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Real-Time High-Resolution Tandem Mass Spectrometry Identifies Furan Derivatives in Exhaled Breath

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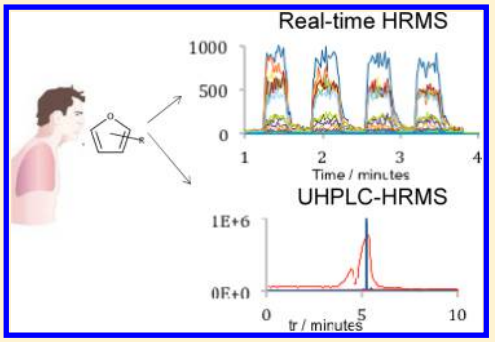
Supporting Information

ABSTRACT: The identification of chemical compounds in exhaled human breath is promising in the search for new biomakers of diseases. However, the analytical techniques used nowadays are not capable of achieving a robust identification, especially in real-time analysis. In this work, we show that real-time high-resolution tandem mass spectrometry (HRMS/MS) is suitable for the identification of biomarkers in exhaled breath. Using this approach, we identified a number of furan derivatives, compounds found in the exhalome whose nature and origin are not yet clearly understood. It is also shown that the combination of HRMS/MS with UHPLC allowed not only the identification of the furan derivatives but also the proper separation of their isomeric forms.



Over the past years, exhaled breath has become an increasingly attractive matrix for metabolomic studies[1−3] and has been proposed as a noninvasive source of biomarkers related to different habits (e.g., smoking[4,5]) and diseases, such as lung cancer[6] or chronic obstructive pulmonary disease.[7] Around 900 compounds have been detected in breath so far, comprising a huge range of chemical functionalities and volatilities.[8] However, proper identification of these compounds is still difficult, and efforts need to be made to develop techniques with better identification capabilities.

Furan derivatives are a set of compounds whose presence in breath has been suggested[8] but is not yet clearly understood. Even though some of them have been related to smoking[9] or fungal infection,[10] their source is still unclear[11−13] since they are not part of the known human metabolome. Proper identification of the whole family of furan derivatives in the exhalome would be useful for a better understanding of the origin and function of these compounds, their source (the main hypothesis being that they are related to the interplay between gut microflora and human metabolism[14]), and their possible usefulness as biomarkers of diseases.

GC/MS has been the preferred technique for (off-line) breath analysis over the last decades.[15] However, other techniques have been developed such as proton-transfer-reaction mass spectrometry (PTR-MS)[16,17] and selected-ion flow-tube mass spectrometry (SIFT-MS).[18,19] These allow the analysis of breath in real time, although they cannot achieve a proper identification because the associated mass spectrometers are usually low-resolution, resulting in convoluted spectra,[17] and are not capable of carrying out tandem MS experiments. In a recent publication,[20] we have shown that ultra high performance liquid chromatography coupled with tandem high-resolution mass spectrometry (UHPLC-HRMS/MS) is suitable for proper and robust identification of biomarkers in exhaled breath condensate (EBC) based on MS/MS fragmentation, on chromatographic retention times, and on the comparison of these parameters with reference standards. We also showed that secondary electrospray ionization (SESI) interfaced to high-resolution mass spectrometry (HRMS) allows the real-time analysis of several biomarkers in breath. SESI[21,22] has proven to be able to detect compounds with significantly higher m/z values and lower volatility that those detected by PTR-MS and SIFT-MS and has also shown promising identification capabilities.

Therefore, the aim of this work is to couple SESI with tandem HRMS for identifying biomarkers in exhaled breath, in this case several furan derivatives. Its combination with UHPLC for resolving isomeric forms is also explored.

EXPERIMENTAL SECTION

Breath Analysis in Real Time. For breath analysis with an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), the inlet was interfaced to a low-flow secondary electrospray ionization source (LF-SESI)[23] that allowed the admission of breath samples through a heated tube. Mass spectra from 50 u to 400 u were recorded in positive ion mode. For MS^2 analyses, an isolation window of 1 u was selected. Some experiments were done on a tripleTOF 5600+ (AB Sciex, Concord, ON, Canada). In this case, the inlet was modified with a heated Teflon tube allowing for admission of exhaled breath, which then intercepted a nanoelectrospray plume formed from 0.1% aqueous formic acid. The nanospray

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voltage was set to 3.6 kV in positive ion mode, the declustering potential was set to 20 V, the curtain gas was nitrogen at 2.4 L min^{-1}, and mass spectra from 40 u to 700 u were recorded. For HRMS/MS analysis, product ion scan experiments with TOF MS at high sensitivity were run. The collision energy was set to 30 V with a spread of ±15 V.

EBC Analysis. EBC samples were collected using a home-built device that was constructed following the recommendations of the ATS/ERS task force.[24] Ten subjects were asked to breathe during 10 min through a cold trap formed by an isopropyl alcohol slush bath cooled to −78.5 °C with dry ice. After collection, the EBC samples (1−2 mL each) were quickly thawed and transferred to polypropylene vials where they were frozen at −20 °C until analysis, when they were thawed to 5 °C. Ten μL were transferred to chromatographic vials without any dilution or other sample preparation and were then injected into an ACQUITY UPLC system (Waters, Milford, MA, USA). Separation took place in a C18 ACQUITY column (2.1 mm × 100 mm, 1.7 μm, Waters) with the following conditions: a 10 min gradient was set from 95/5% to 10/90% of a water/acetonitrile mixture modified with 0.1% formic acid. The chromatographic flow was set to 0.4 mL min^{-1}, and the column was thermostatized at 25 °C. For the coupling with the LTQ Orbitrap spectrometer, the eluent from the column was introduced into an Ion Max source in positive ion mode (4 kV). Nitrogen was used as sheath, auxiliary, and sweep gas at flow rates of 30, 10, and 2 (arbitrary units), respectively. The capillary temperature was set to 275 °C. The LTQ Orbitrap mass spectrometer was working at a resolution of 30,000 at m/z 400. Spectra from 50 u to 2000 u were recorded. The peak at 149.0233 u (protonated phthalic anhydride) was used as internal lock resulting in a mass accuracy below 2 ppm. For the coupling with the TripleTOF, the eluent was introduced into a DuoSpray ion source interface that was operated in the positive ion electrospray mode (5.5 kV). The declustering potential was set at 80 V. Nitrogen was used as nebulizer gas, heater gas (450 °C), and curtain gas with pressures set to 90, 60, and 30 psi, respectively. A full scan was run with a mass range from m/z 40 to 500 u and with a 200 ms accumulation time. Automated calibration was performed using an external calibrant delivery system that infused calibration solution prior to each sample.

Data Analysis. Raw spectra were converted to mzXML files (ProteoWizard Software Foundation) and visualized and analyzed using MZMine 2[25] and mMass 5.5.[26] MS^2 fragmentation pathways were built by means of Sirius 2.[27]

Chemicals. Standards of 2-ethylfuran and 2-heptylfuran were obtained from ABCR (Karlsruhe, Germany), 2-furaldehyde was obtained from Acros Organics (Geel, Belgium), and 2,3-dimethylfuran, 2-acetylfuran, furfuryl alcohol, and 2-pentylfuran were obtained from TCI (Eschborn, Germany). Stock solutions were prepared by diluting the appropriate amount of standard in analytical grade methanol. Water, acetonitrile, and formic acid were of LC-MS quality and were obtained from Sigma-Aldrich (Buchs, Switzerland).

RESULTS AND DISCUSSION

Real-Time HRMS Analysis of Breath. Over the past years, several different approaches have been proposed for establishing a suitable technique that allows real-time analysis of breath as well as proper identification of biomarkers. Our group has shown that SESI coupled to HRMS is a powerful approach to achieve these two goals,[21,22] although the identification step needs to be further improved. To show the capabilities of this real-time technique, exhaled breath samples were measured following the procedures described in the Experimental Section. Real-time m/z values were extracted within isolation windows of ±20 ppm and ±2 ppm from mass spectra obtained on the TripleTOF and the Orbitrap instruments, respectively. Hundreds of features were found, several of them corresponding to compounds previously found in exhaled breath such as major[8] (e.g., acetone, aldehydes, and several other VOCs) and minor[28,29] (e.g., fatty acids, nonvolatile compounds) metabolites. Regarding the target compound, up to 16 features were found to correspond with the expected $[M + H]^+$ ions of furan derivatives (Table 1 and Figure 1). As can be seen, the higher sensitivity of the TripleTOF instrument allowed the detection of more of these features, whereas the higher mass accuracy of the Orbitrap instrument allowed a more confident assignment of the molecular formulae, improving the identification. This identification was further improved by the good fits found for isotopic patterns. It can also be noted that the intensity of the signal, i.e., the concentration of furan derivatives in breath (assuming similar ionization efficiencies and degrees of fragmentation), decreased with the length of the alkyl chain. This trend is similar for both instruments with the exception of C1-furan and acetyl-furan that showed higher abundances in the TripleTOF than in the Orbitrap. This fact is caused by an interfering compound in the isolation window of the TripleTOF that is circumvented by the higher mass accuracy of the Orbitrap (Supporting Information, S2−S3).

Table 1. Furan Derivatives Found in the Real-Time HRMS Analysis of Exhaled Breath[a]

	furan derivatives			
	real-time triple TOF		real-time Orbitrap	
R	$[M + H]^+$	acc/ppm	$[M + H]^+$	acc/ppm
C1	83.0479	15	83.0491	0.5
C2	97.0639	9	97.0648	0.1
C3	111.0796	8	111.0805	0.5
C4	125.0947	11	125.0961	0.1
C5	139.1105	9	139.1118	0.4
C6	153.1260	9	153.1274	0.1
C7	167.1418	7	167.1430	0.2
C8	181.1574	7	181.1588	0.6
C9	195.1752	4	195.1741	1.2
C10	209.1880	10		
C11	223.2047	4		
C12	237.2212	1		
formyl	97.0278	6	97.0283	1.1
acetyl	111.0425	14	111.0439	1.4
acetone	125.0584	10	125.0595	1.6
hydroxymethyl	99.0423	18	99.0440	0.6

[a]R stands for the alkyl chain substituting the furan aromatic ring. acc is the mass accuracy calculated as the ratio of the m/z measurement error to the true m/z.

It should be highlighted that this approach allowed the detection in breath of the whole homologous series of alkylfurans for the first time. This could be of great interest for the study of these compounds as biomarkers and for assessing the source of these compounds as byproducts of fungi or bacteria in guts. It should also be noticed that the series expands up to 237 u, far beyond the mass range of other real-time techniques such as PTR-MS and SIFT-MS (~150 u). In

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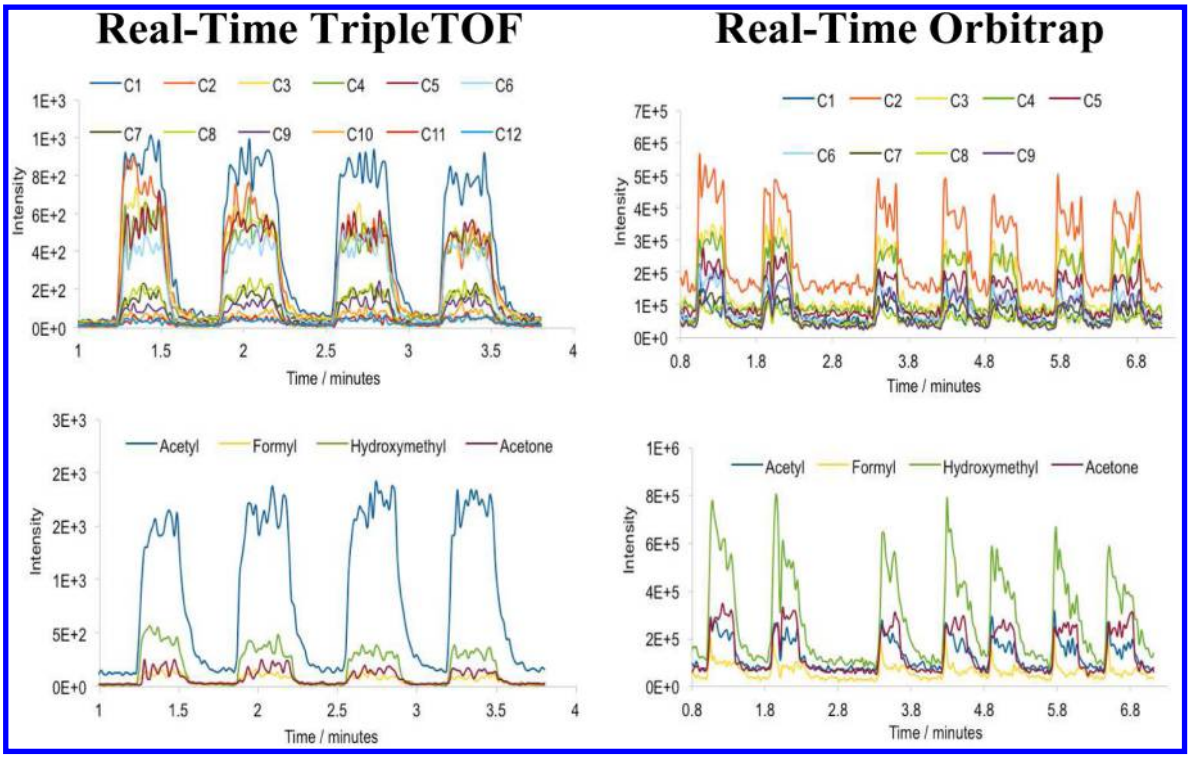

Figure 1. *m/z* features corresponding to the $[M + H]^+$ adducts of several furan derivatives extracted from four (real-time TripleTOF, isolation window: ± 20 ppm) and seven (real-time Orbitrap, isolation window: ± 2 ppm) consecutive breath exhalations.

Table 2. HRMS/MS Product Ions Obtained from the Analysis of the Furan Derivatives

	MS/MS spectra of furan derivatives (normalized intensities of fragments)						
fragments/R-	dimethyl	ethyl	pentyl	heptyl	formyl	acetyl	hydroxymethyl
C_4H_7			5	9			
C_5H_7		28	11	15			44
C_6H_7	100	100	52	48			
C_6H_9			17	50			
C_7H_9			100	100			
C_7H_{11}			24	16			
C_8H_{11}				78			
C_9H_{13}			36	36			
$C_{11}H_{15}$				16			
C_4H_5O	45	36	9	12	100	100	
C_5H_7O			50	52			
C_6H_9O			34	29			
$C_7H_{11}O$			12	17			
$C_8H_{13}O$				13			
$C_9H_{15}O$				7			
C_5H_5O							14
C_4H_7O							100

seven consecutive exhalations, relative standard deviation values ranged from 8% to 14%.

Real-Time HRMS/MS Analysis of Breath. It is widely accepted that a proper chemical identification cannot be achieved by mass spectrometry relying only on molecular mass alone, even with the great mass accuracy available on modern high-resolution instruments, since the number of isobaric compounds is large. One way of increasing the confidence of the identification is to acquire tandem MS spectra that allow not only the study of fragmentation pathways but also the comparison of these spectra with those from standards. Here, we show for the first time that this approach can be readily implemented online with SESI, developing in that way a real-time HRMS/MS analysis of breath, a technique that has never been previously demonstrated.

To achieve this, HRMS/MS experiments of the furan derivatives detected were run in the TripleTOF spectrometer. The duty cycle of this instrument (300 ms per precursor ion) allowed the tandem MS analysis of all 19 compounds studied in a single breath exhalation (20−30 s). These HRMS/MS spectra were compared with those obtained from standards (Table 2) and were used for the generation of fragmentation pathways (Figure 2 and Supporting Information, S4−S9). These fragmentation pathways showed strong similarities, as expected

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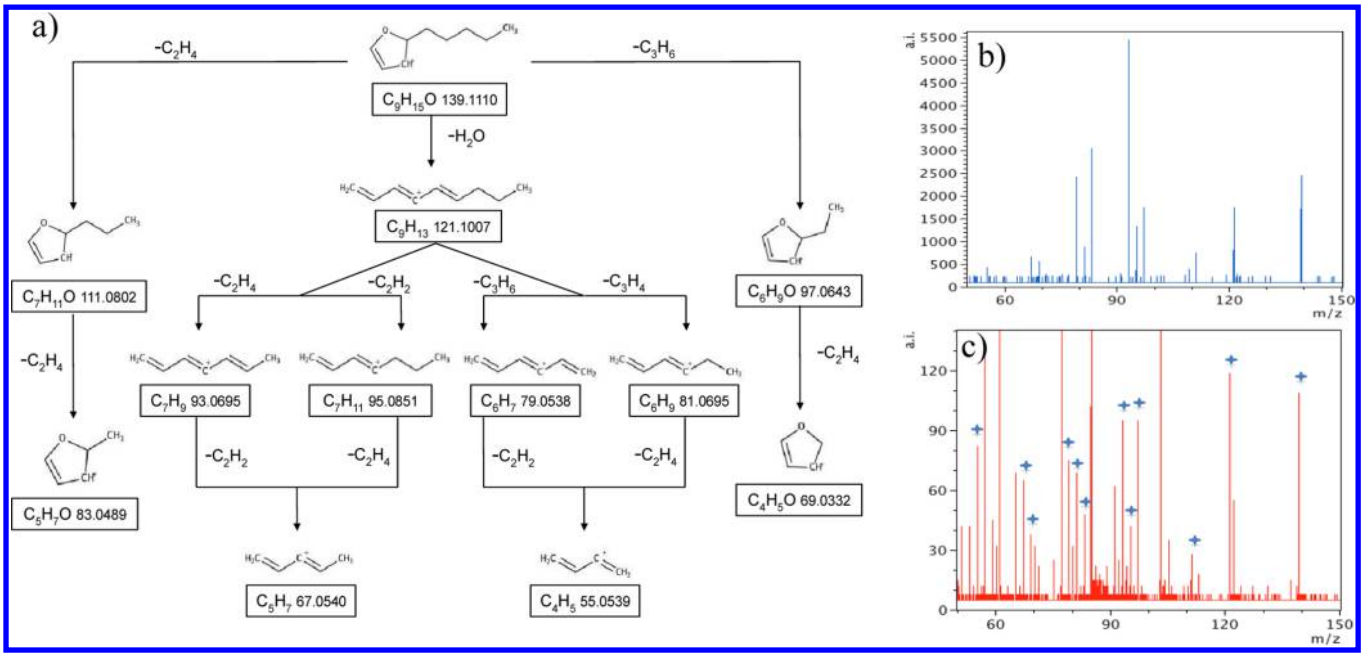

Figure 2. HRMS/MS fragmentation pathway (a) and spectra from a standard (b) and from exhaled breath (c) for the compound identified as pentylfuran. A star symbol marks a match with the standard.

for a homologous series. Alkylfurans showed two main routes of fragmentation, the loss of alkenes of different length from the alkyl chain, finally resulting in the protonated furan ion $[C_4H_5O]^+$, and the loss of water that resulted in the formation of the corresponding unsaturated alkenyl ions that underwent subsequent fragmentation by losses of alkene and alkyne fragments leading to the $[C_6H_7]^+$ and $[C_7H_9]^+$ ions as main products from short and long side chains, respectively. Other furan derivatives such as formyl- or acetyl-furan only showed the loss of the side chain, whereas the fragmentation pathway of hydroxymethylfuran followed a different route. The comparison of HRMS/MS spectra from standards with those obtained from real-time breath analysis showed a clear match with all the fragments from the standards detected in breath (Figure 2). Not surprisingly, there were also other peaks in breath tandem mass spectra that should arise from other compounds isolated in the first stage apart from the furan derivatives (Supporting Information, S2−S3). It should also be highlighted that this comparison with standards confirmed not only the corresponding compounds but also the whole homologous series, because common fragmentation pathways could be found in breath for all the furan derivatives (Supporting Information, S10−S16).

UHPLC-HRMS(/MS) Analysis of EBC. The technique used here for the analysis of breath allowed for real-time identification of several furan derivatives, some of them never identified in breath before. However, the main disadvantage of this approach is that structural isomers are not separated, and the distinction between them based only on tandem MS spectra is not always feasible. Therefore, to improve the identification of structural isomers, we added a separation step by coupling UHPLC to HRMS(/MS), even though with this approach it is not possible to run analyses in real time. Ten EBC samples were collected and analyzed as described in the Experimental Section. Extracted ion chromatograms were plotted, with an isolation window of ±2 ppm, for the previously identified furan derivatives. Chromatographic peaks were found for all the compounds studied with molecular masses higher than 130 u (Table 3). No signals were found for compounds with a mass below 130 u, a disadvantage of the analysis of EBC by means of UHPLC-HRMS that has been previously reported.[20] This may be related to the EBC sampling method, which typically has lower recoveries for more volatile compounds, i.e., those in the low m/z range. As can be seen in Figure 3, at least two or three structural isomers were found for C5 to C12 alkylfurans. These chromatograms were compared with those obtained from standards (Table 3) showing perfect retention time matches for the n-pentyl- and n-heptylfuran chromatographic peaks (Figure 3). These matches allowed us to propose that the last peak of each chromatogram in Figure 3 can be identified as the corresponding member from the homologous series of unbranched alkylfurans. This assignment is strengthened by the fact that these peaks showed a consistent increase in their

Table 3. Chromatographic Peaks, Corresponding to Furan Derivatives, Found in the Analysis of EBC and of Standards by UHPLC-HRMS[a]

	UHPLC-HRMS			
	furan derivatives			standards
R-	t_r/min	$[M + H]^+$	acc/ppm	t_r/min
C2				4.2
C5	4.4/**5.2**	139.1118	0.4	5.2
C6	4.9/**5.7**	153.1274	0.1	
C7	5.3/**6.3**	167.1431	0.3	6.3
C8	5.9/**6.8**	181.1587	0.1	
C9	6.5/6.8/**7.3**	195.1743	0.2	
C10	6.0/7.1/**7.9**	209.1900	0.1	
C11	6.3/7.4/**8.2**	223.2054	1.1	
C12	6.8/7.9/**8.7**	237.2209	1.7	
formyl				1.4
acetyl				2.6

[a]The peaks in bold were assigned to the homologous series of unbranched alkylfurans.

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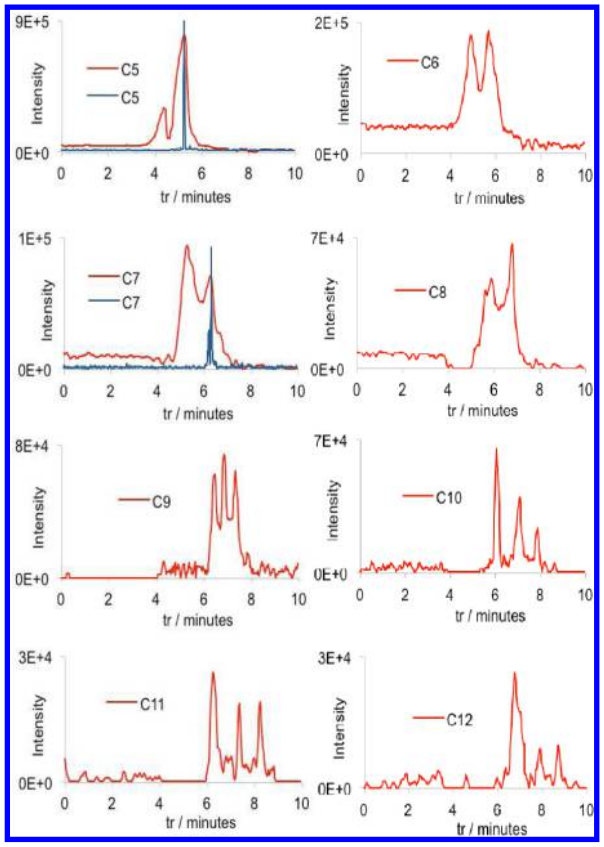

Figure 3. Extracted ion chromatograms for the furan derivatives obtained by the analysis of EBC samples (red) and standards (blue) by UHPLC-HRMS.

retention times with the length of the alkyl chain, an increase that ranged from pentylfuran at 5.2 min to dodecylfuran at 8.7 min. The fact that the peaks from EBC are broader than those from standards may be the result of two different processes, the close elution of unresolved isomers in the EBC samples and the pH difference between the acidic mobile phase and the basic EBC samples. Regarding the less retained peaks of each chromatogram, they very likely arise from isobaric branched alkylfurans. It should be noticed that branched alkanes, when separated by means of reverse-phase liquid chromatography, are usually slightly less retained than the corresponding unbranched ones.[30] However, a proper identification of these branched compounds was not possible because of the unavailability of commercial standards.

Quantification of Furan Derivatives in EBC and Exhaled Breath. One of the main disadvantages of the technique proposed here is its lack of quantitative information, especially when comparing to PTR-MS and SIFT-MS. One way to address this problem is to quantify target compounds in EBC samples by UHPLC-HRMS and then convert these values to gas-phase concentrations using the following conversions:[31] 1.8 ± 0.5 mL EBC ≙ 119 ± 25 L breath ≙ 15 min; we assume a 100% recovery in the EBC sampling step. Following this approach, mean concentrations of 75 pptv and 10 pptv were found for C5-furan and C7-furan, respectively. These values are in good agreement with those found previously for C2-furan (250−3200 pptv)[32] and C5-furan (37 pptv),[33] taking a decrease of the concentration with the length of the alkyl chain into account. Due to the uncertainties associated with this approach, conclusions can only be made about the order of magnitude of the gas-phase concentrations of these compounds: these are in the range of 10−100 pptv for the long-chain alkylfurans and 100−1000 pptv for the short-chain ones.

CONCLUSIONS

We showed that real-time HRMS/MS is a powerful analytical technique not only for the analysis of exhaled breath in real time but also for the proper identification of biomarkers found in the exhalome, as demonstrated by applying this technique to the identification of furan derivatives, a set of compounds related with gut microflora whose presence and origin in breath is not yet clearly understood. In addition, the combination of HRMS/MS with UHPLC, even though it cannot be applied in real time, allowed the identification of different isobaric structural isomers.

ASSOCIATED CONTENT

Supporting Information

Schemes of the SESI sources, real-time breath HRMS isolation windows, HRMS/MS spectra and fragmentation pathways from standards and HRMS/MS spectra from exhaled breath. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b01509.

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Notes

The authors declare no competing financial interest.

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