs analysis. Polymer bags with breath samples can be stored at room temperature, however, they should be heated prior to the extraction.

SPME is a fast and simple pre-concentration method, which can be easily applied in breath analysis of VSCs. Coupled with mass selective detector working in SIM mode it provides satisfactory LODs for all investigated VSCs. In addition to this SPME requires relatively small amounts of breath-gas to perform extraction. This feature is especially valuable when the volume of breath sample is limited, or when analysis is aimed at the determination of VSCs in a single exhalation. SPME has, however, some disadvantages. Poor stability of VSCs in SPME vials and emission of CS_2 and COS from rubber septa complicates the automation of this analytical step. Consequently, SPME has to be performed immediately after

filling the extraction vessel. The matrix effects manifested by the losses of mercaptans during the fiber desorption in the presence of oxygen and decrease of extraction efficiency for humid samples significantly affect the sensitivity of the method. Whereas, the former effect is difficult to compensate, the latter one can be circumvented by the aforementioned drying method.

Sorbent trapping is an alternative sample enrichment method that can be applied in analysis of breath VSCs. The two-bed sorbent tube containing Tenax TA and one of the carbon molecular sieves (e.g. Unicarb, Carboxen 1000) is the optimal one for trapping of five investigated sulphur species. H_2S requires special materials (e.g. cold trap filled with silica gel) and has to be analysed separately. The obtained LODs (ppt level) are excellent and can be easily improved by the use of larger sample volume. Consequently, in case of SPE/TD FPD can successfully replace the mass selective detector. This method, however, is time- and effort-consuming. Stabilities of VSCs in sorbent tubes are very poor, consequently, trapped VSCs have to be analysed immediately after extraction. Moreover, due to this effect automation of the method seems to be difficult. SPE can be recommended for breath studies aimed at very low concentration (low ppt level) where maximum sensitivity is required.

Acknowledgments

This project was financially supported by the European Commission (project BAMOD, no. LSHC-CT-2005-019031), by the Jubiläumsfonds of the Austrian National Bank (project number 12760) and by the Polish Ministry of Science and Higher Education (project no. N N404 2793 33) over the time period of 2007–2009.

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