ELSEVIER

Journal of Chromatography B, 739 (2000) 183–190

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of isoprene in human expired breath using solid-phase microextraction and gas chromatography–mass spectrometry

Radomír Hyšpler[a,*], Šárka Crhová[b], Jiří Gasparič[b], Zdeněk Zadák[a], Marie Čížková[c], Věra Balasová[c]

[a]*Medical Faculty, Charles University, Šimkova 870, Hradec Králové 500 01, Czech Republic*
[b]*Faculty of Pharmacy, Charles University, Heyrovského 1203, Hradec Králové 500 05, Czech Republic*
[c]*Laboratory of Organic Analysis, Public Health Department of Karviná, Těreškovové 2206, Karviná 734 01, Czech Republic*

Abstract

An analytical method for determination of isoprene in expired breath as a marker of body cholesterol synthesis was developed with a special emphasis on breath sampling. Patients were breathing controlled air using respiratory masks for 2 min (washout period) and then their expired breath was collected in 8-l Tedlar bags. The bags were heated to 40°C and the solid-phase microextraction fiber Carboxen–polydimethylsiloxane 75 μm was inserted through the septum. Extraction time was 10 min. Analytes were desorbed in the GC injector for 2 min at 270°C. Analyses were performed on a Q-PLOT column and fragment ions 68, 67 and 53 were quantified. The concentration range was 1–40 nmol/l, limit of detection was 0.25 nmol/l, the calibration curve was linear. Precision, expressed as RSD, was 5.5–12.5%. These tests are non-invasive, feasible and relatively inexpensive.

Keywords: Breath sampling; Cholesterol synthesis; Isoprene

1. Introduction

Isoprene (2-methyl-1,3-butadiene) is a reactive aliphatic hydrocarbon. It is a colorless liquid, b.p. 35°C (101.325 kPa). It is synthesized by nearly all animals as precursor of many important compounds and is present among the hydrocarbon metabolites in human breath in the highest concentration. Isoprene originates from the decomposition of dimethylallyldiphosphate, a member of cholesterol and isoprenoids synthetic pathway [1]. This pathway is controlled by the key enzyme β-hydroxy-β-methylglutaryl-CoA-reductase. This enzyme creates mevalonic acid. Reactions follow by which isopentenyldiphosphate and dimethylallyldiphosphate are formed from mevalonic acid. The activity of the key enzyme can be controlled by drugs called statins, the application of which lowers the concentration of isoprene in breath [2]. That is why isoprene in expired breath is understood as a marker of cholesterol synthesis and its concentration corresponds with the key enzyme activity changes during the day. Cholesterol is synthesized mainly during the night hours due to high demands on energy supply. Isoprene concentration measurement should improve the care of patients suffering from disorders in cholesterol metabolism, such as hypercholesterolemia, which is a risk factor for atherosclerosis development and its complications are cause of most

*Corresponding author. Tel.: +420-49-5832-241; fax: +420-49-5832-001.
E-mail address: rhyspler@lfhk.cuni.cz (R. Hyšpler)

0378-4347/00/$ – see front matter
PII: S0378-4347(99)00423-5

deaths in Europe, diabetes, critical illnesses and so on.

In connection with a systematic investigation of hypercholesterolemia [2,3] and its monitoring a method for determination of isoprene in expired breath was desirable. Several, mostly gas chromatographic methods, have been described in the literature for this purpose using different detection methods [flame ionization detection (FID), UV detection, photoionization detector (PID) or mass spectrometry (MS)] [4–6], proton transfer MS [1] or selected ion flow tube [7]. Most authors used preconcentration procedures to obtain sufficient accuracy and precision. This may be done at ambient temperature in cartridges containing adsorbing resin or at low temperatures in a gas sampling loop. All authors have pointed out that it is necessary to pay attention to the sampling procedure. The circadian changes of isoprene content in the breath [8,9], the influence of smoking [10], the influence of food intake and the quality of the air in which the patient was inspiring before the measurement should be taken into account. The isoprene content given by these authors changes in the range of 1.6 to 10.33 nmol/l [3,11,12], others presented higher mean values 14.5 nmol/l [4,9] or 48.5 nmol/l [13]. Isoprene concentration in the breath of children is much lower [1], in the case of adults the concentration does not change with the age [14]. All the methods mentioned above suffer from many disadvantages, such as sensitivity to moisture and carbon dioxide present in breath or low reproducibility. Due to this fact we attempted to develop a reproducible, inexpensive method for routine work.

2. Experimental

2.1. Chemicals and materials

Deionised water, acetone, ethanol were from Merck (Darmstadt, Germany). Helium 6.0 and pure air 2.1 were from Aga (Hamburg, Germany). Tedlar bags (8.1 l, 0.05 mm thickness) equipped with PTFE-coated septa and Tygon tubings were from Chromatography Research Supplies (Addison, IL, USA). Respiratory masks, wrapped respiratory tubings (22 mm I.D.) and valves were from Juncalor (Hradec Králové, Czech Republic). Standard mixtures (isoprene in nitrogen in concentrations 2.38 and 39.7 nmol/l) were purchased from Siad (Milan, Italy).

2.2. Equipment

For solid-phase microextraction (SPME) a Carboxen–polydimethylsiloxane (PDMS) 75 μm fiber was obtained from Supelco (Bellefonte, PA, USA). The fibers were mounted into a manual holder (obtained from Supelco). Prior to first use, they were conditioned in the GC injector in a helium stream following the instructions of the manufacturer.The split/splitless injector was equipped with a 0.75 mm I.D. SPME glass liner (Supelco).

The GC–MS analysis itself was performed on a TurboMass (electron ionisation quadrupole mass spectrometer) coupled with an AutoSystem XL gas chromatograph (purchased from Perkin-Elmer, Norwalk, CT, USA). The separation was performed using a Q-PLOT capillary column (Supelco) of 30 m×0.32 mm I.D. Data acquisition and processing were performed by TurboMass software (Perkin-Elmer).

2.3. Breath sampling and standard preparation

Breath sampling was realized using respiratory masks, wrapped respiratory tubing (22 mm I.D.), one-way valves and 8.1-l Tedlar bags. Patients were instructed to breath moderately without deep breaths. They inspired controlled air from the gas cylinder through the fixed mask and after 2 min of breathing (washout period) the Tedlar bag was connected and the expired gas was collected into the bag and the time necessary to obtain 8-l samples recorded.

Standard application was performed by slow inflation of the Tedlar bag by the certified calibration standard and enriched with deionised water through rubber septum to saturate it with water vapor (0.32 ml per 8 l).

2.4. Extraction of the sample on SPME fiber

The sample bag was thermostatted at 40°C for 5 min (preheating time). The SPME needle was in-

serted through a rubber septum coated with PTFE into the gas sample and the fiber exposed for 10 min.

2.5. Gas chromatographic and mass spectrometric conditions

The fiber with extracted analytes was inserted into the split/splitless injector of GC–MS system and heated up to 270°C, where the compounds were thermally desorbed in splitless mode for 2 min and afterwards the splitter was opened and the fiber was removed. The inlet pressure was 100 kPa, carrier gas (helium) velocity was 37 cm/s and the oven was programmed at 40°C (for 2 min), ramped at 20°C/min to 160°C (2 min). The transfer line and ionisation source were heated to 150°C and ionisation energy was 70 eV.

Mass fragments (m/z) 53, 67, 68 (dwell 0.2 s) were monitored in selected ion monitoring (SIM) mode and their sum was used for quantitation (Fig. 1).

2.6. TO-14[1] GC–MS reference method

The breath sample, after one SPME extraction and analysis was transferred from the Tedlar bag into a pre-evacuated canister by suction. The canister was simply connected to an Aerocan (Tekmar-Dohrmann, Cincinnati, OH, USA) equipped with an additional trap filled with sodium hydroxide and sodium sulfate to collect moisture and carbon dioxide. A vacuum pump transferred a definite sample volume (50–500 ml) to the cryotrap, cooled by liquid nitrogen to −165°C. Analytes were transferred to cryofocuser cooled to −165°C by heating the trap to 200°C. The injection onto the chromatographic column of the GC–MS HP 5972 system (Hewlett-Packard, Palo Alto, CA, USA) was done by a quick temperature rise (1 min) on the cryofocuser from −165 to +100°C. The analytical column was a Vocol (Supelco) of 105 m×0.53 mm I.D., stationary phase film 3 μm. The oven was programmed at 35°C (5 min), ramped at 8°C/min to 180°C. Qualitative and quantitative analyses were performed using the MS detector HP 5972 and ChemStation software. The same mass fragments (m/z) were monitored as in the case of SPME analysis.

3. Results and discussion

3.1. Sampling procedure

The method used to obtain a proper sample, is of extreme importance. Breathing is a dynamic process and is continuously changing according to the physical and psychological status of the person.

From the point of view of gas exchange, a notable property of the final airways is that they are blind. Therefore a complete replacement of the air during ventilation is impossible. The gas exchange regions are reached only by way of gas mixing. It is not possible to obtain so called ''alveolar air'' without invasive procedures. The total surface area of the alveoli for diffusion of isoprene is not constant even in one patient – in adult man it is about 35 m^2 in expiration and 100 m^2 in maximum inspiration, and has an impact on isoprene levels and is another factor making reproducibility difficult.

This is probably the reason why we experimentally found the ''single breath techniques'' [6] to be irreproducible and the collection of relatively large samples over a period of several minutes to be necessary. The humidity and temperature of air in airways are also important factors. Humidity in the inspired air reaches near saturation very quickly, but because of gas mixing, even the temperature of expired breath does not reach body temperature. This fact can have a large impact on the concentration of volatiles with boiling point at about body temperature, such as isoprene.

To standardize the sampling procedure and to get rid of many environmental contaminants a lung washout is often employed [15]. It is performed by breathing air free of evaluated substances. Because isoprene in the environment of our laboratory does not reach high values – the background is 0.042 nmol/l – a washout time of 2 min was found to be satisfactory and also is short enough not to discomfort patients. We collected whole expired gas –

[1]Certified US Environmental Protection Agency method ''Determination of Volatile Organic Compound in Ambient Air using SUMMA Passivated Cannister Sampling and Gas Chromatographic Analysis''. SUMMA canisters are commercially available canisters with passivated electropolished inner walls.

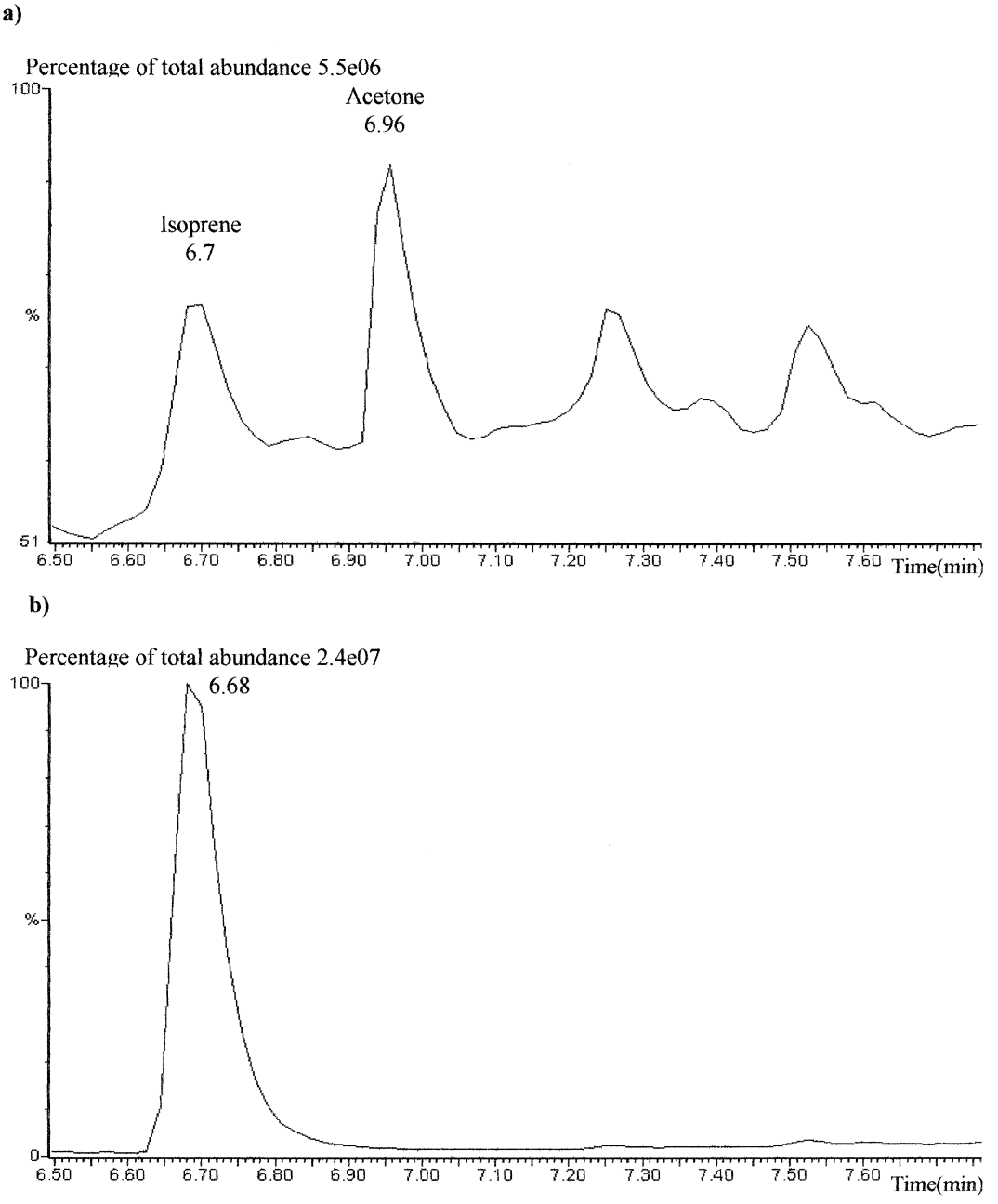

Fig. 1. (a) GC–MS scan chromatogram of real breath sample. (b) GC–MS selected ion monitoring chromatogram of ions 53, 67, 68 of the same real breath sample.

the patient is breathing the controlled air from the tank and compensatory bag via the respiratory mask and expired air goes into the collection bag. These systems are not very comfortable for patients, but ensure reasonable reproducibility and precision. This may be demonstrated on isoprene monitoring of a laboratory staff member during one working day (Fig. 2a) starting at 6:00 after arrival to the labora-

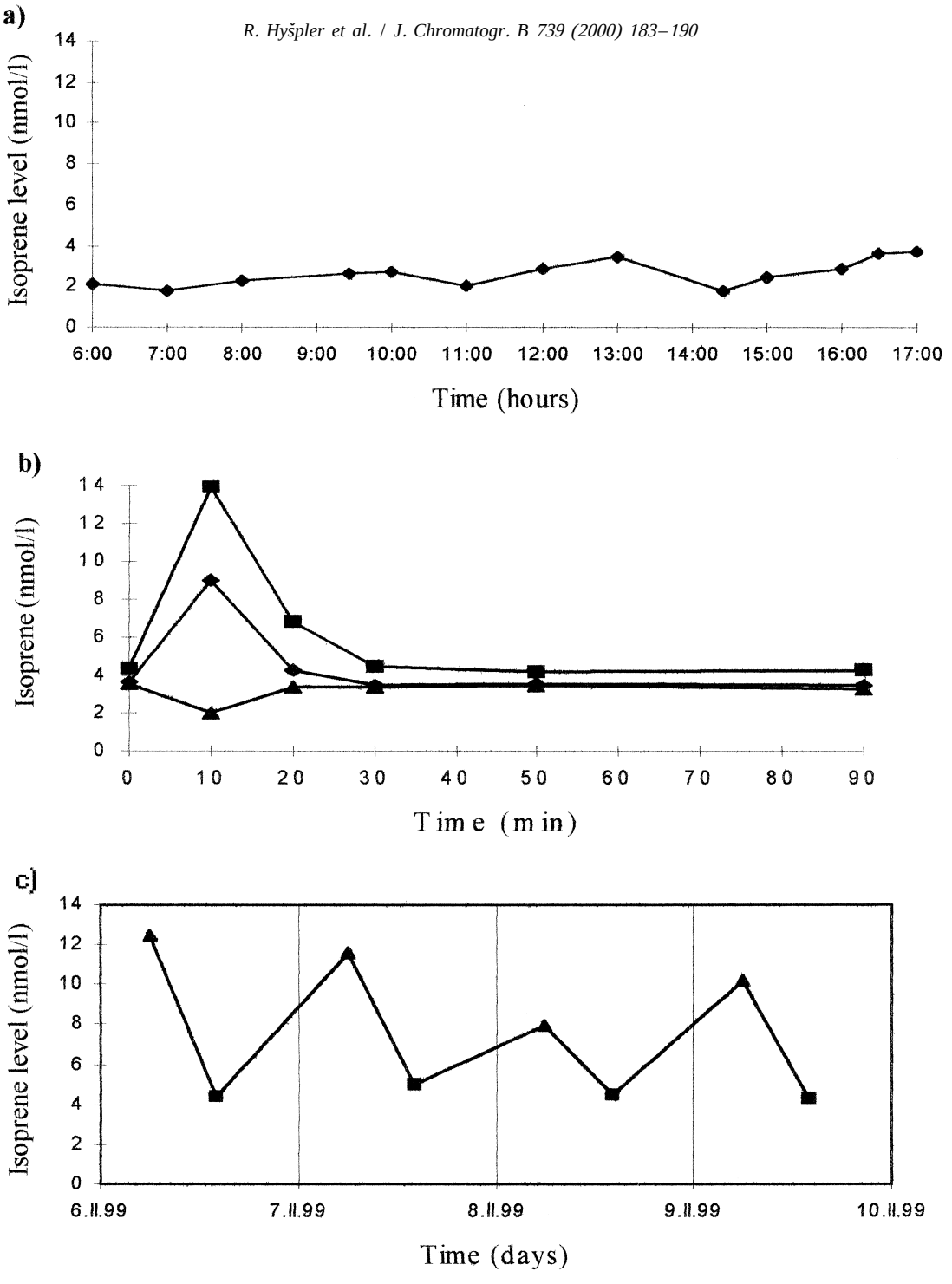

Fig. 2. (a) Isoprene levels of a laboratory staff member during the working day starting at 6:00 after the arrival to laboratory. (b) Effect of some external factors on isoprene levels in breath. Influence of smoking one cigarette during the first 10 min of the experiment on isoprene levels – heavy smoker (■), non-smoker (♦) and influence of light exercise during the first 10 min (▲). (c) Isoprene levels of a fasting patient reducing his weight in four consecutive days. Isoprene levels were measured at 6:00 in bed after awaking (▲) and at 14:00 after 1 h resting in bed (■).

tory. Patients are not allowed to smoke at least 1 h prior sampling, because isoprene is present in cigarette smoke in large quantities and it accumulates in the lungs (Fig. 2b). Patients should be also resting for at least 30 min after physical activity to make measurement in resting conditions possible (Fig. 2b).

Circadian variation is characteristic and a long-time known parameter for isoprene production. According to our experiences, night time values are even more valuable for interpretation. Therefore we used the following sampling scheme (time): 6:00 – patients were woken up just before sampling and these samples represented the night-time levels and 14:00 – patients rested in bed at least 1 h before sampling and their samples represented the day-time levels.

An example of these results is presented in Fig. 2c to show circadian variation of isoprene excretion. This patient was reducing his weight by total fasting – the reason for the decrease of night-time levels, but day-time levels are surprisingly unaffected.

3.2. SPME method – extraction time and temperature

According to manufacturer's instructions, the Carboxen–PDMS 75 μm fiber was chosen due to large affinity of non-polar trace-level compounds towards the fiber. Both temperature and humidity can have a large effect on extraction [16] and their values must be kept within tight limits. Due to the large affinity of compounds towards the Carboxen–PDMS fiber, the equilibrium was not reached even after 6 h according our time–sorption profile.

Therefore, for practical reasons, we used a 10-min adsorption time at 40°C. This temperature was chosen to ensure evaporation of all condensed water and isoprene in expired gas as well as standards. Higher temperatures could damage the bag material and in the case of lower temperatures water condensation on bag walls and the fiber itself occur. It was found experimentally using repeated extractions that approximately 4% of total amount of isoprene in the 8.1-l sample is extracted. This percentage is dependent on sample volume. During the extraction at 40°C no visible condensation was observed on the inner walls of the bags. There is always a water peak present in the chromatogram as water is adsorbed onto the SPME fiber, but it is well separated from isoprene. Carryover of less than 1% was found. The fiber was pre-desorbed every day in the morning prior to analyses to eliminate passive sampling during the night.

3.3. Calibration and linear range

The method was calibrated using external standards. The calibration curve was obtained by dilution of standard gases with pure air in gas-tight bulbs above water saturated with sodium chloride. A calibration curve was linear with a correlation coefficient of at least 0.990 over the range of 1–40 nmol/l. Great attention was paid to the purity of the sampling material and the laboratory background. Sampling and the preparation of calibration mixtures were realized in a room separated from the analytical laboratory. It was very important to clean sampling bags and canisters properly. Canisters were cleaned by heating and washing using clean air. The Tedlar bags were cleaned by triple wash with clean air at ambient temperature. The blank analyses of canisters or bags were performed and the history of their use was noted.

3.4. Precision, accuracy and limit of detection

The precision of the analyses was calculated as relative standard deviation (RSD) 4.81–14.60% (Table 1). The higher value was reached in the case of real breath samples. Accuracy was measured by comparison of our results with our reference method. Sampling to SUMMA passivated canisters was possible after one SPME extraction and analysis, because only a minor part of the isoprene content had been adsorbed (see Section 3.2). A special care had to be paid to very slow suction and gas tight connections, because condensation of analytes may occur what could seriously impair the results obtained.

The limit of detection was estimated on the basis of the signal-to-noise ratio to be greater than 5. It was 0.25 nmol/l (5.7 ppbv) for the SPME method, 0.02 nmol/l (0.45 ppbv) for the TO-14 method.

Table 1
Method validation

	Method	
	SPME–GC–MS	TO-14 GC–MS
RSD of standard gas concentration 2.38 nmol/l ($n=7$)	14.60%	4.64%
RSD of standard gas concentration 39.7 nmol/l ($n=7$)	4.81%	3.25%
Correlation coefficient	0.9961	0.9983

3.5. Effect of relative humidity and other compounds on extraction

The Carboxen–PDMS fiber shows a decreased extraction by 35% of an amount extracted in a water vapor-saturated atmosphere compared with the extraction of a dry standard.

The presence of other compounds in the breath could affect the extraction by active-sites competition. Ethanol and acetone are present in the breath in relatively large quantities, other compounds are present only in traces. We spiked bags with different concentrations of ethanol and acetone dissolved in water, but no differences in adsorption occurred. We have found no effect of other compounds present in human breath (metabolic ethanol, acetone) on extraction, so that their presence in the calibration standards was not necessary.

3.6. Storage time

GC–MS analyses using our equipment are difficult to carry out at the patient's bed-side and samples must be transported to the analytical laboratory. Therefore, it was essential to know how long the sample can be stored in the bags. According to our experiments, the samples are stable for 6 h. The samples cannot be stored overnight probably due to diffusion of isoprene through the bag wall or adsorption onto it. The isoprene concentration of samples stored in canisters was found to be unchanged for at least two weeks (longer periods were not tested).

4. Conclusion

The method presented enables one to determine the isoprene content in human breath in the concentration range of 0.25–40 nmol/l. Maximum care must be given to the breath sampling, taking into consideration the parameters that can affect the results, i.e., time of sampling, influence of smoking, physical activity and so on. The advantages of the method involve the non-invasivity of sampling (multiple sampling is possible) and relative cost-effectiveness (100–150 analyses per fiber). Certain disadvantages could be the relative labor-intensity (early-morning sampling is necessary, throughput of three analyses per hour including sampling). The method presented is currently used in medical investigation and is being evaluated.

Acknowledgements

Supported by grants of IGA Nos. 4095-3, 4221-3 and GACR No. 203/99/1165.

References

[1] J. Taucher, A. Hansel, A. Jordan, R. Fall, J.H. Futrell, W. Lindinger, Rapid. Commun. Mass. Spectrom. 11 (1997) 1230.
[2] B.G. Stone, T.J. Besse, W.C. Duane, C.D. Evans, E.G. DeMaster, Lipids 28 (1993) 705.
[3] S. Mendis, P.A. Sobotka, D.E. Euler, Free Radic. Res. 23 (1995) 117.

[4] M. Phillips, J. Greenberg, J. Awad, J. Clin. Pathol. 47 (1994) 1052.
[5] E.R. Mohler, P. Reaven, J.E. Stegner, N.S. Fineberg, D.R. Hathaway, J. Chromatogr. B 685 (1996) 201.
[6] C. Grote, J. Pawliszyn, Anal. Chem. 69 (1997) 587.
[7] P. Spanel, D. Smith, Med. Biol. Eng. Comput. 34 (1996) 409.
[8] E.G. DeMaster, H.T. Nagasawa, Life Sci. 22 (1977) 91.
[9] A. Cailleux, P. Allain, Life Sci. 44 (1989) 1877.
[10] D.E. Euler, S.J. Dave, H. Guo, Clin. Chem. 42 (1996) 303.
[11] A.W. Jones, V. Lagesson, C. Tagesson, J. Chromatogr. B 672 (1995) 1.
[12] S. Mendis, P.A. Sobotka, D.E. Euler, Clin. Chem. 40 (1994) 1485.
[13] A. Cailleux, X. Moreau, A. Delhumeau, P. Allain, Biochem. Med. Metab. Biol. 49 (1993) 321.
[14] D. Gelmont, R.A. Stein, J.F. Nead, Biochem. Biophys. Res. Commun. 99 (1981) 1456.
[15] C.M.F. Kneepkens, G. Lepage, C.C. Roy, Free Radic. Biol. Med. 17 (1994) 127.
[16] M. Chai, J. Pawliszyn, Environ. Sci. Technol. 29 (1995) 693.