Optimization of a breath analysis methodology to potentially diagnose transplanted kidney rejection

Nicoletta De Vietro
University of Bari “Aldo Moro”
Antonella Maria Aresta (antonellamaria.aresta@uniba.it)
University of Bari “Aldo Moro”
Arcangelo Picciariello
University of Bari “Aldo Moro”
Donato Francesco Altomare
University of Bari “Aldo Moro”
Alessia Digilio
University of Bari “Aldo Moro”
Jolanda Palmisani
University of Bari “Aldo Moro”
Gianluigi De Gennaro
University of Bari “Aldo Moro”
Carlo Zambonin
University of Bari “Aldo Moro”

Article

Keywords: ReCIVA®, GC-MS, VOCs, CKD, human breath

Posted Date: September 8th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2033197/v1

Abstract

Chronic kidney disease may result in end stage renal disorder and increased mortality rate. Up to date, kidney transplantation represents the only definitive treatment to restore normal life expectancy. Nevertheless, there is a high risk of organ rejection in the short-medium term after surgery. This paper proposes a new ReCIVA®-GC-MS technology to potentially predict the rejection of the transplanted kidney during the first year after surgery by analyzing human breath. Twenty VOCs, recognized by literature as targets or as representative of the major classes of molecules essential to the identification of chronic kidney disease affected patients and/or healthy subjects, were selected and employed for this methodology optimization. The breath profile of healthy subjects was considered as target in case of restored kidney function after transplantation. Calibration curves, linearity concentration range, limit of detection and quantification of selected molecules were estimated as well as the intra-day and inter-day reproducibility of the method. To test the applicability of the GC-MS developed methodology, the breath of healthy subjects and chronic kidney disease affected patients was ReCIVA® sampled, and then analyzed. Sixty-seven molecules were identified, and between these, thirteen of twenty selected compounds were quantified, confirming the robustness of the optimized protocol.

1. Introduction

Kidney failure is a major global health concern, recognized as wide public health problem. In most cases the only strategy to improve chronic kidney disease (CKD) affected patients' quality of life and life expectancy is kidney transplant.

In clinical practice, blood and urine tests, glomerular filtration rates, imaging, and kidney biopsies are used to detect chronic kidney failure. Some of these methods are complex, expensive, invasive, time-consuming, require skilled technicians, and may cause pain in some individuals.

In recent years, exhaled breath analysis has captured the interest of scientists and clinicians, providing important information regarding crucial biochemical changes linked to certain pathologies [1–7]. Exhaled breath, in fact, comprises, in addition to condensates (EBCs; cytokines, H_2O_2, isoprostanes, leukotriene), and volatile inorganic compounds (e.g., O_2, NO, CO_2), organic compounds (VOCs) [8-10] which are produced by cellular metabolism, enter the blood, travel to the lungs, and are finally exhaled through the respiratory tract. When a person suffers from a certain disease, the exhaled air components' changes can provide useful clues for clinical diagnosis and monitoring.

Breath analysis involves collecting breath samples of subjects, their analysis, and data processing. The advantages of breath analysis are that it is safe, non-invasive, reproducible, acceptable for patients, easy to operate, and fast. Another benefit is that samples are readily obtained, and compared to blood and urine collection, breath analysis is less time-consuming and requires a smaller sample [7]. Therefore, breath analysis is unique compared to traditional technologies, making it a research hotspot in the field of disease diagnosis, even though it is an old technique for diagnosing physical conditions. Hippocrates

(460–370 BCE) first described it in his "*treatise on respiratory aromas and diseases*". Over the past thirty years, scientists have identified thousands of different breath organic compounds, employing emerging analytical techniques, including proton transfer reaction mass spectrometry (PTR-MS) [11-14], proton reaction transfer time-of-flight mass spectrometry [15-18], selected ion stream tube mass spectrometry (SIFT-MS) [19-23], laser spectroscopy [24, 25], ion mobility spectrometry [26-28] sensor array [29], and electronic nose technology [30-33], even if the gold standard for detecting respiratory biomarkers is a combination of gas chromatography and mass spectrometry (GC-MS) [3, 34-36]. However, independently from the diagnostic technique employed, several studies have demonstrated that breath analysis may be a promising strategy for detection and follow-up of kidney disease.

Nitrogen containing VOCs such as ammonia and amines have been shown to be elevated in the breath of subjects with renal failure [37-39]. From ancient times, in fact, a fishy-like smell of exhaled breath was attributed to renal disorders [40], and ammonia and trimethyl amine (TMA) were used as useful biomarkers for real-time monitoring of the hemodialysis efficacy [38-39]. Other than for the nitrogen-containing compounds, little is known about other classes of VOCs such as: sulfur compounds, ketones, alkenes, aliphatic hydrocarbons, with short and long chain (e.g., propane, butane, pentane, hexane, decane, etc.), organic acids (e.g., acetic acid, butanoic acid, etc.), benzene derivates (e.g., toluene, xylenes, etc.), halogen containing, alcohols whose concentrations in human breath change in response to the onset of specific pathologies [41-43] and that have been proved to be essential in the fingerprint breath profile of healthy subjects [44-48].

This study aims to optimize a ReCIVA®-GC-MS protocol which could be potentially useful, in the next future, for the analysis of the breath of kidney transplanted patients to eventually find a pattern of VOCs able to predict organ rejection.

The experimental procedure was set up employing a mixture of standard compounds recognized as target or selected as representative of the major classes of molecules essential to the detection of CKD. Moreover, supposing that in case of successful transplantation and acceptance by the organism of the transplanted organ we expect an evolution of the breath profile towards that of a healthy subject, representative molecules of the major classes of compounds characterizing healthy breath was also incorporated into the study. Standard solutions of different concentrations were adsorbed onto biomonitoring sorbent tubes and then thermally desorbed, before being injected and analyzed by a GC-MS to test, for each selected compound, the linearity, the intra-day, and the inter-day reproducibility of the developed method.

The biomonitoring sorbent tube desorption conditioning time and storage were also optimized.

Finally, the breath of twenty healthy subjects and of six CKD affected patients was sampled by ReCIVA® before undergoing kidney transplant by living organ donor, and then analyzed employing the optimized protocol.

2. Experimental Results And Discussion

To test the experimental conditions set, in terms of the ability to detect and separate the twenty selected molecules, each analyte was individually analyzed. Specifically, 50 ng of each compound was added to a previously conditioned biomonitoring sorbent tube according to the procedure described (§ 3.2).

It was demonstrated that the operating conditions used were able to adequately detect each molecule without any overlap between them. The analysis of each standard compound was repeated five times and the retention time (RT) of each compound was recorded (**Table 1**). Table 1, reports for each molecule the characteristic fragment ions at m/z (mass-to charge) ratios employed for substance quantification in XICs mode from SCAN chromatogram. Only acetonitrile was quantified from SCAN chromatogram.

At the end of each analysis, the conditioning procedure of the biomonitoring sorbent tube used was repeated and the relative chromatogram was acquired, to verify that the cleaning method carried out was successful. The biomonitoring sorbent tube thus cleaned was capped, sealed, and stored as described (§ 3.2).

Table 1. Selected VOCs RTs and m/z ratios.

Common compound name	*RT (min.)*	*m/z*
2-butanone	13.8±0.2	72, 57, 43
3-heptanone	22.9±0.2	114, 85, 72
1-Octine	18.4±0.2	95, 81, 67
Acetonitrile	11.4±0.3	-
Benzaldehyde	25.5±0.3	106, 77, 51
Butanoic acid	16.7±0.3	88, 73, 60
Butanol	15.5±0.2	56, 43, 41
Decan	26.2±0.3	142, 85, 57
Dichloromethane	13.2±0.2	84, 49
Dimethyl sulfoxide	25.0±0.2	78, 63, 45
Dodecane	28.3±0.3	170, 85, 57
Ethyl ether	8.4±0.4	74, 59, 43
Ethylenediamine	12.2±0.4	60, 59, 43
Hexanal	19.6±0.2	82, 72
Hexanoic acid	22.4±0.2	87, 60
Octanal	20.8±0.2	84, 57
Octanol	28.0±0.3	84, 70
Propanal	17.9±0.2	59, 57
Propylamine	10.7±0.2	59, 41
Toluene	17.5±0.2	91, 65

The optimized analytical conditions were tested with linear regression analysis of peak area versus analyte amount, adding the biomonitoring sorbent tubes with aliquots of each standard compound in quantities between 5 and 100 ng. Each measurement was repeated three times. **Table 2** reports the linear ranges, the equations of the obtained calibration curves, and LOD and LOQ for all selected molecules.

Table 2. Calibration curves equations, correlation coefficients (R^2), linear ranges, LOD and LOQ values for selected VOCs.

Common compound name	*Equation*	*R^2*	*Linear range (ng)*	*LOD*		*LOQ*	
				Tube (ng)	*Breath[a] ($pg \cdot mL^{-1}$)*	*Tube (ng)*	*Breath[a] ($pg \cdot mL^{-1}$)*
2-Butanone	$y=2 \cdot 10^7 x-2 \cdot 10^8$	0.9764	16-100	1.7	3.4	5.7	11.4
3-Heptanone	$y=4 \cdot 10^6 x-1 \cdot 10^7$	0.9719	16-100	2.1	4.2	6.9	13.8
1-Octine	$y=3 \cdot 10^6 x-2 \cdot 10^7$	0.9789	30-100	1.8	3.6	6.2	12.4
Acetonitrile	$y=2 \cdot 10^7 x-1 \cdot 10^8$	0.9889	35-100	1.1	2.2	3.6	7.2
Benzaldehyde	$y=2 \cdot 10^6 x+3 \cdot 10^7$	0.9536	18-100	2.7	5.4	8.9	17.8
Butanoic acid	$y=6 \cdot 10^6 x-2 \cdot 10^7$	0.9936	45-100	1.0	2.0	3.5	7.0
Butanol	$y=3 \cdot 10^7 x-3 \cdot 10^8$	0.9952	16-100	1.0	2.0	3.2	6.4
Decane	$y=1 \cdot 10^6 x-1 \cdot 10^7$	0.9948	28-100	1.2	2.4	3.9	7.8
Dichloromethane	$y=7 \cdot 10^6 x-2 \cdot 10^7$	0.9883	20-100	1.3	2.6	4.3	8.6
Dimethylsulfoxide	$y=4 \cdot 10^6 x-3 \cdot 10^7$	0.9780	22-100	1.7	3.4	5.8	11.6
Dodecane	$y=1 \cdot 10^6 x-3 \cdot 10^6$	0.9947	28-100	1.0	2.4	3.4	6.8
Ethyl ether	$y=9 \cdot 10^6 x-6 \cdot 10^6$	0.9917	18-100	1.1	2.2	3.6	7.2
Ethylenediamine	$y=3 \cdot 10^7 x-3 \cdot 10^8$	0.9668	40-100	2.4	4.8	8.1	16.2
Hexanal	$y=604904x-5 \cdot 10^6$	0.9961	12-100	0.7	1.4	1.4	2.8
Hexanoic acid	$y=3 \cdot 10^6 x-3 \cdot 10^7$	0.9923	20-100	1.2	2.4	4.0	8.0
Octanal	$y=102130x-348975$	0.9860	23-100	1.4	2.8	4.8	9.6
Octanol	$y=921372x-6 \cdot 10^6$	0.9844	25-100	1.5	3.0	5.1	10.2
Propanal	$y=2 \cdot 10^6 x-7 \cdot 10^6$	0.9952	12-100	0.7	1.4	2.4	4.8
Propylamine	$y=1 \cdot 10^8 x+2 \cdot 10^8$	0.9916	23-100	1.4	2.8	4.6	9.2
Toluene	$y=5 \cdot 10^7 x-3 \cdot 10^8$	0.9835	27-100	1.6	3.2	5.4	10.8

[a] Breath volume sampled: 500 mL

For all considered analytes, a good linearity is ensured in the quantitative range explored with the R^2 values resulting always greater than 0.9719, and the linearity range is correct for the significant determination of considered analytes. Estimated LOD and LOQ values, moreover, are in line with those reported in literature. Grabowska-Polanowska et al., for example, reported for alkane (e.g., pentane and hexane) LOD of about few dozen of $pg \cdot mL^{-1}$ and LOQ values of a maximum of 200 $pg \cdot mL^{-1}$ [48]. Similar values were recorded also for nitrogen and sulfur containing compounds, and ketones [48-49].

The reproducibility of the investigated analytical procedure was evaluated in terms of intra-day (n = 3) and inter-day (n = 3, over 7 days) RSD %, using standard solutions of considered analytes at amount

levels equal to five, ten and twenty times the respective LOQs values. Experimental results showed in Table 3.

Table 3. Intra-day and inter-day mean RSD % values for selected VOCs.

	*LOD*5*	*LOQ*5*	*LOD*10*	*LOQ*10*	*LOD*20*	*LOQ*20*
Compound common name	*Intra-day RSD % (n=3)*	*Inter-day RSD % (n=21)*	*Intra-day RSD % (n=3)*	*Inter-day RSD % (n=21)*	*Intra-day RSD % (n=3)*	*Inter-day RSD % (n=21)*
2-butanone	5±1	12±1	6±1	10±1	3±1	11±1
3-heptanone	6±1	10±1	4±1	11±3	5±1	10±3
1-Octine	7±1	12±2	6±2	11±3	8±2	11±2
Acetonitrile	3±1	14±2	3±1	14±2	4±1	13±3
Benzaldehyde	7±2	13±2	8±3	12±3	5±2	13±2
Butanoic acid	5±1	11±1	3±1	10±2	5±1	12±2
Butanol	7±1	11±1	5±1	10±1	6±2	11±1
Decane	4±1	8±1	6±1	8±1	4±1	8±1
Dichloromethane	4±1	9±1	6±2	8±1	3±1	8±1
Dimethyl sulfoxide	7±1	12±2	8±2	12±2	6±2	10±1
Dodecane	4±1	8±1	4±1	8±2	3±1	7±1
Ethyl ether	6±1	10±2	5±2	9±3	6±1	9±1
Ethylenediamine	3±1	8±1	5±2	7±3	4±1	8±1
Hexanal	6±1	15±2	8±3	15±3	7±2	14±3
Octanal	7±2	15±3	7±2	14±3	7±2	14±3
Octanol	7±2	9±1	7±2	8±2	7±1	9±2
Propanal	7±1	10±1	6±2	11±3	5±1	11±2
Propylamine	6±1	13±2	6±1	12±3	5±1	12±3
Toluene	4±1	9±1	3±1	9±2	4±1	9±1

Mean RSD % values ≤ 8 (intra-day) and ≤ 15 (inter-day) were always obtained for all the analytes at all levels of concentration.

Finally, to evaluate the in vivo application of the ReCIVA®-GC-MS method developed up to now, the breath of twenty healthy subjects and six CKD affected patients sampled before undergoing kidney transplant from a living donor was analyzed. Figure 1 shows the chromatograms, acquired in SCAN mode, of the breath of a healthy subject (Fig. 1(A)) and of a CKD affected patient (Fig. 1(B)), selected as examples.

Seventy-four VOCs were detected (S/N ≥ 3) overall, sixty-seven of them identified, and reported in Table 4.

Table 4. List of molecules detected (S/N ≥ 3) in the breath of twenty healthy subjects and six CKD affected patients before undergoing kidney transplant.

Peak n°	*RT (min)*[a]	*Common compound name*	*Match (%)*	*Probability (%)*	*Standard identity confirmation*[b]	*Healthy subjects*	*CKD patients*
1	4.2	Unidentified				yes	
2	5.7	Carbon dioxide	891	90		yes	yes
3	6.4	2,4-Dimethyl pentane	930	91		yes	yes
4	6.5	Hexene	879	89		yes	yes
5	6.6	Sulfur dioxide	878	87		yes	yes
6	6.7	Difluoro methyl-silane	801	52		yes	yes
7	6.8	Trimethyl silylanol	773	55		yes	yes
8	6.9	Ethane, 1,2-diethoxy	801	61		yes	yes
9	7.0	1-Pentene-4-methyl	822	54		yes	yes
10	7.2	2-propane	833	60	yes	yes	yes
11	7.6	1,1,1,1-Trifluoro trimethyl-silylanol	828	56		yes	yes
12	7.9	Cyclobutanolo	903	78		yes	yes
13	8.3	Trichloro-monofluoro-methane	822	57		yes	yes
14	8.9	1,3-Pentadiene	954	75		yes	yes
15	9.1	2-Propanol-1-methoxy	930	80		yes	yes
16	9.7	Unidentified				yes	yes
17	10.1	2-Pentene	915	85		yes	yes
18	10.2	2-Butanol-3-methyl	907	84		yes	yes
19	10.3	2-Methyl pentanal	839	58		yes	yes
20	10.5	Cyclopentane	903	88		yes	yes
21	10.7	**Propylamine**		55	yes		yes
22	10.8	2,3-Dimethyl pentane		66		yes	yes
23	10.9	Hexane	913	92	yes	yes	yes
24	11.0	4-Methyl-2-pentyne	877			yes	yes
25	11.4	**Acetonitrile**	920	90	yes		yes
26	11.5	Unidentified				yes	yes
27	11.6	Benzene	938	89	yes	yes	yes
28	11.8	Methoxy-acetonitrile	888	56			yes
29	12.2	**Ethylenediamine**	815	52	yes		yes
30	12.4	Unidentified				yes	yes
31	12.9	1,3,5-Trifluoro benzene	852	57		yes	yes
32	13.2	**Dichloromethane**	931	93	yes	yes	yes
33	13.5	Hexamethyl disiloxane	828	81		yes	yes
34	13.6	Xylitol	876	83		yes	yes
35	13.7	Phenol	913	92	yes	yes	yes
36	13.8	**2-Butanone**	948	96	yes	yes	yes
37	14.1	Heptene	899	88	yes	yes	yes

38	14.3	3-Hexanol	866	77		yes	yes
39	14.9	Acetic acid	915	67		yes	yes
40	15.9	2-Propanol-1-methoxy	838	52		yes	yes
41	16.4	1,4-Dioxane	828	51		yes	yes
42	16.6	2-Pentanone	903	89		yes	yes
43	16.7	**Butanoic acid**	933	97	yes	yes	yes
44	17.4	Cyclotrisiloxane hexamethyl	807	58		yes	yes
45	17.5	**Toluene**	938	97	yes	yes	yes
46	18.2	Unidentified	907	70			yes
47	18.4	2-Hexanone	881	68		yes	yes
48	19.6	**Hexanal**				yes	yes
49	19.8	Methyl isobutyl ketone	902	76		yes	yes
50	20.00	Hexanoic acid, methyl ester	874	83		yes	yes
51	20.1	Nonane	934	54	yes	yes	yes
52	20.3	Pentanoic acid, methyl ester	879	79		yes	yes
53	20.5	Pentanoic acid	809	54	yes	yes	yes
54	22.0	Di(isobutyl)acetone	815	58		yes	yes
55	22.4	**Hexanoic acid**	879	79	yes	yes	yes
56	22.9	**3-Heptanone**	918	82	yes	yes	yes
57	23.00	Heptanoic acid, methyl ester	988	83		yes	yes
58	23.5	Eptane, 2,2,4,6,6-pentamethyl	888	55		yes	yes
59	23.9	Tetrasiloxane, decamethyl	848	51		yes	yes
60	24.8	Limonene	915	92		yes	yes
61	25.1	Butanoic acid, dimethyl ester	855	74		yes	yes
62	25.5	**Benzaldehyde**	933	95	yes	yes	yes
63	25.9	Octanoic acid, methyl ester	832	68		yes	yes
64	26.2	**Decane**	932	55	yes	yes	yes
65	26.8	Benzoic acid, methyl ester	815	54		yes	yes
66	27.5	1-Decanol-2-esil	877	53		yes	yes
67	27.8	Ibruprofen	984	83			yes
68	28.3	**Dodecane**	928	54	yes	yes	yes
69	29.0	Unidentified				yes	yes
70	29.6	Silane, ethyl-dimethyl-phenyl	813	62		yes	yes
71	29.8	4-Phenyl benzofurane	822	56		yes	yes
72	30.5	Tri-tetra-contane	812	56		yes	yes
73	30.7	Hexestrol	828	52			yes
74	31.5	Unidentified				yes	yes

[a]Values expressed as mean (s.d.).

[b]Authenticated using the NIST library and standard injection.

The breath of the two populations considered are characterized by the presence of the same substances except for nitrogen containing compounds (acetonitrile, ethylenediamine, propylamine), which were present only in CKD affected patients exhaled breath. As reported in literature, nitrogen-based substances are an indication of renal failure. In fact, as previously underlined, ammonia and amines have been shown to be elevated in the breath of subjects affected by CKD [37-39].

In general, higher levels of aldehyde compounds are expected in the breath of CKD patients. These compounds can originate from membrane phospholipids during peroxidation processes by reactive oxygen species. Oxidative stress has been related to chronic renal failure [41]. Therefore, aldehydes can be considered as biomarkers of oxidative stress [41]. For istance, Hermanns et al. induced renal oxidative damage in rats by daily injecting ferric nitrilotriacetate, for thirteen days, and estimated the concentration of acetone and seven aldehydes in the urine, finding that acetaldehyde and propanal significantly increased much earlier than the classic chemical-clinical parameters of renal damage. On the other hand, the urinary excretion of acetone, butanal, formaldehyde, hexanal, malonedialdehyde and pentanal was increased at the same time or shortly before that of the urinary parameters [43].

As shown in Table 4, alkanes characterize the exhaled breath of both groups analyzed and the C6-C12 compounds. Alhamdani et al. found significantly higher levels of these compounds in hemodialysis patients compared to controls [42], suggesting that alkanes may be useful for monitoring the organism's response to the transplanted organ.

Breath analysis of healthy subjects and CKD affected patients allowed to highlight the presence of thirteen out of the twenty VOCs selected to optimize the experimental method and which were: 2-butanone, 3-heptanone, hexanal, acetonitrile, benzaldehyde, butanoic acid, decane, dichloromethane, dodecane, ethylenediamine, hexanoic acid, propylamine, and toluene. The other fifty-four molecules identified, common to both populations, belong to the same classes of which the twenty selected compounds are representative.

Traces of drugs were also found in two CKD affected patients' breath, such as: hexestrol (antitumor drug) and ibuprofen (nonsteroidal anti-inflammatory drug). A contamination of limonene and xylitol, compounds frequently used by food industry as seasoning, was revealed in some breath samples analyzed.

Finally, the concentration range of the thirteen selected target compounds were estimated for both healthy subjects' and/or CKD affected patients' population. The experimental results are reported in **Table 5**.

Table 5. Concentration range of the thirteen selected VOCs revealed in the exhaled breath samples for both healthy subjects and/or CKD affected patients' population.

Common compound name	*pg·mL^{-1} in exhaled breath*	
	Healthy subjects	*CKD affected patients*
2-Butanone	20-70	10-30
3-Heptanone	LOD-12	10-40
Acetonitrile	n.d.	7-20
Benzaldehyde	n.d.-50	n.d.-LOD
Butanoic acid	LOD-60	LOD-15
Decane	n.d.-50	25-40
Dichloromethane	LOD-20	LOD-15
dodecane	n.d.-60	40-70
Ethylenediamine	n.d.	13-30
Hexanal	n.d.-50	35-150
Hexanoic acid	n.d.-60	90-120
Propylamine	n.d.	n.d.-LOD
Toluene	n.d.-20	5-20

From this experimental evidence, it was possible to conclude that the ReCIVA®-GC-MS protocol developed could be advantageously exploited to analyze, and follow-up the breath of CKD affected patients, before and after undergoing kidney transplantation, to potentially identify target substances to allow early diagnosis in a simple, fast, economical, and non-invasive way, of the incoming rejection processes of the donated organ.

3. Materials And Methods

3.1 VOCs GC-MS analyses

Following the previously optimized protocols [46], VOCs collected in stainless steel inert biomonitoring sorbent tubes, able to retain C_4-C_{30} compounds (*Markes International,* Llantrisant, UK), were desorbed with a thermal desorber (Unity-xr, *Markes International*), directly connected to the gas chromatograph with a heated transfer line. The tube was heated for 10 min at 220 °C and the desorbed VOCs were directly transferred in the gas chromatograph injector at 200 °C, operating in split mode (50 % in and 50 % out), utilizing helium as carrier gas, at the linear velocity of 0.5 cm·s^{-1}. The separation and quantification of desorbed VOCs was performed with a gas chromatograph (Clarus 680, *PerkinElmer*, Massachusetts, USA) coupled with a quadrupole mass spectrometer (Clarus SQ 8T, *PerkinElmer*, Massachusetts, USA). A 60 m x 0.25 mm i.d., 1.4 µm film thickness, capillary column Rtx®-VMS (*Restek*, Bellefonte, PA) was utilized with the following oven temperature program: 50 °C for 5 min, then ramped 10 °C·min^{-1} to 160 °C, 5 min at 160 °C, ramped 10 °C·min^{-1} to 220 °C, and 5 min at 220 °C. The temperatures of the transfer line and the ion source of quadrupole were 280 °C and 220 °C, respectively. The MS was performed at 70 eV electron impact ionization energy, in full-scan mode (SCAN) with scan range 40–250 amu. SCAN

monitoring mode was used for compound identification and quantification in the case of acetonitrile. Quantification of the other selected analytes was made from extracted ion chromatogram (XIC) obtained in SCAN mode. The Clarus SQ8 GC-MS software (*PerkinElmer*) allowed acquisition and elaboration data.

To prevent memory effects, after each analysis, two empty ReCIVA® biomonitoring sorbent tubes (without adsorbent phase) were analyzed to remove eventual residues of the previous sample from the thermal desorber and analysis apparatus.

After each use, biomonitoring sorbent tubes were conditioned at 340 °C for 3h, as recommended by producer, capped, sealed with parafilm, and stored at 8 °C.

3.2. Linear regression test, LOD and LOQ of the GC-MS method

After reviewing the literature, twenty VOCs, recognized as target or as representative of the major class of molecules essential to elaboration of CKD affected patients and/or healthy subjects' breath were selected, and reported in **Table 6**.

Table 6. Selected VOCs.

Common compound name	*Molecular class*			*CAS*		*Bibliographic ref.*
		Healthy subjects	*CKD patients*	*number*	*M.W. ($g \cdot mol^{-1}$)*	
2-Butanone	Ketone	*		78-93-3	72.11	[44-47]
3-Heptanone	Ketone	*		106-35-4	114.19	[44-47]
1-Octyne	Alkyne	*		629-05-0	110.20	[44]
Acetonitrile	Nitrogen compound		*	75-05-8	41.05	[37-39, 44]
Benzaldehyde	Aldehyde, benzene compound	*	*	100-52-7	106.12	[41-47]
Butanoic acid	Acid	*		107-92-6	88.11	[44-47]
Butanol	Alcohol	*		71-36-3	74.12	[44-47]
Decane	Alkane	*	*	124-18-5	142.29	[42, 44-47]
Dichloromethane	Chlorine compound	*	*	75-09-2	84.93	[44]
Dimethyl sulfoxide	Sulfur compound	*	*	67-68-5	78.13	[44, 48]
Dodecane	Alkane	*	*	112-40-3	170.33	[44-47]
Ethyl ether	Ether	*		60-29-7	74.12	[44]
Ethylene diamine	Amine		*	107-15-3	60.10	[37-39, 44]
Hexanal	Aldehyde	*	*	66-25-1	100.16	[41-47]
Hexanoic acid	Acid	*		142-62-1	116.16	[44-47]
Octanal	Aldehyde	*	*	124-13-0	128.21	[41-47]
Octanol	Alcohol	*		111-87-5	130.23	[44-47]
Propanal	Aldehyde	*	*	123-38-6	58.08	[41-47]
Propylamine	Amine		*	107-10-8	59.11	[37-39, 44]
Toluene	Benzene compound	*	*	108-88-3	92.14	[45-47]

Stock solutions (1 $mg \cdot mL^{-1}$) of each chosen volatile molecule (purity ≥ 97 %; *Sigma-Aldrich*, Milan, Italy) were prepared in methanol (purity ≥ 98 %; *Sigma-Aldrich*), except for hydrocarbons which were solubilized in hexane (purity ≥ 98 %; *Sigma-Aldrich*), stored at 8 °C, and diluted to prepare working solutions.

Working solution (1 µL), containing authentic standards (5, 10, 15, 25, 50 and 100 ng·mL^{-1}), was added into a biomonitoring sorbent tube which was then analyzed following the procedure described above. The identification of VOCs was performed with the MS database of the National Institute of Standards and Technology (NIST).

The proposed GC-MS method was tested by linear regression analysis, plotting the peak area against the amount (ng) of each analyte in biomonitoring sorbent tube.

Limits of detection (LOD) and quantification (LOQ) were determined by LOD ≅ (3·sda)/b and LOQ ≅ (10·sda)/b, where sda is the standard deviation of the y intercept and b is the slope of the regression line. The reproducibility, as intra-day (n=3) and inter-days (n=3 over 7 days) percentage relative standard deviation (RSD %) were calculated at three levels of concentration (five, ten, and twenty times the LOQ values, in the test biomonitoring sorbent tube) analyzing solutions prepared daily by the same working solutions stored at 8 °C.

3.3 Exhaled breath sampling and analyses

After obtaining written informed consent, the breath of twenty healthy subjects and six CKD affected patients, enlisted to undergo kidney transplant from a living donor, was sampled to test the here proposed ReCIVA®-GC-MS protocol. Exhaled breath was collected with the ReCIVA® Breath Sampler (*Owlstone Medical*, Cambridge, UK). The device was connected to a breath-sampling kit (mask and sorbent tubes), ensuring reproducible collection of VOCs during real-time monitoring of the patient's breathing. The patients' exhaled breath was captured into four stainless steel inert biomonitoring sorbent tubes. The apparatus comprised infrared carbon dioxide detection with pressure sensors, permitting the selection of different volumes and fractions of the exhaled breath. A mask manufactured from medical grade silicone, which included a high-efficiency, low-resistance bacterial filter, was fixed on the device before each sampling. This was connected to a medical air canister via a plastic pressure reducer, set to 15 L·min^{-1}. A USB cable connected the ReCIVA® breath sampler to a laptop installed with breath-sampling software (*Owlstone Medical*), designed to ensure accurate monitoring of breathing air pressures (partial pressure of CO_2). The subjects were kept fasted for at least 4 h before breath sampling. Sampling was always performed in the same room, aerated for 30 min before the procedure. Patients were instructed to keep the mask securely adhered to the face and to breathe normally the air released by the medical air canister. After 60 s ReCIVA® device washout with pure air (purity ≥ 99 % per cent; *SOL Group*, Monza, Italy), patient breath was collected for 10 min under PC-dedicated program control [45, 46]. At the completion of sampling, the biomonitoring sorbent tube was removed, capped with plastic caps, sealed with parafilm, stored at 8 °C, and then delivered to the chemistry department within 24 h for GC-MS analysis, following the experimental procedure optimized.

3.4 Ethical Approval

All methods were carried out in accordance with relevant guidelines and regulations. The experimental protocol was approved by the ethics committee of the Azienda Ospedaliero-Universitaria Policlinico, Bari, Italy, and performed in compliance with the Declaration of Helsinki. All of patients recruited provide written informed consent before breath-testing.

4. Conclusions

In the next future, the breath of other CKD affected patients, before kidney transplant by living donor and during the following months, will be sampled by ReCIVA® device and then GC-MS analyzed, following the so validated protocol. The breath will be sampled and analyzed at regular intervals of time, over a year after surgery, considering this the ultimate time for eventually observing the rejection of the transplanted organ. Other thirty-five patients, at least, will enter the study to eventually find qualitative and/or quantitative differences in the pattern of VOCs expired by patients that undergo organ rejection with respect to subjects that won't suffer this complication. If this hypothesis will be confirmed, it will possible, employing the here optimized method, to predict in a simple, inexpensive, fast, and non-invasive way, the organ rejection by the organism of the patient under observation.

Declarations

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgements

The authors thank Dr Agnese Dezi, Dr Giovanni Tomasicchio and Dr Giuseppe Lucarelli for linguistic revision of the paper and technical support. This work was supported by the University of Bari "Aldo Moro" and Azienda Ospedaliero-Univesitaria Policlinico, Bari, Italy.

Author contributions

Conceived and designed experiments: N. De Vietro, A. Picciariello, A.M. Aresta and D.F. Altomare. Analysed the data: A.M. Aresta, N. De Vietro Alessia Di Gilio, Provided technical support: J. Palmisani. Provided supervision and interpretation of results: C. Zambonin, G. De Gennaro. Wrote the manuscript: N. De Vietro, A.M. Aresta and A. Piccialriello. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to A.M. Aresta.

References

[1] Hepner, G.W., Uhlin, S.R., Lipton, A., Harvey, H.A., G., Rohrer, V. Abnormal aminopyrine metabolism in patients with hepatic neoplasm: detection by breath test *JAMA* **236(14)**, 1587–1590 (1976).

[2] Kharitonov, S.A. *et al.* Exhaled nitric-oxide is increased in asthma *Chest.* **107(3),** S156–S157 (1995).

[3] Phillips, M. *et al.* Volatile organic compounds in breath as markers of lung cancer: a cross-sectional study *Lancet.* **353(9168)**, 1930–1933 (1999).

[4] Borrill, Z.L., Roy, K., Singh, D. Exhaled breath condensate biomarkers in COPD *Eur. Respir. J.* **32(2)**, 472–486 (2008).

[5] Nakhleh, M.K. et al. Diagnosis and classification of 17 diseases from 1404 subjects via pattern analysis of exhaled molecules *ACS Nano.* **11(1)**, 112–125 (2017).

[6] Spanel, P., Smith, D. Volatile compounds in health and disease *Curr. Opin. Clin. Nutr. Metab. Care* **14(5)**, 455–460 (2011).

[7] Amann, A. *et al*. Analysis of exhaled breath for disease detection in *Annual review of analytical chemistry*, (Ed. Annual Reviews) **7**, 455-482 (Palo Alto, 2014).

[8] Miekisch, W., Schubert, J.K. From highly sophisticated analytical techniques to life-saving diagnostics: technical developments in breath analysis *TrAC Trends Anal. Chem.* **25 (7)**, 665–673, (2006).

[9] Mazzone, P.J. Analysis of volatile organic compounds in the exhaled breath for the diagnosis of lung cancer *J. Thoracic Oncol*. **3 (7)**, 774–780 (2008).

[10] Agapiou, A., Amann, A., Mochalski, P., Statheropoulos, M. K., Thomas, C.L.P. Trace detection of endogenous human volatile organic compounds for search, rescue, and emergency applications *TrAC-Trends Anal. Chem.* **66**, 158–175 (2015).

[11] Hansel, A. *et al.* Proton-transfer reaction mass-spectrometry – online trace gas analysis at the ppb level *Int. J. Mass Spectrom.* **149**, 609–619 (1995).

[12] Blake, R.S., Monks, P.S., Ellis, A.M. Proton-transfer reaction mass spectrometry, *Chem. Rev*. **109(3)**, 861–896 (2009).

[13] Yuan, B. *et al.* Proton-transfer-reaction mass spectrometry: applications in atmospheric sciences *Chem. Rev.* **117(21)**, 13187–13229 (2017).

[14] Nenadis, N., Heenan, S., Tsimidou, M.Z., Van Ruth, S. Applicability of PTR-MS in the quality control of saffron *Food Chem*. **196**, 961–967 (2016).

[15] Blake, R.S, Whyte, C., Hughes, C.O., Ellis, A.M., Monks, P.S. Demonstration of proton-transfer reaction time-of-flight mass spectrometry for real-time analysis of trace volatile organic compounds *Anal. Chem.* **76(13)**, 3841–3845 (2004).

[16] Brock, B. *et al.* Monitoring of breath VOCs and electrical impedance tomography under pulmonary recruitment in mechanically ventilated patients *J. Breath Res.* **11(1)**: 016005; doi: 10.1088/1752-7163/aa53b2 (2017).

[17] Stadler, S., Stefanuto, P.H., Brokl, M., Forbes, S.L., Focant, J.F. Characterization of volatile organic compounds from human analogue decomposition using thermal desorption coupled to comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry *Anal. Chem*. **85(2)**, 998–1005 (2013).

[18] Trefz, P., Schubert, J.K., Miekisch, W. Effects of humidity, CO_2 and O_2 on real-time quantitation of breath biomarkers by means of PTR-ToF-MS *J. Breath Res.* **12(2)**: 026016; doi: 10.1088/1752-7163/aa9eea (2018).

[19] Smith, D., Spanel, P. Selected ion flow tube mass spectrometry (SIFT-MS) for on-line trace gas analysis, *Mass Spectrom. Rev*. **24(5)**, 661–700 (2005).

[20] Boshier, P.R., Marczin, N., Hanna, G.B. Repeatability of the measurement of exhaled volatile metabolites using selected ion flow tube mass spectrometry *J. Am. Soc. Mass Spectrom*. **21(6)**, 1070–1074 (2010).

[21] Chin, S.T., Romano, A., Sophie L.F. Doran, S.L.F., Hanna, G.B. Cross-platform mass spectrometry annotation in breathomics of oesophageal-gastric cancer *Sci. Rep.* **8**: 5139; doi: 10.1038/s41598-018-22890-w (2018).

[22] Hicks, L.C. *et al.* Analysis of exhaled breath volatile organic compounds in inflammatory bowel disease: a pilot study *J. Crohns Colitis* **9(9)**, 731–737 (2015).

[23] Alkhouri, N. *et al.* Isoprene in the exhaled breath is a novel biomarker for advanced fibrosis in patients with chronic liver disease: a pilot study *Clin. Translat. Gastroenterol.* **6**: e112; doi: 10.1038/ctg.2015.40 (2015).

[24] Mastrigt, E. *et al.* Exhaled breath profiling using broadband quantum cascade laser-based spectroscopy in healthy children and children with asthma and cystic fibrosis *J. Breath Res*. **10(2)**:026003; doi: 10.1088/1752-7155/10/2/026003 (2016).

[25] Tuetuencue, E., Mizaikoff, B. Cascade laser sensing concepts for advanced breath diagnostics *Anal. Bioanal. Chem*. **411(9)**, 1679-1686 (2019).

[26] Handa, H. *et al.* Exhaled breath analysis for lung cancer detection using ion mobility spectrometry *PLoS ONE* **9(12)**: e114555; doi: 10.1371/journal.pone.0114555 (2014).

[27] Hagemann, L.T. *et al.* Hybrid analytical platform based on field-asymmetric ion mobility spectrometry, infrared sensing, and luminescence-based oxygen sensing for exhaled breath analysis *Sensors* **19(12)**: 2653; doi.org/10.3390/s19122653 (2019).

[28] Brodrick, E., Davies, A., Neill, P., Hanna. L, Williams, E.M. Breath analysis: translation into clinical practice *J. Breath Res.* **9(2)**: 027109; doi:10.1088/1752-7155/9/2/027109 (2015).

[29] Konvalina, G., Haick, H. Sensors for breath testing: from nanomaterials to comprehensive disease detection *Acc. Chem. Res*. **47(1)**, 66–76 (2014).

[30] Hanson, C.W., Thaler, E.R. Electronic nose prediction of a clinical pneumonia score: biosensors and microbes *Anesthesiology* **102(1)**, 63–68 (2005).

[31] Gupta, A., Singh, S.T. Yadava, R.D.S. Mems sensor array-based electronic nose for breath analysis – a simulation study *J. Breath Res.* **13(1)**: 016003; doi: 10.1088/1752-7163/aad5f1 (2019).

[32] van de Goor, R., van Hooren, M., Dingemans, A.M., Kremer, B., Kross, K. Training and validating a portable electronic nose for lung cancer screening *J. Thoracic Oncol.* **13(5)**, 676–681 (2018).

[33] Wilson, AD. Application of electronic-nose technologies and VOC-biomarkers for the noninvasive early diagnosis of gastrointestinal diseases *Sensors (Basel)* **18(8)**: 2613; doi: 10.3390/s18082613 (2018).

[34] Labows, J.N., McGinley, K.J., Webster, G.F., Leyden, J.J. Headspace analysis of volatile metabolites of pseudomonas aeruginosa and related species by gas chromatography-mass spectrometry *J. Clin. Microbiol.* **12(4)**, 521–526 (1980).

[35] Raymer, J.H., Thomas, K.W., Cooper, S.D., Whitaker, D.A., Pellizzari, E.D. A. Device for sampling of human alveolar breath for the measurement of expired volatile organic-compounds *J. Anal. Toxicol*. **14(6)**, 337–344 (1990).

[36] Thomas, K.W., Pellizzari, E.D., Cooper, S.D. A canister-based method for collection and GC/MS analysis of volatile organic-compounds in human breath *J. Anal. Toxicol*. **15(2)**, 54–59 (1991).

[37] Davies, S., Spanel, P., Smith, D. Quantitative analysis of ammonia on the breath of patients in end-stage renal failure *Kidney Int*. **52(1)**, 223–238 (1997).

[38] Narasimhan, L.R., Goodman, W., Patel, C.K. Correlation of breath ammonia with blood urea nitrogen and creatinine during hemodialysis *Proc. Natl. Acad. Sci. USA* **98(8)**, 4617-4621 (2001).

[39] Endre, Z.H. *et al.* Breath ammonia and trimethylamine allow real-time monitoring of haemodialysis efficacy *Physiol. Meas.* **32**, 115–130 (2011).

[40] Simenhoff, M.L., Burke, J.F., Saukkonen, J.J., Ordinario, A.T., Doty, R. Biochemical profile or uremic breath *N. Engl. J. Med*. **297**, 132–135 (1977).

[41] Giustarini, D., Dalle-Donne, I., Tsikas, D., Rossi, R. Oxidative stress and human diseases: origin, link, measurement, mechanisms, and biomarkers *Crit. Rev. Clin. Lab. Sci.* **46**, 241–81 (2009).

[42] Alhamdani, M.S., Al-Kassir, A.H., Jaleel, N.A., Hmood, A.M., Ali, H.M. Elevated levels of alkanals, alkenals and 4-HO-alkenals in plasma of hemodialysis patients *Am. J. Nephrol*. **26**, 299–303 (2006).

[43] Hermanns, R.C.A. *et al.* Urinary excretion of biomarkers of oxidative kidney damage induced by ferric nitrilotriacetate *Toxicol. Sci*. **43(2)**, 241–249 (1988).

[44] de Lacy Costelo, B. *et al.* A review of the volatiles from the healthy human body *J. Breath Res*. **8**: 014001; doi: 10.1088/1752-7155/8/1/1014001 (2014).

[45] De Vietro, N. *et al.* Relationship between cancer tissue derived and exhaled volatile organic compound from colorectal cancer patients. Preliminary results *J. Pharm. Biomed. Anal*. **180**, 113055-113065 (2020).

[46] Altomare, D.F. *et al*. Chemical signature of colorectal cancer: case–control study for profiling the breath print *BJS open access* **4(6)**, 1189-1199 (2020).

[47] De Vietro, N., Aresta, A.M., Picciariello, A., Rotelli, M.T., Zambonin, C. Determination of VOCs in surgical resected tissues from colorectal cancer patients by solid phase microextraction coupled to gas chromatography–mass spectrometry *Appl. Sci*. **11(15)**: 6910; doi: 10.3390/app11156910 (2021).

[48] Grabowska-Polanowskaa, B. *et al*. Detection of potential chronic kidney disease markers in breath using gas chromatography with mass-spectral detection coupled with thermal desorption method *J. of chromatography A* **1301**, 179-189 (2013).

[49] Grabowska-Polanowskaa, B., Skowrona, M., Miarka, P., Pietrzycka, A., Śliwkaa, I. The application of chromatographic breath analysis in the search of volatile biomarkers of chronic kidney disease and coexisting type 2 diabetes mellitus *J. of Chromatography B* **1060**, 103-110 (2017).

Figures

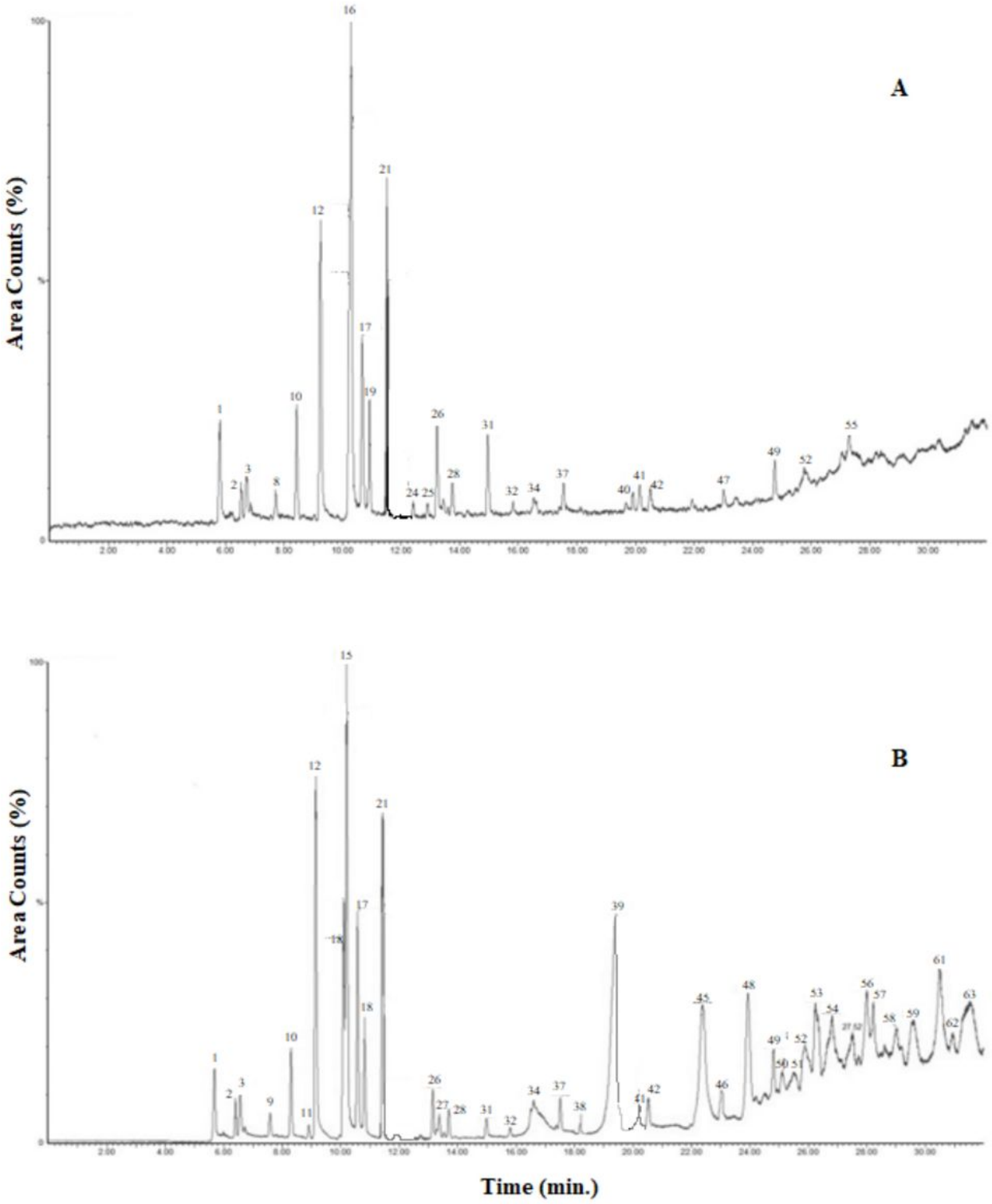

Figure 1

Breath chromatographic profile (SCAN mode) of a healthy subject (A) and of a CKD affected patient before **undergoing** kidney transplant (B).