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Research article

Investigation of different approaches for exhaled breath and tumor tissue analyses to identify lung cancer biomarkers

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ABSTRACT

Development of early noninvasive methods for lung cancer diagnosis is among the most promising technologies, especially using exhaled breath as an object of analysis. Simple sample collection combined with easy and quick sample preparation, as well as the long-term stability of the samples, make it an ideal choice for routine analysis. The conditions of exhaled breath analysis by preconcentrating volatile organic compounds (VOCs) in sorbent tubes, two-stage thermal desorption and gas-chromatographic determination with flame-ionization detection have been optimized. These conditions were applied to estimate differences in exhaled breath VOC profiles of lung cancer patients and healthy volunteers. The combination of statistical methods was used to evaluate the ability of VOCs and their ratios to classify lung cancer patients and healthy volunteers. The performance of diagnostic models on the test data set was greater than 90 % for both VOC peak areas and their ratios. Some of the exhaled breath samples were analyzed using gas chromatography coupled with mass spectrometry (GC-MS) to identify VOCs present in exhaled breath at lower concentration levels. To confirm the endogenous origin of VOCs found in exhaled breath, GC-MS analysis of tumor tissues was conducted. Some of the VOCs identified in exhaled breath were found in tumor tissues, but their frequency of occurrence was significantly lower than in the case of exhaled breath.

1. Introduction

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer death [1]. Despite the latest achievements in the fields of medicine and pharmacy in the last few decades, new anticancer drugs have still failed to decrease mortality. These types of treatments can prolong life and alleviate patients' suffering, but they are unfortunately unable to cure the disease completely.

In the case of early diagnosis, the efficiency of treatment can be dramatically improved. Lung cancer has the highest mortality rate among all types of cancer [2] mainly due to the lack of ability to diagnose it at early stages. Computed tomography (CT) and biopsy are the main methods for lung cancer diagnosis, but they are invasive and harmful. Therefore, there is an urgent need to develop a noninvasive, simple to use and accurate tool for lung cancer diagnosis.

Determination of VOCs in exhaled breath is a promising way to identify new lung cancer biomarkers [3, 4]. This approach is suitable for clinical diagnosis because sampling is noninvasive and comfortable for patients. Various analytical methods can be applied for the determination of VOCs (volatile organic compounds) in exhaled breath. Among them, GC-MS (gas chromatography coupled with mass spectrometry) remains the most efficient and convenient method for the identification of potential biomarkers. In this case, sorbent tubes with different types of sorbents [5, 6, 7, 8, 9] or solid phase microextraction (SPME) fibers [10, 11, 12, 13, 14] are applied to preconcentrate VOCs from an exhaled breath sample.

Other techniques for detecting VOCs in exhaled breath include ion mobility spectrometry (IMS) [15, 16] and proton transfer reaction mass spectrometry (PTR-MS) [17, 18]. In addition, a diverse range of sensor systems, such as functionalized gold nanoparticle (GPN) sensor arrays [19], metal oxide semiconductor sensors [20], sensor arrays based on organically stabilized spherical GNPs and single wall carbon nanotubes capped with polycyclic aromatic hydrocarbon [8], quartz microbalance sensor arrays [21] or colorimetric sensor arrays [22], are applied.

In general, a plethora of approaches for lung cancer biomarker identification in exhaled breath have been proposed. At the same time, a

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large variability across sample preparation techniques and analysis conditions, even considering only GC-MS, can be seen, which can partially explain the wide-ranging list of biomarkers and the lack of correlation among results obtained by different research groups (Table 1). Data obtained from analysis can be treated in several ways: by using the absolute value of the amount of VOCs or their ratios. The former approach can cause inconsistency of the results because it does not allow differences in the concentrations due to metabolic features to be estimated. In addition, reproducibility is limited by the sensitivity of the chromatographic system. The latter approach can take these factors into account and reduce the risk of obtaining incorrect data.

In addition to these problems, an exhaled breath sample contains exogenous compounds, which can be mistakenly attributed to biomarkers. Tumor tissue headspace analysis can provide indirect evidence of the endogenous origin of exhaled breath VOCs. Some efforts have already been made in this field [7, 25], but the results are incomparable due to dissimilar sampling techniques and analysis conditions.

In this work, a preconcentration procedure, the conditions of two-stage thermal desorption and gas chromatography with flame ionization detector (GC-FID), and GC-MS analyses were optimized to study lung VOC exhaled breath profiles of cancer patients and healthy volunteers for the identification of potential lung cancer biomarkers. Different approaches to statistical data analysis for the discrimination of lung cancer patients and healthy volunteers were investigated. Optimized conditions were also applied to the analysis of tumor tissues to verify the endogenous origin of putative biomarkers.

2. Materials and methods

2.1. Human subjects and sampling of exhaled breath

Two groups of participants took part in the study: 75 healthy volunteers and 75 lung cancer patients. The patients were recruited from Scientific Research Institute – S.V. Ochapovsky Regional Clinical Hospital No. 1, Krasnodar. Patients with other morbidities in addition to lung cancer were not included in the study. Healthy participants were defined as healthy from their annual medical examination report. The medical diagnosis of each patient was confirmed by biopsy (65 patients – endobronchial biopsy, 7 patients – transbronchial biopsy, and 3 patients – transtorachal biopsy). All the samples from lung cancer patients except 6 were collected during chemotherapy courses conducted under the following schemes: 28 patients – carboplatin AUC + paclitaxel (PC); 11 patients – etoposide + cisplatin (EP); 7 patients – cisplatin + vincristine (PV); 6 patients – carboplatin AUC + topotecan; 3 patients – etoposide + doxorubicin + cisplatin (EAP); 3 patients – rituximab, gemcitabine, oxaliplatin (GEMOX); 2 patients – cyclophosphan + doxorubicin + cisplatin (CAP); 1 patient – cisplatin + gemcitabine (PG); 1 patient – hycamtin + doxorubicin + carboplatin + zoledronic acid; 1 patient – vincristine + cyclophosphane + doxorubicin + etoposide; 1 patient – hycamtin + carboplatin; 1 patient – docetaxel + vinorelbine + resorba; 1 patient – carboplatin AUC + docetaxel + lomustine; 1 patient – carboplatin + doxorubicin + vincristine + cyclophosphane; 1 patient – carboplatin + pemetrexed; and 1 patient – paclitaxel + carboplatin AUC + doxorubicin. The remaining 6 samples were collected before the beginning of any treatment. Each participant provided information regarding their smoking status and time since last smoking. A subject was defined as a smoker if his or her last smoking was within 10 days before sampling. Informed consent was obtained from each subject at the time of enrolment. The study was approved by the local ethics committee of State budgetary healthcare institution “Research Institute—Regional Clinical Hospital № 1 named after Professor S.V. Ochapovsky” and conducted in conformity with relevant guidelines and regulations. The information about the participants is presented in Table 2.

The sampling procedure for 75 lung cancer patients and 9 medical personnel as healthy volunteers was carried out in the hospital. The exhaled breath of the remaining healthy volunteers was sampled in Kuban state university. To consider the contribution of exogenous compounds, the ambient air was sampled on the day of exhaled breath sampling. Mixed expiratory breath samples with no restriction of a particular part of breath were collected in 5 L Tedlar (Supelco, Bellefonte, PA, USA) or Mylar (EKAN, Russia) sampling bags previously cleaned by flushing with nitrogen gas. The subjects consumed food no later than 1 h before breath sampling and did not smoke for at least 2.5 h before breath sampling. Breath holding may influence the levels of exhaled volatile molecules, especially acetone [26], and therefore, the sampling procedure was conducted as follows: after a 10-min rest in a sampling room, volunteers were asked to deeply breathe, hold their breath for 10 s and breathe out into the sampling bag in a calm manner, repeating the procedure until filling it.

2.2. Human subjects and sampling of tissues

Seventeen tumor tissue samples and 1 healthy tissue located around a tumor sample were obtained during surgical resection from 17 lung cancer patients (1 sarcoma with poorly differentiated cells patient, 1 patient with adenocarcinoma metastasis in the lung and 15 adenocarcinoma patients). The samples were provided by Scientific Research Institute – S.V. Ochapovsky Regional Clinical Hospital № 1, Krasnodar. The mass of the tissue specimens was 0.1–8.8 g, and the mass of a healthy tissue sample around the tumor was 10.0 g. The samples were collected in 50-mL glass bottles (Simax, Czech) after resection and were transported to the laboratory using an isothermal biological sample medical cooler (Termo-Kont MK, Russia). Diagnosis was confirmed by histopathological examinations. Informed consent was obtained from all participants. The study was approved by the local ethics committee of State budgetary healthcare institution “Research Institute—Regional Clinical Hospital № 1 named after Professor S.V. Ochapovsky” and was conducted in conformity with relevant guidelines and regulations.

Table 1. List of putative biomarkers found by different research groups.

Putative biomarker	Sampling technique (sorbent or fiber type)	Composition of GC column	Ref.
O-toluidine, aniline	Sorbent tube (Tenax TA)	Polyethylene glycol	[5]
Ethanol, octane	Sorbent tube (Tenax TA + Carboxen 569 + Car- boxen 1000)	Divinylbenzene	[7]
Isoprene, 2-methylpentane, pentane, ethylbenzene, thrimethylbenzene, toluene, benzene, heptane, decane, styrene, octane, pentamethylheptane	SPME (CAR-PDMS)	Polydimethylsiloxane	[10]
Acetoin, n-butanol	SPME (CAR-PDMS)	Phenyl methylpolysilox-ane	[11]
Propane, 2-propenal, carbon disulfide, 2-propanol, ethylbenzene	SPME (CAR-PDMS)	Divinylbenzene	[13]
Butane, 3-methyltridecane, 7-methyltridecane, 4-methyloctane, 3-methylhexane, heptane, 2- methylhexane, pentane, 5-methyldecane	Sorption tubes (Carbo-trap C + Carbopack B)	Phenyl methylpolysilox-ane	[23]
Butane, 2-methylbutane, 4-methyloctane, propane, 2-pentanone, propanal, 2,4-dimethylheptane, propene	SPME (CAR-PDMS)	Divinylbenzene	[24]

Table 2. Participant information.

Group	Parameter	Total	Male	Female
Healthy control	Number	75	22	53
	Age, range	18–71	18–69	18–71
	Number of smokers	12	9	3
Lung cancer patient	Number	75	54	21
	Age, range	30–74	30–72	34–74
	Number of smokers	25	25	0
	Type of lung cancer			
	Adenocarcinoma	17	8	9
	Squamous cell carcinoma	13	9	4
	Non-small cell lung cancer	10	5	5
	Small cell carcinoma	33	30	3
	Pleural mesothelioma	1	1	0
	Sarcoma with poorly differentiated cells	1	1	0
	TNM (tumor, nodus, metastasis) stage			
	T1N2M0	5	3	2
	T2N0M0	4	2	2
	T2N0M1	3	1	2
	T2N1M0	4	3	1
	T2N2M0	5	3	2
	T2N2M1	3	3	0
	T3N0M0	4	3	1
	T3N1M0	3	3	0
	T3N2M0	7	6	1
	T3N2M1	4	3	1
	T4N0M1	3	2	1
	T4N1M0	4	4	0
	T4N2M0	8	5	3
	T4N2M1	8	4	4
	T4N3M0	4	4	0
	T4N3M1	6	5	1

2.3. Reagents

Benzene, toluene, acetonitrile, isoprene, butanal, pentanal, 1-methylsulfanylpropane, 1-pentanol, and n-hexane were purchased from Sigma-Aldrich, USA. Ethanol, acetone, 2-propanol, and ethyl acetate were purchased from Vekton, Russia. Ethyl ester was obtained from Medhimprom, Russia, and o-xylene, m-xylene, and p-xylene were purchased from Ecohim, Russia.

2.4. Preconcentration of VOCs in sorbent tubes and GC analysis

Sorbent tubes with the Chromosorb 106 (60/80 mesh), Tenax TA (35/60 mesh), and Porapak N (50/80 mesh) sorbents (Chromatec, Russia) and a multibed sorbent (Tenax GR, Carbopack B, Carbosieve SIII) (Chromatec, Russia) were used for VOC preconcentration. Sorbent tubes were conditioned according to the manufacturer's recommendations before the preconcentration.

Exhaled breath VOCs were transferred from the sampling bag by pumping through a sorbent tube with the help of an aspirator PV-2 (Chromatec, Russia). All exhaled breath samples were stored at room temperature and were processed within 6 h.

The preconcentration of VOCs from tissue samples was carried out in the static mode. For this purpose, a sample was transferred to a 0.5- to 1-L conical flask (Simax, Czech) closed with a silicone plug. A silicone hose with a cap at the end was passed through the plug. The tissue headspace air VOCs were transferred from the flask by pumping through the sorbent with the help of an aspirator. The air from an empty flask was sampled in parallel to estimate the influence of exogenous compounds.

Exhaled breath samples of 75 lung cancer patients and 75 healthy volunteers were analyzed using a gas chromatograph with flame ionization detection (FID) (Chromatec crystal 5000.2, Russia) combined with a thermal desorber TD2 (Chromatec, Russia). Separation of the compounds was performed on capillary columns: Agilent HP-FFAP (50 m × 0.32 mm, 0.5 μm), SGE CR-5 (30 m × 0.32 mm, 1.0 μm), SGE Equity 1701 (30 m × 0.32 mm, 0.25 μm), and Bruker CP-Porabond-Q (50 m × 0.32 mm, 0.45 μm). The acquisition of chromatographic data was performed by means of the Chromatec Analytic (Chromatec, Russia) software. A gas chromatograph (Chromatec crystal 5000.2, Russia) coupled with a quadrupole mass spectrometer (Chromatec MSD, Russia) combined with a thermal desorber TD2 (Chromatec, Russia) was used for the identification of exhaled breath VOCs of 20 lung cancer patients and 20 healthy volunteers as well as for the analysis of tissue samples. The GC was equipped with a Supelco Supel-Q PLOT (30 m × 0.32 mm) column (an analog of Bruker CP-Porabond-Q). The acquisition of chromatographic data was performed by means of the Chromatec Analytic (Chromatec, Russia) software and the mass spectrum library NIST 2017, Version 2.3 (Gatesburg, USA). The samples were analyzed under the conditions optimized for each particular column and sorbent (Table 3).

2.5. Statistical analysis

The exhaled breath VOC profiles were quantified on the basis of peak areas. Peak area was calculated as the difference between the compound peak area in the sample and ambient air. Negative values of subtraction were set to zero.

Statistical analysis was conducted only with respect to compounds occurring in more than 50 % of samples: isoprene, acetone, acetonitrile,

Table 3. TD, GC-FID and GC-MS operation modes.

	Parameter				Value
TD	Carrier gas				Nitrogen
	Valve temperature, °C				150
	Transition line temperature, °C				180
	Desorption temperature, °C	Tenax TA			250
		Multi - bed			250
		Chromosorb 106			220
		Porapak N			150
	Desorption time, min				5
	Initial trap temperature, °C				–10
	Final trap temperature, °C	Tenax TA			250
		Multi - bed			250
		Chromosorb 106			220
		Porapak N			150
	Trap heating time, min				2
GC-FID	Injector temperature, °C				250
	FID temperature, °C				250
	Split ratio				1:17
	Temperature program				
	Column	Heating rate, °C/min	Temperature, °C	Time, min	Carrier-gas flow rate, mL/min
	HP-FFAP	0	41	3	1.55
		7	180	15	
	CR-5 and Equity TM 1701	0	35	3	1.25
		7	260	0	
	CP-Porabond-Q	0	150	1	1.42
		6	220	0	
		4	270	7	
GC-MS	Carrier gas			Helium	
	Injector temperature, °C			250	
	Split ratio			1:10	
	Ion source temperature, °C			200	
	Transfer line temperature, °C			250	
	Scan mode			full	
	Scan range, amu			29–250	
	Electron impact ionization, eV			70	
	Temperature program				
	Column	Heating rate, °C/min	Temperature, °C	Time, min	Carrier-gas flow rate, mL/min
	Supelco Supel-Q PLOT	0	50	0	1.30
		10	150	0	
		6	220	7	
		4	250	0	

dimethyl sulfide, ethyl ester, butanal, 2-butanone, hexane, benzene, 2-pentanone, pentanal, 1-methylthio-propane, 1-pentanol, and toluene. The ratios of the compound peak areas to the main ones occurring in almost all the samples (acetone, isoprene, and acetonitrile) were also considered.

Statistical analysis was performed using StatSoft STATISTICA (version 10). The distribution of normality was estimated by using the Kolmogorov–Smirnov test. Because the distribution data were not normal, the nonparametric Spearman's rank correlation test with a significance level of $p = 0.05$ was applied to evaluate the correlation coefficient to determine the strength of the relationship between the peak areas (ratios) and disease. The peak areas and ratios, the correlation coefficients of which were statistically significant excluding duplicative ratios, were applied to build the diagnostic models using neural networks.

Multilayer perceptron neural networks with one hidden layer were used to create the diagnostic models. One thousand different neural network topologies were tested for peak areas, and 1000 topologies were tested for ratios. The best neural networks were chosen for the peak areas and ratios. The input value of one model represented the peak areas of 8 compounds, and in the case of the other model – 8 ratios. The hidden layer of each neural network consisted of 6 neurons, and the output layer contained 2 neurons, which determined whether the input data belonged to the healthy or lung cancer group. The Broyden – Fletcher – Goldfarb – Shanno algorithm was applied to train the neural networks. The activation function of the hidden layer was sigmoid. Softmax was the output layer activation function.

3. Results

3.1. Optimization of preconcentration and analysis procedures

Preconcentration of analytes in sorbent tubes with subsequent two-stage thermal desorption followed by gas chromatographic determination was applied to identify lung cancer biomarkers. Exhaled breath is a complex object consisting of a wide range of compounds. Analysis of exhaled breath necessitates the selection and optimization of the preconcentration and analysis conditions.

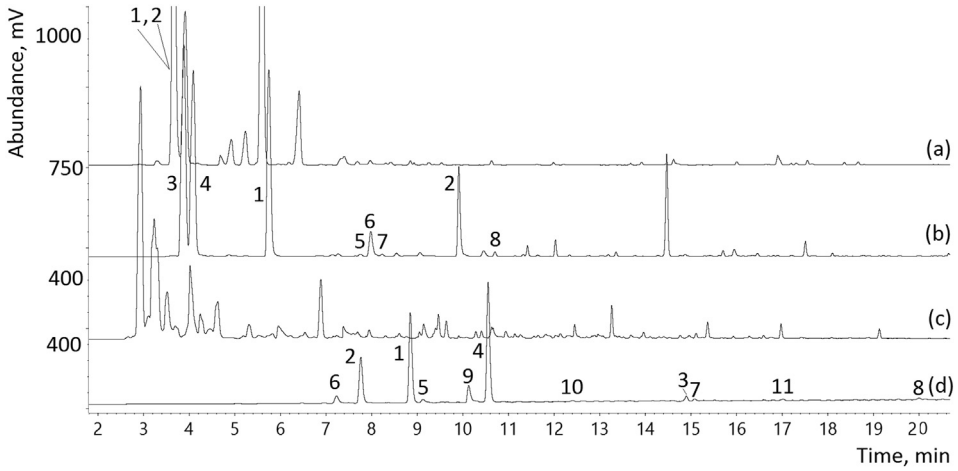

Figure 1. Chromatograms of an exhaled air sample preconcentrated in a sorbent tube with the Tenax TA sorbent using various chromatographic columns (a – CR-5, b – HP FFAP, c – Equity TM 1701, d – CP-Porabond-Q; 1 – acetone, 2 – acetonitrile, 3 – hexane, 4 – isoprene, 5 – 2-porpanol, 6 – ethanol, 7 – benzene, 8 – toluene, 9 – ethyl ester, 10 – 2-butanone, 11 – pentanal).

A study of the efficiency and selectivity of the applied chromatographic columns with respect to the components of exhaled breath using GC-FID was conducted. The following chromatographic columns with different physicochemical properties were studied: a midpolar column, Supelco Equity TM 1701; a nonpolar column, SGE CR5; a polar column, Agilent HP-FFAP; and a PLOT column, Varian CP-Porabond-Q. A 5-L sample of exhaled breath was pumped through a Tenax TA sorbent tube at a flow rate of 200 mL/min.

The Equity TM 1701 column was not sufficiently efficient to separate endogenous low-molecular-weight compounds, and therefore, the use of this column for the analysis of exhaled breath was impractical. The CR-5 nonpolar column was not able to separate small polar compounds such as acetone and acetonitrile, which are the main components of exhaled breath and therefore valuable analytes for further study. The opposite efficiency was observed in the case of the HP FFAP column due to its inability to separate such substances as alkanes and alkenes, which can also be endogenous. The use of this column may lead to the loss of significant information. The CP-Porabond-Q column has heightened retention parameters for various VOCs and is able to separate volatile light hydrocarbons and solvents. As a result, this column is the most suitable for the analysis of exhaled breath because it can separate the widest range of VOCs (Figure 1).

The sorption properties of four types of sorbents, namely, Porapak N, Tenax TA, Chromosorb 106 and a multibed sorbent, were studied for the selection of the most efficient to preconcentrate exhaled breath VOCs. A 1-L air volume from the same sample of exhaled breath with a volume of 5 L was preconcentrated by using each sorbent at a flow rate of 50 mL/min and analyzed by means of a CP-Porabond-Q column.

The Porapak N sorbent was the least suitable for the exhaled breath VOCs. The use of the multibed sorbent in the case of preconcentrating a larger sample volume is questionable because some peak splitting and shape distortion were detected, which can be explained by the differences in the desorption temperatures for the sorbents contained in the multibed sorbent tubes. The capacities of the Chromosorb 106 and Tenax TA sorbents were comparable in relation to the VOCs to be determined, but for the Chromosorb 106 sorbent, a system peak was observed in the chromatograms. This peak can overlap the peaks of other VOCs present in exhaled breath and have the same retention time. The Tenax TA sorbent was selected for the preconcentration because it was the most stable (Figure 2).

To provide the preconcentration of VOCs with no breakthrough, two sorbent tubes were constructed in two beds to the sampling bag by use of a silicone tube, and the sample volume passed through the tubes at a flow rate of 50 mL/min was varied. All the analytes, excluding ethanol, were

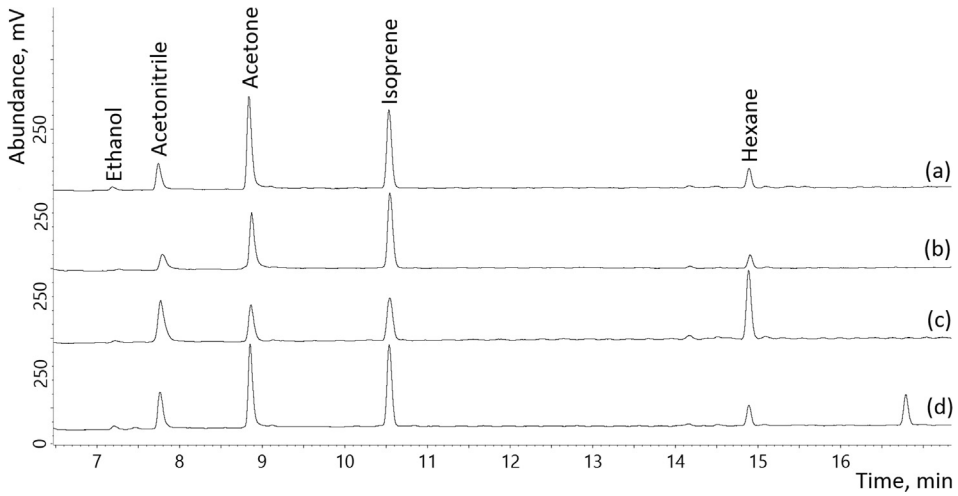

Figure 2. Chromatograms of exhaled air samples obtained on the CP-Porabond-Q column using sorbent tubes with the following sorbents: a – Tenax TA, b – Porapak N, c – a combined sorbent, d – Chromosorb 106.

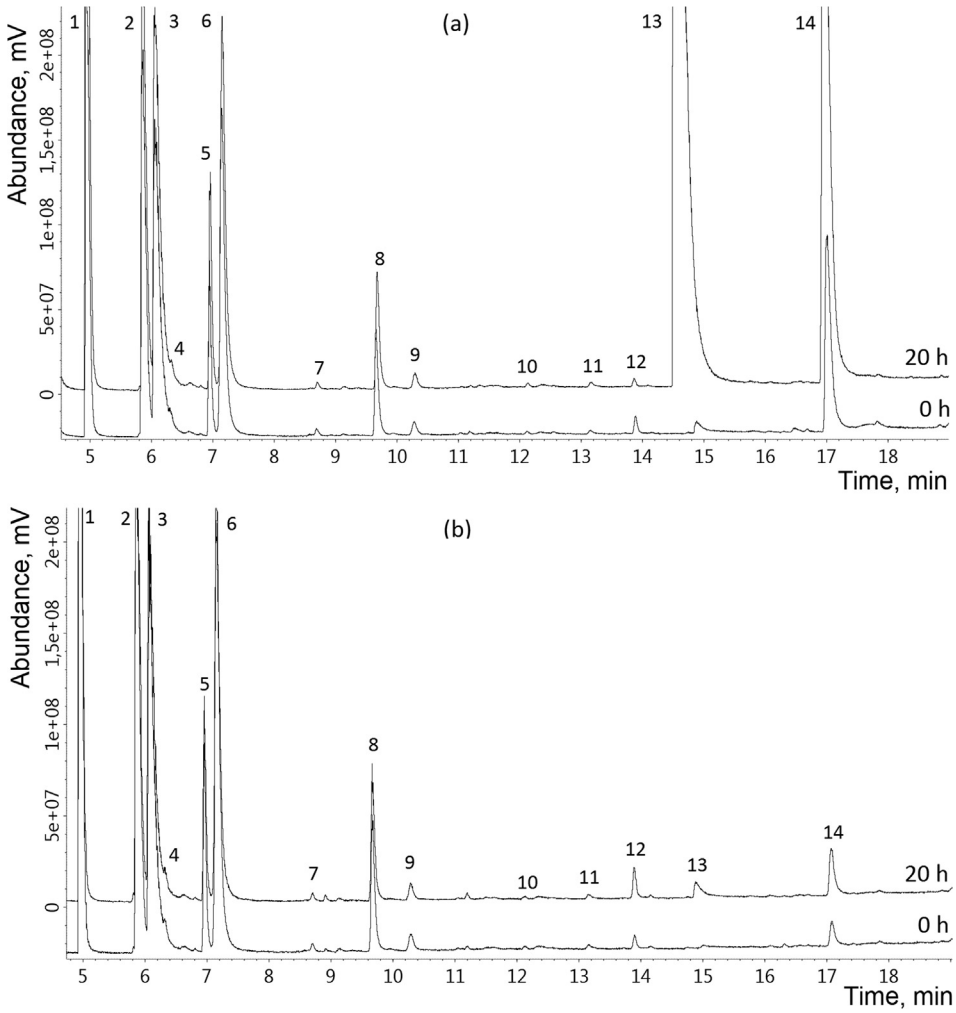

Figure 3. GC-MS chromatograms of exhaled breath sampled by using a – a Tedlar sampling bag or b – a Mylar sampling bag, with preconcentration immediately after filling and 20 h later (1 – acetonitrile, 2 – acetone, 3 – isopropanol, 4 – dimethyl sulfide, 5 – ethyl ester, 6 – isoprene, 7 – 2-butanone, 8 – hexane, 9 – benzene, 10 – heptane, 11 – toluene, 12 – hexanal, 13 – N,N-dimethylformamide, 14 – phenol).

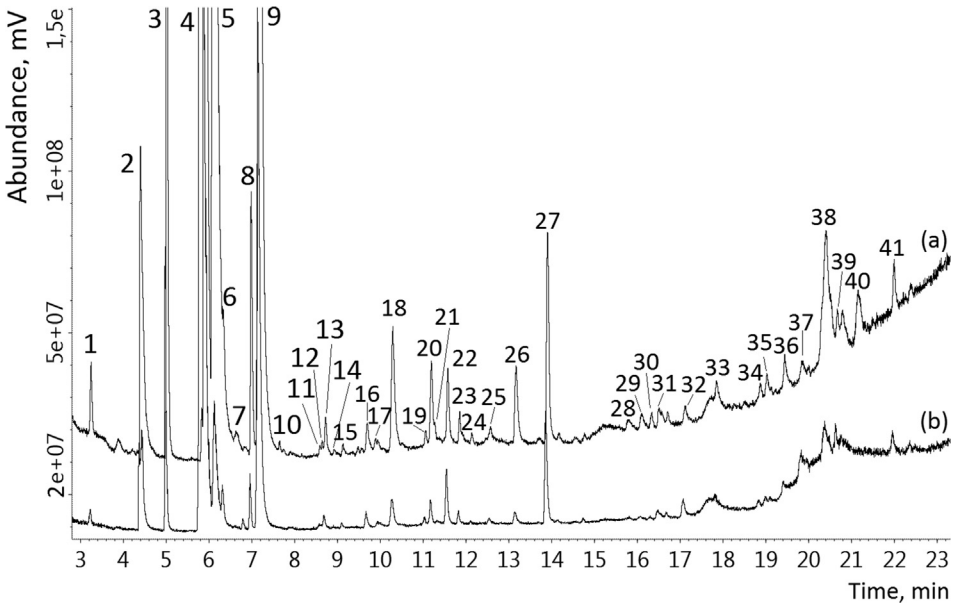

Figure 4. GC-MS chromatograms of exhaled breath samples from: a – a lung cancer patient, b – a healthy volunteer (1 – acetaldehyde, 2 – ethanol, 3 – acetonitrile, 4 – acetone, 5 – 2-propanol, 6 – dimethyl sulfide, 7 – methyl acetate, 8 – ethyl ester, 9 – isoprene, 10–1,4-penthadiene, 11 – butanal, 12–2,3-butandione, 13 – 2-butanone, 14 – dimethyl carbonate, 15 – ethyl acetate, 16 – hexane, 17 – 3-methyl-3-penten-1-yne, 18 – benzene, 19 – 2-pentanone, 20 – pentanal, 21 – furane, 2,5-dimethyl, 22 – 1-methylthio-propane, 23 – 1-methylthio-propene, 24 – heptane, 25 – 1-pentanol, 26 – toluene, 27 – hexanal, 28 – ethylbenzene, 29 – m-xylene + p-xylene, 30 – 3-heptanone, 31 – 2-heptanone, 32– phenol, 33 – benzaldehyde, 34 –6-methyl-5-hepten-2-one, 35 – benzene, 1,4-dichloro-, 36 – octanal, 37 – 2-ethyl-1-hexanol, 38–1,2-nonadiene, 39–1,1-(1,4-phenylene)bis-ethanone, 40 – nonanal).

preconcentrated with no breakthrough upon pumping a 0.5-L sample. An attempt to increase the sample flow rate per tube to 100 and 200 mL/min was made to accelerate the preconcentration procedure, and it did not affect the breakthrough, which is why a flow rate of 200 mL/min was chosen. The optimization stage has previously been described in detail [28].

3.2. The comparative analysis of different sampling bag types

Mylar and Tedlar sampling bags are the most frequently used for exhaled breath analysis. The possibility of sample pollution by compounds from the sampling bag material was studied. For this purpose, the following experiment was conducted: The Mylar and Tedlar bags were filled with nitrogen. The air from the bags was sampled immediately after filling and 2 h later. The samples were analyzed using GC-MS. The intensities of phenol and N,N-dimethylacetamide increased in both sampling bag types after 2 h of storage, but the intensities of these compounds were higher in the case of the Tedlar sampling bag.

Exhaled air sample degradation over time using different sampling bag types was studied. The Mylar and Tedlar bags were filled by a volunteer. The sample was preconcentrated immediately after sampling and 2 and 20 h later. The intensities of the main compounds were shown to not be changed during this period of time, but increases in the intensities of phenol and N,N-dimethylacetamide were observed in both sampling bag types. Note that both the Mylar and Tedlar sampling bags were able to retain analytes for over 20 h, but the pollution of a sample by compounds from the latter sampling bag itself was more substantial in comparison to the former (Figure 3).

3.3. GC-MS and GC-FID analysis of exhaled breath

The GS-MS method was applied to identify the compounds in the exhaled breath VOC profile. Exhaled breath of 20 lung cancer patients and 20 healthy volunteers was analyzed by this method. Typical GC-MS chromatograms of exhaled breath samples from a lung cancer patient and a healthy volunteer are shown in Figure 4.

To identify the putative lung cancer biomarkers, the exhaled breath of 75 lung cancer patients and 75 healthy volunteers was analyzed using GC-FID. Typical GC-FID chromatograms of exhaled breath samples from a lung cancer patient and a healthy volunteer are shown in Figure 5.

Nonparametric Spearman's rank correlation test was applied to evaluate the correlation coefficient to determine the strength of the relationship among the peak areas, their ratios and disease. The peak areas and ratios, the correlation coefficients of which were statistically significant excluding duplicative ratios (Table 4), were applied to build the diagnostic models using neural networks.

Neural networks were built for the peak areas and their ratios. The input value of one model represented the peak areas of 8 compounds: acetonitrile, acetone, butanal, hexane, benzene, pentanal, toluene, and 2-butanone; in the case of the other model, 8 ratios: toluene/acetonitrile, 1-methylthio-propane/acetonitrile, 1-pentanol/acetonitrile, hexane/acetonitrile, butanal/isoprene, pentanal/isoprene, 2-butanone/isoprene, and benzene/acetone. The dataset was divided into three datasets: training (104 samples), control (20 samples) and test (26 samples). Based on the established models, the sensitivity, specificity and overall accuracy of these diagnostic models based on the peak areas (ratios) were 98.1 % (92.3 %), 88.4% (92.3 %) and 93.3 % (92.3 %) on training data; 90.0 % (100.0 %), 90.0 % (90.0 %) and 90.0 % (95.0 %) on control data (20 samples); and 100.0 % (100.0 %), 100.0 % (92.3 %) and 100.0 % (96.2 %) on test data (26 samples). The model based on peak areas correctly classified all lung cancer patients and healthy volunteers. The model based on peak area ratios mistakenly identified one healthy volunteer as a lung cancer patient.

3.4. Evaluation of the influence of different conditions on the extraction effectiveness of VOCs from tissue samples

While studying the tissue VOC profiles, in most cases, tissues are held at a temperature close to body temperature (37 °C [7, 27] or 30 °C [29]) before preconcentration. However, an increase in temperature can enhance the extraction efficiency because the boiling points of many VOCs are higher than 37 °C. Alternately, the increase in temperature can lead to matrix degradation and, subsequently, the extraction of compounds nonspecific for normal body functioning. Taking the above-mentioned factors into account, sarcoma with poorly differentiated cells type of tumor tissue №1 (8.8 g, Table 5) and healthy tissue around this tumor (10.0 g) samples were separated into two parts. The first parts of the tumor and healthy tissue samples were transferred to separate 1-L flasks and held at 37 °C for 10 min, and the other parts were held at 50 °C for 10 min. The headspace above the tissue (0.5 L) was preconcentrated at a flow rate of 200 mL/min after heating. The decision to increase the temperature to 50 °C, on the one hand, was caused by the potential increase in the extraction of VOCs from a sample; on the other hand, there was a possibility that matrix degradation at this temperature would not lead to the appearance of VOCs not typical for the normal functioning of the body.

As shown in Figure 6, the intensities of most VOCs were greater while keeping the temperature at 50 °C, which is why further research was carried out in this manner.

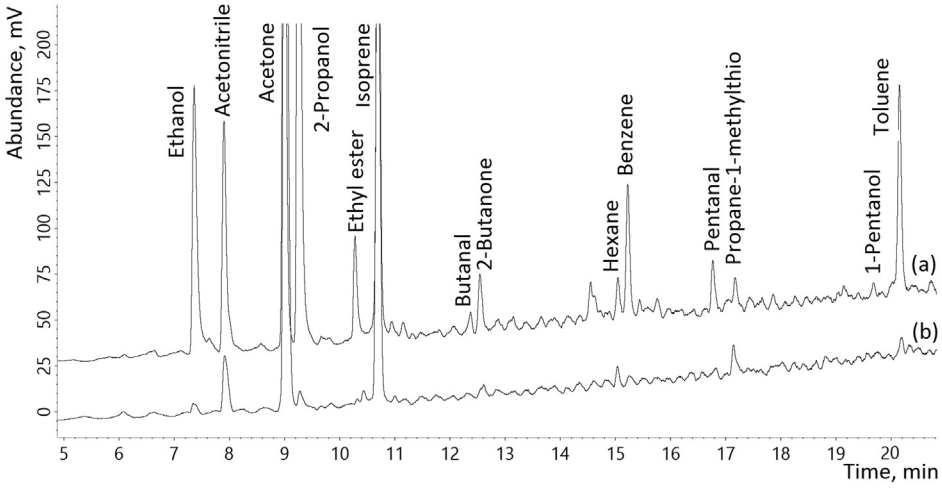

Figure 5. GC-FID chromatograms of exhaled breath samples from a – a lung cancer patient and b – a healthy volunteer.

Table 4. Compounds and ratios considered for the creation of diagnostic models (Bold compounds and ratios were selected for the creation of the diagnostic models).

Compound	Correlation coefficient	p-value
Acetonitrile	**0.448496**	**0.000000**
Isoprene	**0.192608**	**0.018206**
Butanal	**0.316623**	**0.000079**
2-Butanone	**0.222787**	**0.006139**
Hexane	**0.169770**	**0.037805**
Benzene	**0.346182**	**0.000014**
Pentanal	**-0.254887**	**0.001645**
Toluene	**0.352271**	**0.000010**
Acetone	0.071285	0.385249
Dimethyl sulfide	-0.085348	0.329368
Ethyl ester	0.035052	0.671029
2-pentanone	0,130203	0,116149
1-methylthio-propane	-0.068330	0.414704
1-pentanol	-0.065624	0.449947
Toluene/Acetonitrile	**0.240154**	**0.003075**
Toluene/Acetone	0.191099	0.022186
Toluene/Isoprene	0.204875	0.014559
1-Methylthio-propane/Acetonitrile	**-0.235910**	**0.003658**
1-methylthio-propane/Acetone	-0.086865	0.299546
1-methylthio-propane/Isoprene	-0.119538	0.153202
1-Pentanol/Acetonitrile	**-0.175377**	**0.031024**
1-pentanol/Acetone	-0.072789	0.401921
1-pentanol/Isoprene	-0.103077	0.234930
Hexane/Acetonitrile	**-0.272251**	**0.000751**
Hexane/Acetone	0.114926	0.161477
Hexane/Isoprene	0.054536	0.507065
Butanal/Isoprene	**0.284360**	**0.000421**
Butanal/Acetone	0.269258	0.003211
Butanal/Acetonitrile	0.191003	0.036632
Pentanal/Isoprene	**-0.267306**	**0.000944**
Pentanal/Acetone	-0.240296	0.003645
Pentanal/Acetonitrile	-0.206439	0.012568
2-Butanone/Isoprene	**0.170724**	**0.036726**
2-Butanone/Acetonitrile	0.012936	0.880489
2-Butanone/Acetone	0,137283	0,107265
Benzene/Acetone	**0.367153**	**0.000004**
Benzene/Isoprene	0.357303	0.000024
Benzene/Acetonitrile	0.278503	0.000993
Ethyl ester/Acetonitrile	-0,131106	0,132713
Ethyl ester/Acetone	0.035978	0.662828
Ethyl ester/Isoprene	-0.040920	0.619787
Dimethyl sulfide/Acetonitrile	-0.159535	0.068023
Dimethyl sulfide/Acetone	-0.072053	0.410417
Dimethyl sulfide/Isoprene	-0.094539	0.279861
2-pentanone/Acetonitrile	0.002641	0.976011
2-pentanone/Acetone	0.142191	0.085503
2-pentanone/Isoprene	0.101921	0.217628

A decrease in the flow rate was observed when 0.5 L of headspace of 1-L flasks was passed through a sorbent tube at a flow rate of 200 mL/min, which resulted in the failure of aspirator operation by increasing air vacuuming in the flask during sampling. Thus, 0.3 L of headspace above tissue samples №2–5 (0.8, 0.9, 0.4, and 1.1 g, respectively; Table 5) was preconcentrated from 1-L flasks. The intensities of most of the sample VOCs were almost equal to the intensities of an ambient air sample excluding some of them.

To increase the efficiency of the extraction of VOCs from a sample, the heating time at 50 °C was increased to 15 min (sample №6; 0.1 g; Table 5) and 30 min (sample №7; 0.3 g; Table 5). In the case of 30-min heating, a significant increase in the intensities of compounds such as isopropanol and sevoflurane was observed compared to the samples analyzed before. The presence of isopropanol can be caused by the use of different disinfectants in hospitals [18]. Sevoflurane is an anesthetic used in surgery [30]. Taking into account that the mass of the sample is relatively small (0.3 g), detector congestion is possible with further increases in isopropanol and sevoflurane intensities induced by the use of a long heating time for samples with larger masses. Thus, further investigations were conducted by preconcentrating the samples after heating for 15 min.

Table 5. VOCs identified in tissue samples.

Compound \ Sample N№	Sarcoma with poorly differentiated cells	Adenocarcinoma metastasis	Adenocarcinoma															%
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
Acetonitrile	-	+	+	-	-	+	-	-	-	+	+	-	-	-	+	+	+	8
Acetone	+	+	+	-	+	+	+	-	+	-	+	-	+	-	+	-	+	11
Isopropyl alcohol	+	+	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	14
Sevoflurane	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	17
Isoprene	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
1-Propanol	+	-	-	+	-	-	-	+	-	-	-	-	-	+	-	+	+	6
2-Methyl-2-propanol	-	-	-	-	-	+	+	-	+	-	+	+	-	-	-	-	-	5
Hexafluoroisopropyl alcohol	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	16
Ethyl ether	+	+	-	+	+	-	-	-	-	+	-	-	-	-	+	-	-	6
Methacrolein	+	+	-	-	-	+	-	-	+	-	+	-	-	-	-	-	+	6
Methyl vinyl ketone	+	+	-	-	-	+	-	-	+	-	+	-	-	-	-	-	+	6
Butanal	+	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	4
2-Butanone	+	-	-	-	-	+	+	+	+	-	+	+	-	-	-	-	+	8
Dimethyl carbonate	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	2
Ethyl acetate	+	-	-	-	-	+	+	+	+	-	+	+	-	-	-	+	+	9
Hexane	-	+	-	-	-	-	+	-	-	+	+	-	-	-	-	+	+	6
1-Butanol	+	+	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-	5
Benzene	+	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	4
2-Bromo-hexane	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	2
2-Pentanone	+	-	-	-	+	-	-	-	+	-	-	-	+	-	+	-	+	6
Pentanal	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	5
Heptane	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	2
1-Pentanol	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	3
toluene	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	3
2-hexanone	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	2
Hexanal	+	+	-	-	+	-	-	-	-	-	+	-	+	-	+	-	+	7
Butyl acetate	+	+	+	-	-	-	+	+	-	-	-	-	-	-	+	-	-	6
o-xylene	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	2
m-xylene + *p*-xylene	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	3
3- Heptanone	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	3
2-Heptanone	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	3
Heptanal	+	-	-	-	-	-	+	-	-	-	-	-	+	-	+	-	-	4

An attempt to increase the efficiency of VOC extraction from a sample by decreasing the flask volume to 0.75 L (samples N№ 8–10; 1.0, 1.5, and 0.2 g, respectively; Table 5) was also made. Failure of the aspirator was not observed in this case, but the results were not improved.

The decrease in the flow rate can allow a larger volume of the tissue headspace to be pumped without failure of the aspirator operation. Additionally, under such conditions, the sampling procedure takes a long time, allowing for the additional extraction of analytes from the sample in the case of tissue headspace supersaturation by the analytes. Samples N№ 11–15 (1.4, 1.6, 7.6, 4.5, and 2.8 g, respectively; Table 5) were analyzed after 15 min of heating at 50 °C in the 0.5-L conical flask and preconcentrating 0.375 L of the tissue headspace at a flow rate of 50 mL/min. Samples N№ 11, 13, and 15 contained more VOCs than samples N№ 12 and 14.

Samples N№ 16 and 17 (4.5 and 2.8 g, respectively; Table 5) were cut into small pieces in an attempt to increase the extraction of analytes: sample N№ 16 contained more VOCs than sample N№ 17.

Mainly, the intensities of VOCs in the tissue samples were lower than in the case of exhaled breath, and many VOCs observed in the exhaled breath samples were absent in the tissue samples.

4. Discussion

The search for lung cancer biomarkers in exhaled breath has been conducted by different research groups, but the conditions of analysis vary among them. It was essential to investigate different approaches for the sampling, preconcentration and analysis procedures to apply those that provided the determination of the widest list of VOCs that might be potential biomarkers. The optimized conditions of GC-FID and GC-MS detection were applied to the analysis of exhaled breath samples of lung cancer patients and healthy volunteers. GC-MS was used to identify compounds occurring in exhaled breath profiles. None of the compounds corresponded to only one of the analyzed groups (lung cancer patients or healthy volunteers). Thus, it is impossible to conduct lung cancer diagnosis by stating the existence of some unique biomarkers. However, the differences in VOC concentrations can be significant. GC-FID is more suitable for routine analysis because its exploitation parameters are easier in regard to the implementation of this type of analysis in clinical practice. At the same time, the sensitivity of this method allows it to obtain information regarding macro compounds present in exhaled breath. Statistical analysis was conducted in relation to not only VOC peak areas but also their ratios. The performances of the models were high for both VOC peak areas and their ratios, but the use of the peak area ratios allows it to identify certain general trends while leveling the influence of individual metabolism features varying from one person to another.

To date, the biochemical pathways of many VOCs observed in exhaled breath generation have still not been studied sufficiently. An attempt to investigate whether the alterations in VOC levels are connected with tumor activity was conducted by the determination of VOCs excreted by

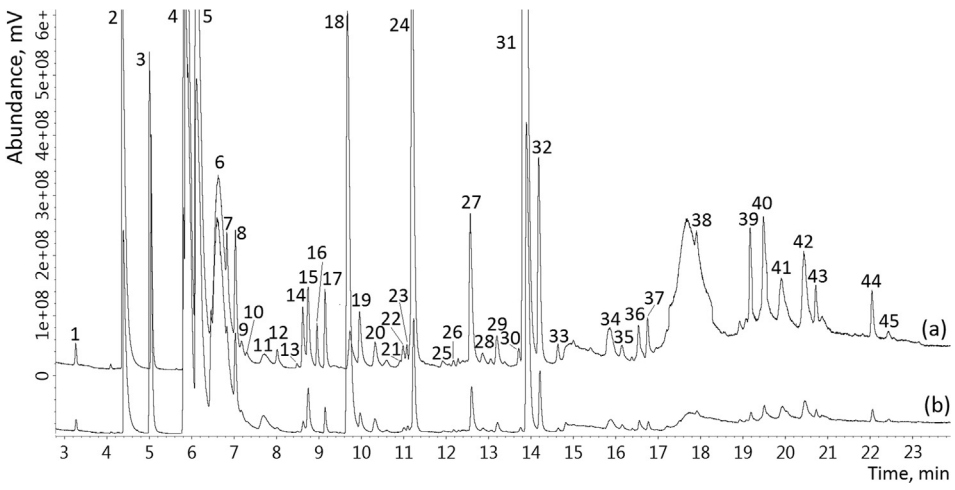

Figure 6. GC-MS chromatograms of sarcoma with poorly differentiated cells tissue sample after a 10-min heating at 37 (a) and 50 °C (b). (1 – acetaldehyde, 2 – ethanol, 3 – acetonitrile, 4 – acetone, 5 – 2-propanol, 6 – sevoflurane, 7 – methyl acetate, 8 – ethyl ester, 9 – pentane, 10 – 1-propanol, 11 – hexafluoroisopropyl alcohol, 12 – methacrolein, 13 – methyl vinyl ketone, 14 – butanal, 15 – 2-butanone, 16 – dimethyl carbonate, 17 – ethyl acetate, 18 – hexane, 19 – 1-butanol, 20 – benzene, 21 – 1-pentene-3-one, 22 – 1-pentene-3-ol, 23 – 2-pentanone, 24 – pentanal, 25 – 3-methyl-3-buten-1-ol, 26 – heptane, 27 – 1-pentanol, 28 – (E)-2-pentenal, 29 – toluene, 30 – 2-methyl-pentanal, 31 – hexanal, 32 – butyl acetate, 33 – ethyl formate, 34 – cyclohexanone + o-xylene, 35 – m-xylene + p-xylene, 36 – 2-heptanone, 37 – heptanal, 38 – benzaldehyde, 39 – 2-pentyl-furan, 40 – octanal, 41 – 2-ethyl-1-hexanol, 42 – limonene, 43–1,2-nonandiene, 44 – nonanal, 45 – undecane).

tumor tissues. The conditions of the preconcentration procedure were varied to enhance the level of extracted VOCs. In general, it is difficult to assess whether the attempts to improve the preconcentration conditions affected the result because they were applied to different samples. Variation of sample masses in a wide range might also significantly influence the results.

VOC profiles of tumor tissue and healthy tissue around the tumor samples in the case of sarcoma with poorly differentiated cells had identical qualitative and quantitative compositions excluding dimethyl carbonate, the intensity of which was higher in the case of tumor tissue under both conditions: 37 and 50 °C. This relative similarity can be caused by several reasons. First, the tissue around the tumor was considered to be healthy, but there was a possibility that it contained tumor cells. Second, both samples were collected immediately after a surgery and were stored in one flask until they were transferred to the cooler, which could lead to the mutual penetration of VOCs from the samples and the homogenization of VOC profiles. Third, tissue removal from a living organism and the subsequent ending of all metabolic processes can also lead to the homogenization of VOC profiles from tumor and healthy tissues. The concentration of dimethyl carbonate was higher in the tumor tissue sample. Dimethyl carbonate can be found in hospital ambient air. In the case of exhaled breath, it was observed only at intensities lower than in ambient air.

The intensities of VOCs in tissue samples were lower than in the case of exhaled breath, and many VOCs observed in the exhaled breath samples were absent in the tissue samples. The most frequently occurring VOCs in the samples of exhaled breath and tissues and the percentage of samples in which these VOCs were observed are summarized in Table 6.

The peak areas of acetone and isoprene were the highest for each exhaled breath sample. Acetone is a product of acetyl-Coa decarboxylation. Isoprene is the main molecule of cholesterol synthesis [31]. Based on a literature survey, the alterations in acetone and isoprene concentrations caused by lung cancer are ambiguous: in one case, the concentrations of these compounds were decreased in lung cancer patients [18], but they were increased in other cases [21]. Acetone was observed in tissue samples [7, 27], but the difference between the intensity of this compound intensity in healthy and tumor tissue samples was not observed as in the case of exhaled breath. According to our experimental data, the exhaled breath isoprene peak areas were significantly higher in the case of lung cancer patients, but the correlation between acetone peak areas and disease status was not significant. The intensity of acetone was higher than in ambient air in 59 % of tissue samples, but isoprene was not found in tissue samples.

The presence of acetonitrile, benzene and toluene in exhaled breath is mainly associated with smoking [10, 23, 31, 33, 34]; nonetheless, some of these compounds have been noted to be lung cancer biomarkers in the literature [19, 35], and toluene was one of the biomarkers found in tumor tissue samples [27]. As stated in this research, almost all of the exhaled breath samples contained acetonitrile. Benzene and toluene occurred in more than 50 % of samples, not only including smokers. Additionally, the number of smokers among the participants in this study was lower than the number of nonsmoking volunteers. These facts show that the appearance of these compounds in exhaled breath may be caused by other sources. The intensities of benzene and toluene in the majority of tissue samples were found at the ambient air level. However, acetonitrile was found in 7 tissue samples of 17. The endogenous origin evidence of this compound still has not been established, but its metabolism in the body is slow, which is why it excretes through exhaled breath or urine [36]. The presence of acetonitrile in tumor tissues and its increased concentrations in exhaled breath can be explained by the ability of this compound to accumulate in tumor tissues and subsequently excrete.

Saturated hydrocarbons are generated from the peroxidation of polyunsaturated fatty acids by free radicals associated with oxidative stress [37]. Alcohols, aldehydes and ketones are derived from the further oxidation of saturated hydrocarbons. According to data obtained in this work, among saturated hydrocarbons, a statistically significant correlation was observed only for hexane, which is in agreement with other works [12], but hexane was later listed as a pollutant [18].

Some alcohols, particularly ethanol, are listed as lung cancer biomarkers in prior research [7, 38]. In this study, ethanol was found to be weakly retained on a Tenax TA sorbent. Therefore, it cannot be considered as a potential biomarker. Additionally, ethanol concentration in exhaled breath can be caused not only by endogenous origin [18].

Isopropanol can occur in samples from disinfectants used in hospitals. Accordingly, the correct estimation of the isopropanol concentration in ambient air is required. To evaluate the exogenous isopropanol concentration, the sampling procedure should be conducted in the area of its exposure. To investigate the possible exogenous origin of this compound, the exhaled breath of 9 volunteers from the medical stuff was sampled directly in the hospital under the same conditions as the 75 lung cancer patients. The peak areas of isopropanol were found to be higher in the case of sampling in the hospital for both lung cancer patients and medical stuff. However, the peak areas of isopropanol in ambient air were higher than in the exhaled breath of medical stuff as well as lung cancer patients. The subtraction of the ambient air concentration from the exhaled breath led to setting the isopropanol concentrations to zero in all of the samples taken in the hospital. Isopropanol is one of the VOCs present in disinfectants applied in hospitals, which is why its peak area was high in ambient air samples (Figure 7). Several researchers have considered isopropanol to be a lung cancer biomarker [17, 38], but nevertheless, the correct estimation

Table 6. Frequency of VOCs occurring in exhaled breath and tumor tissue samples (%).

Detector	FID	FID	MSD	MSD	MSD
Compound	Lung cancer	Healthy	Lung cancer	Healthy	Tumor tissue
Number	75	75	20	20	17
Acetonitrile	100	97	100	100	41
Acetone	100	100	100	100	59
Isoprene	100	100	100	100	0
Butanal	55	29	70	50	18
2-Butanone	68	49	95	80	47
Hexane	91	88	100	85	29
Benzene	79	43	60	30	24
Pentanal	67	83	95	90	24
1-Pentanol	56	49	90	70	12
Toluene	76	53	85	50	18
1-Methylthio-propane	59	73	95	60	0
Dimethyl sulfide	48	53	100	100	0
2-Pentanone	81	67	90	90	35
Ethyl ether	80	84	90	100	29
Ethyl acetate	39	43	30	60	47
Ethyl benzene	-	-	65	30	0
m-xylene + p-xylene	-	-	60	30	12
o-xylene	-	-	55	60	12
2-Heptanone	-	-	85	85	12
2,3-Butandione	-	-	90	75	29
Hexanal	-	-	100	100	29
Dimethyl carbonate	-	-	60	20	12
Limonene	-	-	75	55	18

of ambient air influence allows for confirmation that the appearance of this compound is mainly caused by exogenous factors, and evaluation of isopropanol endogenous contribution in exhaled breath is difficult.

Previous studies have noted the increase in the concentrations of aldehydes, such as butanal [39, 40], pentanal [18, 32], octanal, and nonanal [39, 41], and ketones, such as 2-butanone [18, 42] and 2-pentanone [24], in lung cancer patients' exhaled breath, and the concentrations of hexanal and heptanal were found to be elevated in tumor tissue samples compared to healthy tissues [27]. Exhaled breath can contain aldehydes due to the peroxidation of polyunsaturated fatty acids, but some aldehydes, such as butanal, can also be ambient air pollutants [41]. It is impossible to quantify formaldehyde and acetaldehyde by applying the analysis conditions optimized in the current paper. However, the exogenous origin of these compounds has been noted in other studies, which questioned the value of their determination [38, 41]. Statistically significant differences between the peak areas of butanal, pentanal and 2-butanone in lung cancer patients and healthy volunteers were observed in this research.

Dimethyl sulfide and 1-methylthio-propane were found to be among the compounds able to distinguish lung cancer patients from healthy volunteers [32]. In our work, these VOCs were found in many exhaled breath samples, but they were inefficient in classifying lung cancer patients and healthy volunteers. Tissue samples did not contain these compounds.

Ethyl ester is referred to as an exogenous compound that exists in hospitals [18]. In our investigation, ethyl ester and ethyl acetate were found in both exhaled breath and tumor tissue samples, but the correlation between their peak areas and disease status was not significant.

The source of ethyl benzene and xylenes in exhaled breath can be ambient air pollution [12] or cigarette smoke [43], but xylene had been

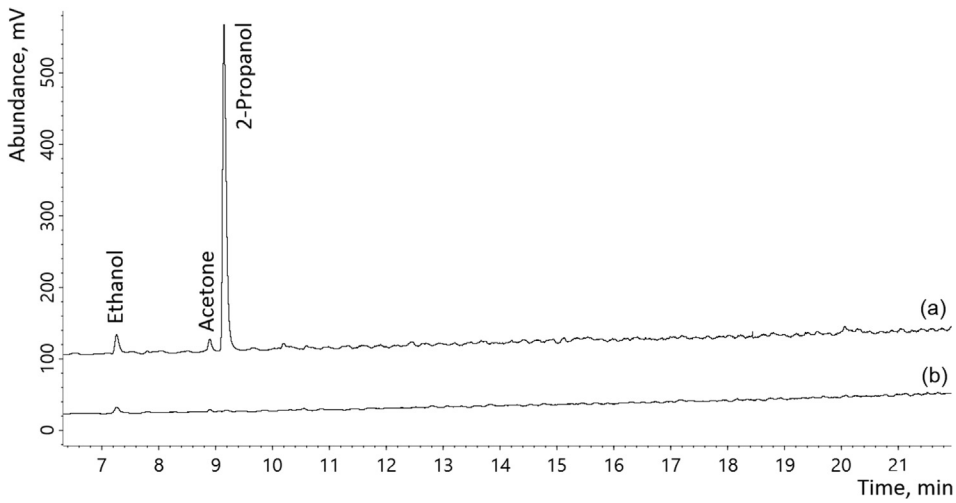

Figure 7. GC-FID chromatograms of ambient air samples obtained from a – a hospital and b – a solvent-free room.

applied for discriminating between patients with lung diseases and healthy volunteers [44]. In our research, the concentrations of these VOCs were observed only by using GC-MS because their abundance was extremely low for detection by GC-FID. Phenol was detected in exhaled breath samples by excretion from sampling bag material [45], which was in agreement with our findings.

Limonene can occur in exhaled breath due to cleaning products, cosmetics and food [18]. 2-Heptanone was also found in many samples during GC-MS analysis. It seems reasonable to estimate the correlation between the concentration of 2-heptanone and disease status after analyzing more exhaled breath samples.

Far from all of the VOCs identified in exhaled breath were found in tumor tissue samples. The question of whether concentration alterations of some VOCs in exhaled breath are caused by the vital activity of a tumor or not is left open. Alternately, biochemical pathways of the generation of many VOCs have not yet been fully understood, and subsequently, a tumor can influence metabolism, resulting in alterations in some VOC concentrations observed in exhaled breath. It seems more pertinent to apply VOC profiles instead of an individual VOC. For this purpose, it is appropriate to use statistical modeling. To ensure the effectiveness and accuracy of the obtained results, it is necessary to apply this approach to a larger cohort of people, which will allow the employment of breath tests in clinical practice. It will also be valuable to implement alternative methods to study the biochemical pathways of VOCs observed in exhaled breath.

Declarations

Author contribution statement

Elina Gashimova, Azamat Temerdashev, Dmitry Perunov: Performed the experiments; Analyzed and interpreted the data.

Vladimir Porkhanov, Igor Polyakov: Conceived and designed the experiments.

Alice Azaryan, Ekaterina Dmitrieva: Performed the experiments; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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