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Identification of 2-Alkenals, 4-Hydroxy-2-alkenals, and 4-Hydroxy-2,6-alkadienals in Exhaled Breath Condensate by UHPLC-HRMS and in Breath by Real-Time HRMS

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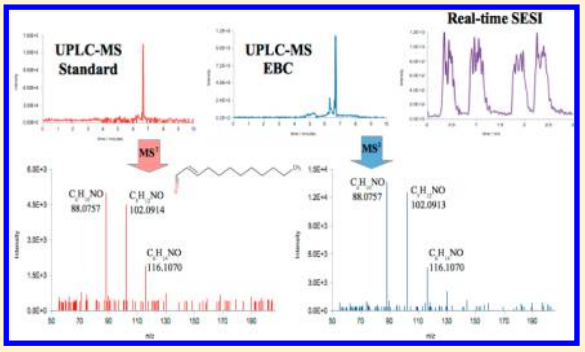
Supporting Information

ABSTRACT: In recent years, breath analysis in real time has become a noninvasive alternative for the diagnosis of diseases and for molecular fingerprinting of exhaled breath. However, the techniques used lack the capabilities for proper identification of the compounds found in the exhalome. Here, we report the use of UHPLC-HRMS as a tool for the identification of several aldehydes (2-alkenals, 4-hydroxy-2-alkenals, and 4-hydroxy-2,6-alkadienals), biomarkers of lipid peroxidation, in exhaled breath condensate of three healthy subjects (N = 3). Some of the aldehydes studied have never been identified before. Their robust identification is based on retention times, on the generation of fragmentation trees from tandem mass spectra, and on the comparison of these parameters with standards. We also show that the identified compounds can be analyzed and confirmed by MS/MS in breath in real time and, therefore, they could be used as biomarkers for the rapid and noninvasive diagnosis of related diseases.



Breath analysis has gained attention in recent years as a noninvasive technique for diagnosis of diseases, such as lung cancer,[1] asthma[2] or chronic obstructive pulmonary disease (COPD)[3] and for molecular fingerprinting of exhaled breath.[4] Although exhaled breath is mainly composed of N_2, O_2, CO_2, and H_2O; a huge set of volatile, semivolatile and nonvolatile compounds (~900 compounds)[5] have been identified in concentrations ranging from hundreds of ppm to ppt. The main source of volatile compounds is the equilibrium reached in the alveoli between blood, containing metabolic products from the whole body, and exhaled breath. However, this fact does not explain the presence of nonvolatile compounds, whose source is supposed to be the formation of aerosol droplets from the airway lining fluid.[6] Nevertheless, the metabolic origin of almost all of these volatile and nonvolatile compounds is still unclear and determining their origins is an active field of research.

The most widely used technique for breath analysis is GC-MS.[7] This technique allows the separation of the compounds in breath and also their identification by comparison with spectral libraries. However, GC-MS cannot be applied in real time and it is not suitable for nonvolatile compounds. Different approaches have been considered to circumvent these problems, mainly proton-transfers-reaction mass spectrometry (PTR-MS),[8] selected-ion flow-tube mass spectrometry (SIFT-MS),[9] and ion mobility spectrometry (IMS-MS).[10] In the past years, secondary electrospray ionization (SESI) has also been successfully applied for the analysis in real time of volatile and nonvolatile compounds in breath.[11] Although these techniques can be applied in an online fashion and show good sensitivity, the fact that there are neither chromatographic separation nor spectral libraries for comparison results in severe difficulties when attempting compound identification. This fact does not prevent these techniques from being proposed as diagnostic tools,[12] but it is clear that an important piece of useful biological information is lost. One alternative to solve this lack of identification is to collect exhaled breath condensate samples and run orthogonal analyses with a technique capable of proper identification, for example, ultra high performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS).

Several aldehydes, mainly, alkanals, 2-alkenals, and 4-hydroxy-2-alkenals, with medium-to-low volatilities, have been revealed as a group of human metabolites produced by the lipid peroxidation of polyunsaturated fatty acids that form part of membrane phospholipids.[13] Their concentrations in biological fluids has been proposed as a way of assessing oxidative stress, and therefore, they are usually listed as biomarkers of such a state.[14] In that sense, malonaldehyde,[15] 4-hydroxy-2-hexenal (4-HHE),[16] and especially 4-hydroxy-2-nonenal (4-HNE)[17] have undoubtedly been the most studied compounds.

The aim of this article is to show the capabilities of UHPLC-HRMS for the proper identification of biomarkers in exhaled

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Table 1. Chromatographic Signals, Found in EBC by UHPLC-HRMS, Identified As 2-Alkenals, 4-Hydroxy-2-alkenals, and 4-Hydroxy-2,6-alkadienals[a]

2-alkenals ($C_xH_{2x-2}O$)							
carbon atoms	t_r (min)	$[M + NH_4^+]$	acc. (ppm)	$[M + H^+]$	acc. (ppm)	$[M + HCOO^-]$	acc. (ppm)
9	5.4	**158.1540**	0.375	141.1274	0.060	185.1182	0.637
10	5.6	172.1696	0.054			199.1339	0.342
11	6.1	186.1852	0.318			213.1496	0.085
12	6.6	**200.2010**	0.545	183.1743	0.228	227.1651	0.168
13	6.8	214.2166	0.276			241.1807	0.905
14	7.6	228.2323	0.478			255.1965	0.268
		ESI+				ESI−	
4-hydroxy-2-alkenals ($C_xH_{2x-2}O_2$)							
carbon atoms	t_r (min)	$[M + NH_4^+]$	acc. (ppm)	$[M + H^+]$	acc. (ppm)	$[M + HCOO^-]$	acc. (ppm)
6	4.1					**159.0662**	0.518
7	4.4					173.0818	0.132
8	4.6					**187.0976**	0.093
9	4.8	**174.1489**	0.257	157.1221	1.312	**201.1132**	0.162
10	5.1	188.1647	1.034	171.1382	1.424	**215.1288**	0.384
11	5.3	202.1801	0.274	185.1538	1.046	229.1444	0.579
12	5.5	216.1958	0.025	199.1694	0.721	243.1601	0.341
13	5.8	230.2115	0.193	213.1850	0.439	257.1756	0.905
14	6.1	**244.2272**	0.386	227.2007	0.631	271.1913	0.675
15	6.4	258.2430	0.946	241.2164	0.802	**285.2069**	0.817
16	6.9	272.2585	0.346	255.2321	0.953	299.2225	0.946
		ESI+				ESI−	
4-hydroxy-2,6-alkadienals ($C_xH_{2x-4}O_2$)							
carbon atoms	t_r (min)	$[M + NH_4^+]$	acc. (ppm)	$[M + H^+]$	acc. (ppm)	$[M + HCOO^-]$	acc. (ppm)
7	4.5					**171.0662**	0.482
8	4.6					185.0818	0.716
9	4.9	**172.1332**	0.030	155.1067	0.283	**199.0974**	0.917
10	4.9	186.1488	0.297	169.1224	0.555	213.1133	0.316
11	5.5	200.1648	1.472	183.1380	0.239	227.1288	0.364
12	5.8	214.1801	0.259	197.1537	0.475	241.1444	0.550
13	5.9	**228.1961**	1.291	211.1693	0.206	**255.1601**	0.325
14	6.1			225.1851	0.859		
15	6.2	**256.2273**	0.759	239.2007	0.600	283.1915	0.060
		ESI+				ESI−	

[a]For m/z features in bold MS^2 or MS^3 spectra were obtained

breath condensate (in this work three groups of aldehydes) and to explore the possibilities of analyzing these compounds in breath in real time.

EXPERIMENTAL SECTION

EBC Sampling and Analysis. EBC samples were collected using a homemade device following the recommendations suggested by the ATS/ERS task force.[18] Three healthy nonsmoking subjects were asked to breathe during 10 min through a cold trap that was cooled to −78.5 °C using an isopropanol/dry ice slush bath. The EBC samples collected (1−2 mL each) were quickly thawed and transferred to polypropylene vials where they were stored at −20 °C until analysis.

For analysis, EBC samples were thawed and kept at 5 °C. Ten microliters were transferred to chromatographic vials without no need for dilution or any other sample preparation procedure, and were then injected into the ACQUITY UPLC system (Waters, MA, USA) where separation took place in a C18 ACQUITY column (2.1 mm × 100 mm, 1.7 μm, Waters, MA, USA). Chromatographic conditions were as follows: 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. The eluent from the column was introduced into an electrospray ionization source (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. Ions were introduced into a LTQ Orbitrap (Thermo Fisher Scientific, MA, USA) mass spectrometer working at a resolution of 30 000 at m/z 400. Spectra from 50 u to 2000 u were recorded. For MS^n analyses, an isolation window of 1 u was selected. Peaks at 149.0233 (protonated phthalic anhydride) and 112.9856 ($[2\times$ formate + Na$]^-$) were used as internal locks in positive and negative mode, respectively, resulting in a working mass accuracy below 1 ppm.

Breath Analysis in Real Time. The inlet of a LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, MA, USA) was modified to set a new low flow secondary electrospray ionization source (LF-SESI)[19] that allowed the admission of breath samples through a heated tube (Poster AS-106; Swiss Chemical Society Fall Meeting 2014 11th

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September, Zurich (Switzerland)). Mass spectra from 50 u to 400 u were recorded in positive-ion mode.

Data Analysis. Raw spectra were converted to mzXML files (ProteoWizard Software Foundation) and visualized and analyzed using MZMine 2 (Okinawa Institute of Science and Technology) and ChemCalc (EPF Lausanne). MS^n fragmentation trees[20] were built by means of Sirius[2] (University of Jena).

Chemicals. Standards of 2-nonenal and 2-dodecenal were obtained from Sigma-Aldrich (MO, USA), and 4-hydroxy-2-hexenal (4-HHE) and 4-hydroxy-2-nonenal (4-HNE) were obtained from Cayman Chemical (MI, USA). Stock solutions were prepared by diluting the appropriate amount of standard in analytical grade methanol. Working solutions were prepared daily by diluting stock solutions in aqueous ammonium formate buffer. Water, acetonitrile and formic acid were of LC-MS quality and were obtained from Sigma-Aldrich.

RESULTS AND DISCUSSION

UHPLC-HRMS Analysis of Aldehydes in Exhaled Breath Condensate. Previous work in our group has shown that breath analysis in real time by means of SESI can be used for monitoring internal body time changes,[21] for establishing an individual breath fingerprint,[4] and for the noninvasive diagnosis of lung diseases.[12] However, it was not possible to properly identify the vast majority of compounds associated with the significant m/z values that allowed the separation between groups.

To show the capabilities of UHPLC-HRMS for solving this lack of identification, three different EBC samples were analyzed according to the procedure detailed in the Experimental Section. Extracted ions chromatograms were plotted, with an isolation window of ±1 ppm, for several 2-alkenals, 4-hydroxy-2-alkenals, and 4-hydroxy-2,6-alkadienals. For almost all the aldehydes from 6 to 16 carbons, chromatographic signals were found for $[M + NH_4]^+$ and $[M + H]^+$ adducts in positive-ion mode ESI and for $[M + HCOO]^-$ adducts in negative-ion mode ESI (Table 1). As can be seen, the main limitation of UHPLC-HRMS is that compounds with less than 6 carbon atoms are not detected, probably because of their high volatilities. On the other hand, the method is capable of detecting aldehydes with up to 16 carbon atoms, compounds that are rarely analyzed by GC-MS or SIFT-MS because of their high molecular weights (~300 u) and their low volatilities. Figure 1 shows the chromatographic separation achieved for the chromatographic signals identified as 4-hydroxy-2-alkenals from C6 to C16.

One of the main advantages of UHPLC-HRMS is the possibility of acquiring tandem mass spectra that greatly increase the structural information obtained and, therefore, allow much better compound identification. Sixteen out of 48 m/z features (highlighted in bold in Table 1) were selected to obtain MS^2 spectra and, in some cases, MS^3 spectra. Tandem mass spectra were used for generating fragmentation trees for the compounds analyzed. Fragmentation trees have been recently proposed as a tool to identify unknown small molecules by means of mass spectrometry.[20]

The fragmentation trees for 2-alkenals showed neutral losses of alkenes that are generally the preferred losses for alkyl chains of different length,[22] these neutral losses being fully compatible with the proposed structures. In addition, the absence of a neutral loss of water suggested that the presence of an alcohol functional group in the molecule is unlikely. Figure 2 shows the fragmentation tree proposed for the m/z feature identified as 2-nonenal.

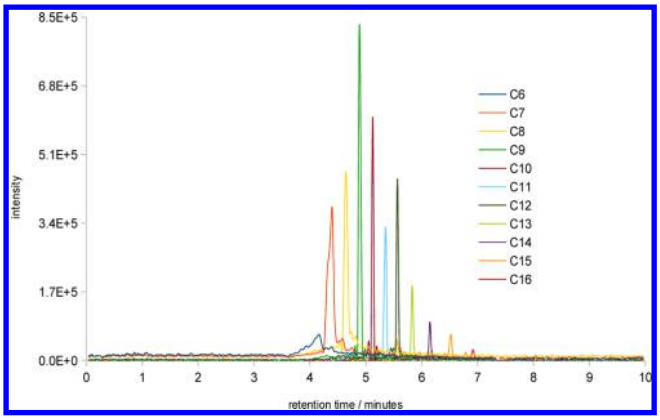

Figure 1. Extracted ion chromatograms for the m/z features identified as the $[M+HCOO]^-$ adducts of 4-hydroxy-2-alkenals from C6 to C16 in exhaled breath condensate.

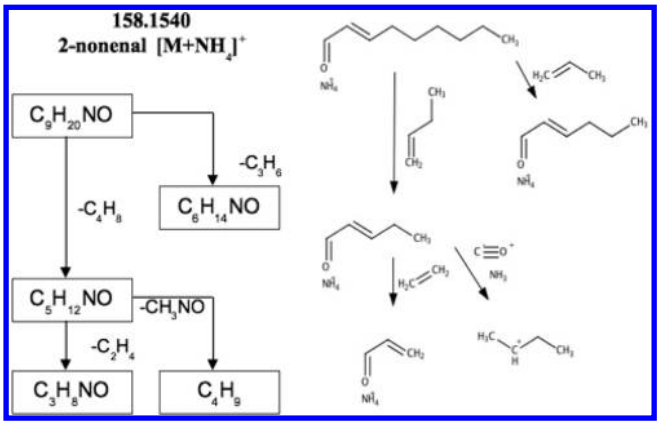

Figure 2. Fragmentation tree and fragmentation pathway obtained from the MS^2 spectrum of the m/z feature 158.1540 identified as the ammonium adduct of 2-nonenal.

In the case of 4-hydroxy-2-alkenals, the positive-ion mode ESI fragmentation trees showed neutral losses of alkenes, like the nonhydroxylated aldehydes, but also very intense neutral losses of water related to the hydroxyl functional group. In negative-ion mode ESI, fragmentation trees showed neutral losses of water, also related to the hydroxyl functional group, and neutral losses of carbon dioxide, that may arise from the formate anion which forms adducts with the aldehydes. The MS^3 spectra obtained from the $[M + HCOO - H_2O - CO_2]^-$ anions showed neutral losses of alkenes and alkynes which are compatible with losses of fragments of different length from the alkyl chains of the fatty aldehydes. Figure 3 shows the fragmentation tree identified as 4-hydroxy-2-nonenal.

Finally, 4-hydroxy-2,6-alkadienals showed, in positive-ion mode ESI, the same main neutral loss of water as 4-hydroxy-2-alkenals and a neutral loss of ammonia that can be explained by the breakdown of the precursor ammonium adduct. The negative-ion mode ESI spectra showed neutral losses of high intensity of carbon dioxide that may arise from the formate adduct.

The fragmentation trees and pathways for all the 16 m/z features studied can be found in the Supporting Information section.

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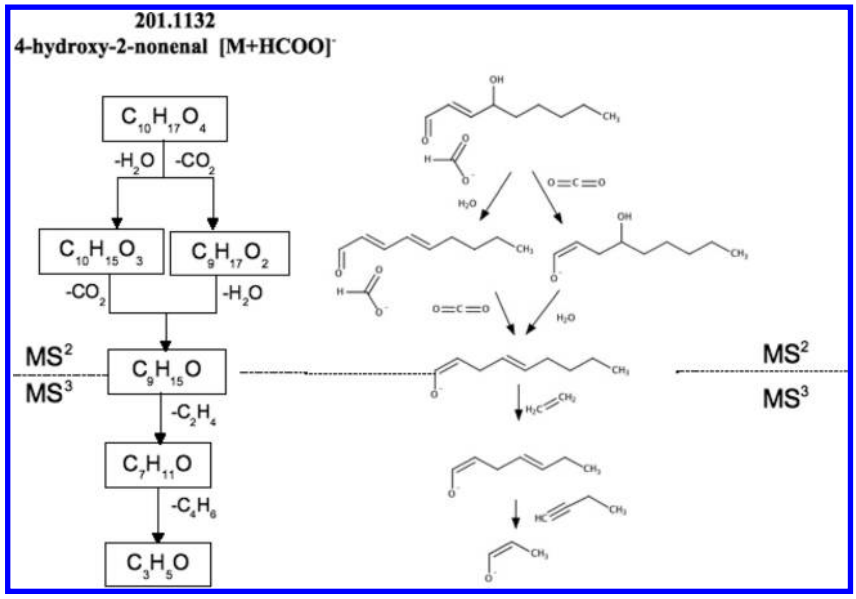

Figure 3. Fragmentation tree and fragmentation pathway obtained from the MS^2 and MS^3 spectra of the *m/z* feature 201.1132 identified as the formate adduct of 4-hydroxy-2-nonenal.

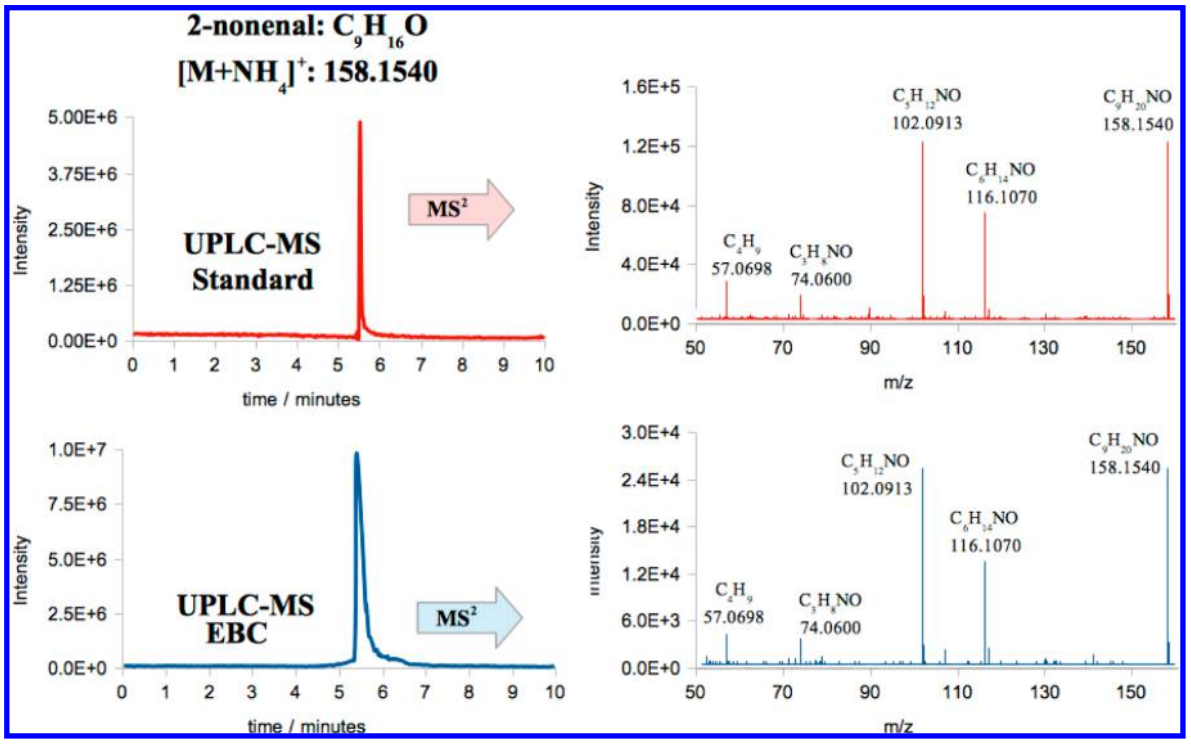

Figure 4. UHPLC-HRMS extracted ion chromatograms and MS^2 spectra obtained in the analysis of an standard of 2-nonenal and a EBC sample.

UHPLC-HRMS Analysis of Standards for Comparison Purposes. To further improve the fidelity of the identification of the groups of aldehydes studied, a set of standards were analyzed by UHPLC-HRMS to compare their retention times and their MS and tandem MS spectra with those obtained with EBC samples. Unfortunately, this approach cannot be used for all the compounds studied since the number of available standards is limited. In this work, we propose the use of four standards for comparison purposes: two of them for comparison between EBC samples and 2-alkenals (C9 and C12) and another two for 4-hydroxy-2-alkenals (C6 and C9). In the case of 4-hydroxy-2,6-alkadienals no standards were available.

MS analyses of the standards (concentration range = 1−2 ng mL^{-1}) showed excellent matches, regarding retention times, for the signals of the $[M+NH_4]^+$ and $[M + H]^+$ adducts of 2-nonenal (Figure 4) and 2-dodecenal. Similarly, the retention times of the $[M+HCOO]^-$ adducts of 4-HHE and 4-HNE (Figure 5) also matched. A very good correlation was also observed for the subsequent MS^2 spectra (Figures 4 and 5) and, in the case of 4-HNE, also for the MS^3 spectra (Figure 5).

These comparisons with standards not only ensure the proper identification of these four compounds but also improve

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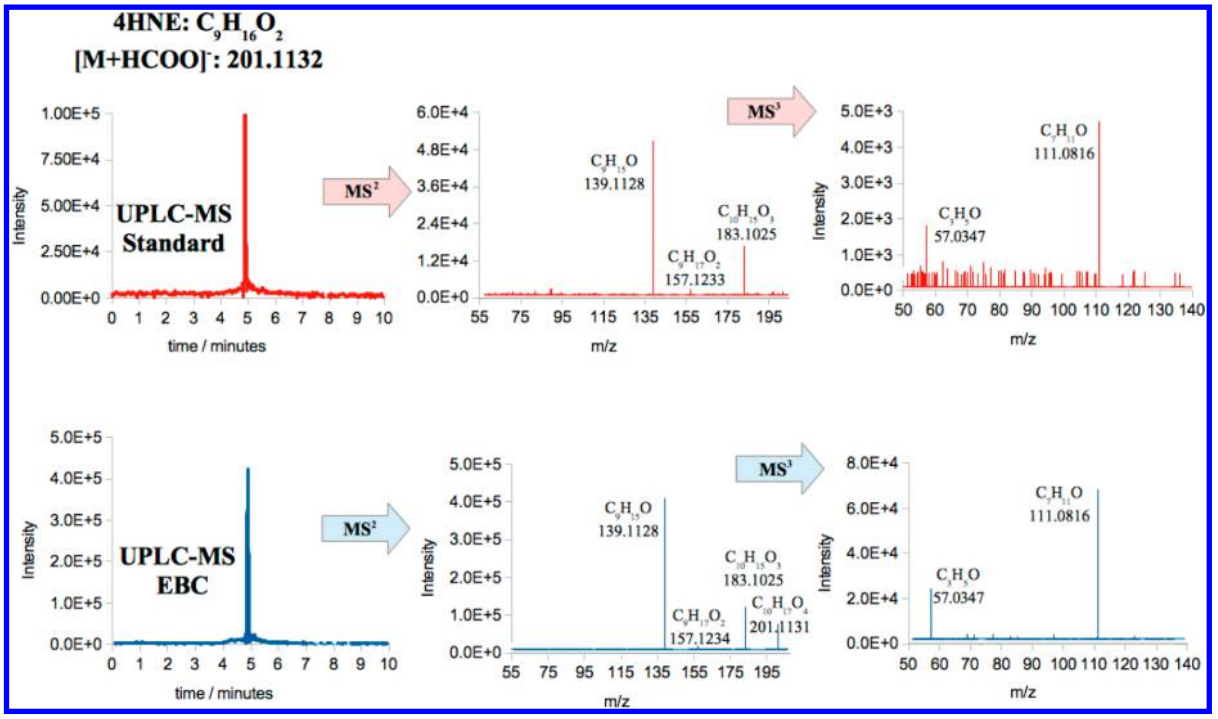

Figure 5. UHPLC-HRMS extracted ion chromatograms and MS^2 and MS^3 spectra obtained in the analysis of an standard of 4HNE and a EBC sample.

Table 2. *m/z* Features, Corresponding to the Identified Aldehydes, Found in Breath Analysis in Real Time[a]

	2-alkenals				4-hydroxy-2-alkenals				4-hydroxy-2,6-alkadienals			
carbon atoms	$[M + NH_4^+]$	acc. (ppm)	$[M + H^+]$	acc. (ppm)	$[M + NH_4^+]$	acc. (ppm)	$[M + H^+]$	acc. (ppm)	$[M + NH_4^+]$	acc. (ppm)	$[M + H^+]$	acc. (ppm)
6					132.1018	0.7960	**115.0752**	1.3560				
7					146.1176	0.3070	129.0908	1.5960	144.1017	1.4230	127.0753	0.4410
8					160.1334	1.2160	143.1067	0.3060	158.1178	0.5770	141.0912	1.3740
9	**158.1539**	0.2570	**141.1273**	0.6490	174.1490	0.8310	**157.1224**	0.5970	172.1332	0.0300	155.1065	1.0070
10	172.1694	1.1080	155.1430	0.2680	188.1642	1.6230	171.1382	1.4240	186.1491	1.3140	169.1222	0.6280
11	186.1856	1.9290	169.1587	0.0490	202.1800	0.7690	185.1538	1.0460	200.1645	0.0270	183.1382	1.3310
12	200.2013	2.0440	183.1745	0.8640	216.1959	0.4370	199.1689	1.7890	214.1803	0.6750	197.1536	0.0320
13			197.1902	1.0560			213.1848	0.4990	228.1954	1.7770	211.1691	0.7410
14			211.2055	0.6720			227.2002	1.5690	242.2112	1.0550	225.1846	1.3610
15					258.2426	0.6030	241.2164	0.8020	256.2267	1.5830	239.2004	0.6540
16					272.2582	0.3460	255.2321	0.9530				
	ESI+				ESI+				ESI+			

[a]For *m/z* features in bold MS^2 spectra were obtained.

the identification for their whole group, keeping in mind that previously obtained fragmentation trees showed related branches (see Supporting Information) that clearly demonstrate that all the compounds belong to a homologous series. This is strengthened by the fact that the retention times also show an increase related with the length of the fatty aldehydes (Table 1).

Real-Time HRMS Analysis of Aldehydes in Breath. The determination of aldehydes in breath such as saturated aldehydes,[23,24] or 4-HHE[25] has been proposed as a diagnostic tool for different diseases related with oxidative stress. In addition, some aldehydes have also been determined in EBC.[26,27] However, most of the aldehydes identified in this work have only been found *in vitro*[28,29] or have never been described before. After checking the capabilities of UHPLC-HRMS for properly identifying these compounds by analyzing exhaled breath condensate, it was the second aim of this work to show that all the aldehydes identified in EBC can also be detected in breath in real time, a technique more suitable for diagnosis purposes.

To achieve this, breath was analyzed in real time as detailed in the Experimental Section. 48 *m/z* features related to the previously identified 2-alkenals, 4-hydroxy-2-alkenals, and 4-hydroxy-2,6-alkadienals were found, in positive-ion mode, and assigned to 2-alkenals from C9 to C14, 4-hydroxy-2-alkenals from C9 to C16 and 4-hydroxy-2,6-alkadienals from C9 to C15 (Table 2). The *m/z* values were assigned to $[M + H]^+$ (Figure 6) and $[M + NH_4]^+$ adducts of the corresponding aldehydes with a mass accuracy below 2 ppm in all cases (Table 2).

To further confirm the identity of the aldehydes detected in real time, MS^2 analyses of some peaks from breath were also run, in real time (highlighted in bold in Table 2). Precursor ions were isolated in the ion trap within a range of ±1 u. Such an isolation window usually contained several peaks apart from

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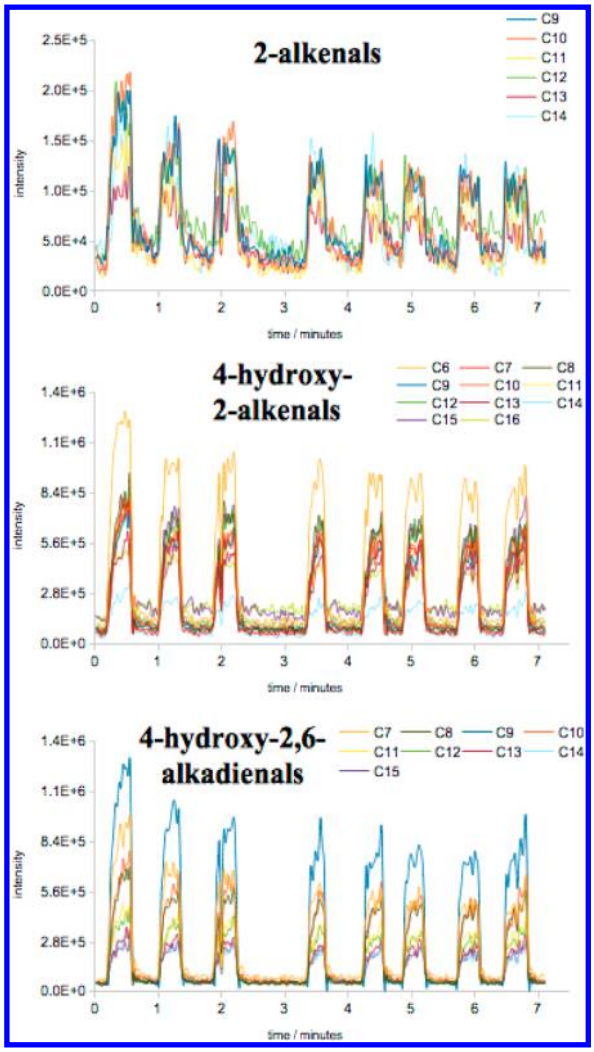

Figure 6. m/z features corresponding to the $[M + H]^+$ adducts of the previously identified aldehydes extracted from eight consecutive analysis of breath in real time, using HRMS.

the targeted aldehyde. Therefore, fragmentation peaks were found that arose not only from the identified aldehydes, but also from other unidentified compounds in the range of ±1 u (Supporting Information). This fact highlights the importance of the UHPLC separation for achieving identification with high confidence. In any case, some peaks were identified as fragments from the $[M + NH_4]^+$ and $[M + H]^+$ adducts of the studied aldehydes and, therefore, it was possible to build fragmentation trees for these compounds. The comparison of these fragmentation trees with those obtained from standards by UHPLC-HRMS correlated very well, as can be seen in Supporting Information for 2-nonenal and 4-HNE, which further confirmed that the compounds detected in real time were the previously identified aldehydes and not any isobaric compounds.

Although the main aim of the real-time HRMS analysis in breath was to show that the compounds previously identified by UHPLC-HRMS could be detected in real-time, it is also important to highlight that the LF-SESI source used in this work did not present any limitation for the analysis of aldehydes with less than 6 carbon atoms showed by the UHPLC-HRMS method. Therefore, this source allowed not only the real-time HRMS breath analysis of the previously identified aldehydes, but also of interesting highly volatile aldehydes such as malonaldehyde ($[M + H]^+ = 73.0283$) and propionaldehyde ($[M + H]^+ = 59.0491$) as can be seen in the Supporting Information.

Finally, to highlight the relevance of the identification of these groups of identified aldehydes, their m/z features were compared with those obtained in a previous work in our group.[12] In that work, 51 m/z features were found to be exhaled at significantly ($p < 0.05$) higher concentrations by COPD subjects than smoking controls. Seventeen of those features (33%) match the ones identified here as aldehydes.

CONCLUSIONS

The use of UHPLC-HRMS has allowed the identification of a set of aldehydes, metabolites of lipid peroxidation, in EBC. This identification has been possible thanks to the capabilities of UHPLC-HRMS for identifying unknowns by means of retention times, tandem mass spectra that allowed the generation of fragmentation trees, and comparison with standards. It was also demonstrated that these compounds can be analyzed, and confirmed by tandem MS, in breath in real time, a technique that could be of great interest for diagnosis of diseases related with oxidative stress, such as COPD or cancer.

ASSOCIATED CONTENT

Supporting Information

Fragmentation trees and pathways, real-time MS^2 spectra, and real-time MS traces. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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