Mass-Spectrometric Detection of Omega-Oxidation Products of Aliphatic Fatty Acids in Exhaled Breath

*Martin Thomas Gaugg, Tobias Bruderer, Nora Nowak, Lara Eiffer, Pablo Martinez-Lozano Sinues, Malcolm Kohler, and Renato Zenobi***.** *Anal. Chem.*, **2017**, *89* (19), pp 10329–10334**. DOI:** 10.1021/acs.analchem.7b02092

https://pubs.acs.org/doi/full/10.1021/acs.analchem.7b02092?src=recsys#

Chemicals and Solvents

Solvents used for UHPLC-MS measurements were water (LC/MS grade, Merck Millipore), methanol (LC/MS grade, Fisher Chemical), and formic acid (≥98%, Aldrich Fine Chemicals). Purchased standards were 8-hydroxyoctanoic acid (≥98.5%, Fluka Chemie AG), 2-hydroxyoctanoic acid (>98%, AlfaAesar), 10-hydroxydecanedioic acid (Apollo Scientific Ltd.), 11-hydroxy-undecanoic acid (96%, Aldrich Fine Chemicals), pentanedioic acid (99%, Aldrich Fine Chemicals), heptanedioic acid (≥99%, Fluka Chemie AG), octanedioic acid (≥98%, Fluka Chemie AG), decanedioic acid (≥95%, Fluka Chemie AG), undecanedioic acid (>97%, Tokyo Chemical Industry), dodecanedioic acid (99%, Acros Organics), and tetradecanedioic acid (99%, Aldrich Fine Chemicals). Since none of the ω-oxoalkanoic acids were commercially available, 10-oxodecanoic acid was synthesized in our lab as described in the literature, and the identity of the product was confirmed by NMR (see Supporting Information in the original publication).

Exhaled Breath Condensate Sampling

EBC was collected using a custom-built sampling device following the guidelines by the ATS/ERS task force. (25) Two healthy, non-smoking subjects were asked to exhale through a Teflon tube connected to a glass cold trap (T = −78.5 °C, isopropanol/dry ice) twice for approximately 45 min, yielding 2 × 8 mL each. The collected EBC samples were pooled (total volume of 32 mL); 0.5 mL was extracted and stored at 4 °C and the remainder lyophilized to dryness (24 h, p = 0.05 mbar, T = −40 °C). The residue was then dissolved in a mixture of 142.5 μL of the previously aliquoted EBC and 7.5 μL of methanol, acidified with 0.1% formic acid, and directly subjected to UHPLC-HRMS/MS analysis.

UHPLC-HRMS/MS Analysis

UHPLC separation was done on an ACQUITY UPLC system (I-Class, Waters, MA, USA) with an ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm, Waters) with a precolumn filter. The flow rate was set to 500 μL min–1 using a binary mixture of solvent A (water with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid). The following gradient was used: 5% B (1 min), 5–95% B (3 min), 100% B (1 min), and 5% B (2 min). The column was kept at 30 °C and the sampler at 5 °C. All standards were dissolved in methanol/water = 5/95 (v/v) with 0.1% formic acid at concentrations of 0.1–10 μg mL–1 and analysed with an injection volume of 10 μL.

A commercial quadrupole time-of-flight mass spectrometer (AB Sciex, Triple TOF 5600+, Concord, ON, Canada) was used to measure a mass range of m/z = 40–500 in negative ion mode, with 1–5 ppm mass accuracy. Product ion scans (PIS) and data dependent acquisitions (DDA) were used for fragment spectra acquisition. The total cycle time was kept at 450 ms to obtain at least 12 points/peak (minimal LC peak width = 6 s) with 80 ms for TOF MS and 320 ms for 1–4 product ion scans acquired with a collision energy CE = 10/40/70 eV.

Real-Time SESI-HRMS Breath Analysis

Real-time breath analysis was performed using a commercial low-flow SESI-ion source coupled to the TripleTOF 5600+ mass spectrometer that recorded spectra from 50 to 350 Da in negative ion mode. To gain additional certainty about compound identity, online MS2 spectra were recorded for selected compounds in breath. The heated breath sampling tube (130 °C) was equipped with a low-Δp mass flow controller (Bronkhorst, Switzerland), which was set to 200 mL min–1. To enable sampling of end-tidal breath, a flow-splitter was installed front-end. A database of online breath measurements of 146 people with no symptoms of lung diseases, which were measured in a similar fashion on a different SESI-source (see SI), was used to analyse correlations between the metabolites. The peak intensities of this control data set were batch corrected using a moving-median single point correction (spanning = 20 samples, measurement date as covariant) for each m/z value, to reduce confounding time drifts.

Online Detection of ω-Oxidation Metabolites in Exhaled Breath

A key strength of online analysis of exhaled breath is the direct detection of vapours without any sample preparation, which reduces confounding influences. It is therefore a promising tool to get insight into metabolism in a rapid, non-invasive, and painless fashion. Using the SESI setup described above, we were able to detect real-time signals of ω-hydroxyalkanoic acids ($C_xH_{2x}O_3$), ω-oxoalkanoic acids ($C_xH_{2x-2}O_3$), and alkanedioic acids ($C_xH_{2x-2}O_4$) with carbon chain lengths of 5–15 in exhaled breath. The detected molecules thereby cover all transformation steps in the degradation of linear aliphatic fatty acids via the ω-oxidation pathway, as shown in Figure 1a.

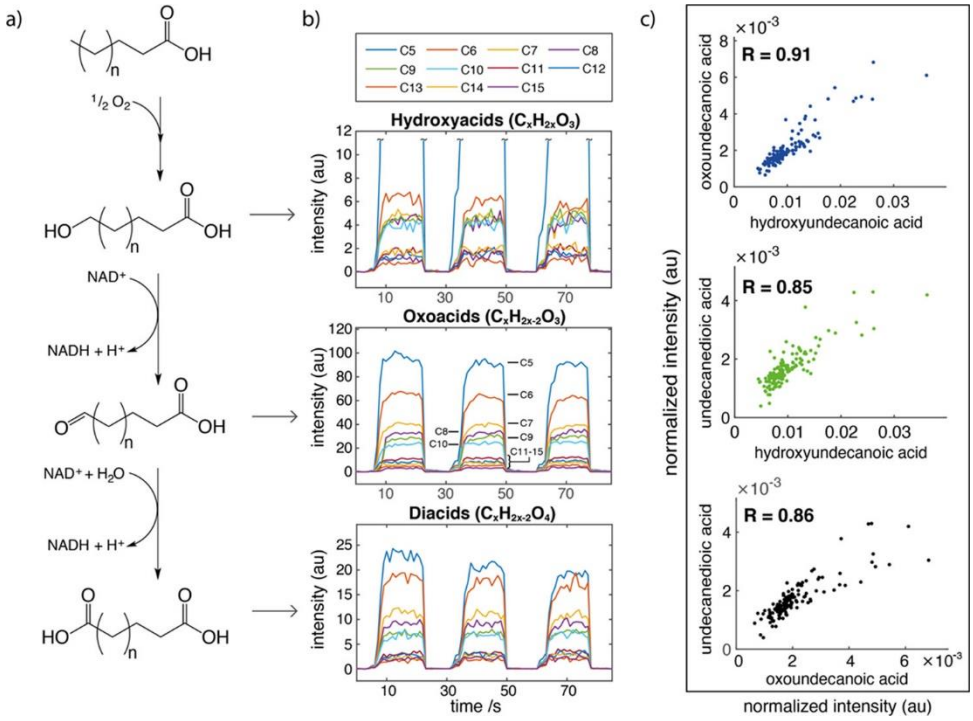

Figure 1. Online detection of ω-oxidation metabolites in exhaled breath. (a) Scheme of the ω-oxidation of linear aliphatic fatty acids, where fatty acids are transformed into ω-hydroxyalkanoic acids, ω-oxoalkanoic acids, and finally alkanedioic acids. (b) Time traces of the corresponding metabolites detected online over the course of three exhalations of a healthy subject as the $[M-H]^-$ ions in negative mode (au = arbitrary units). The signal for hydroxypentanoic acid is cut to improve the visibility of the others. (c) Normalised intensities of hydroxyundecanoic acid, oxoundecanoic acid, and undecanedioic acid for online measurements of 146 healthy subjects plotted against each other and the corresponding Pearson linear correlation coefficient. Although there might be a signal contribution of structural isomers, a strong correlation is observed. The same picture holds true for all carbon chain lengths from 5–15 (see the Supporting Information in original publication).

Figure 1b shows the time traces of these three families of compounds over the course of three exhalations of a healthy subject in negative ion mode. The main factors that determine breath concentration of metabolites are systemic and lining fluid concentrations as well as compound volatility were able to monitor the pharmacokinetics of ketamine (injected i.v.) in mouse breath using SESI-MS, which clearly shows that SESI-MS is capable of detecting vapours of blood metabolites with very low vapor pressure. Despite the low volatility of the compounds studied, a comparison of the estimated vapor pressure at 37 °C of ketamine (v.p. = 2.84 × 10–4 mmHg) with the compounds in this study (e.g., 8-oxooctanoic acid: v.p. = 3.63 × 10–4 mmHg) supports that these also reflect systemic metabolites (vapor pressures calculated with SPARC, ARChem). It is visible that the signal intensity decreases with increasing chain length, which is in agreement with decreasing vapor pressure and perhaps decreasing systemic abundance.

Dicarboxylic acids and ω-oxoacids have been reported to be constituents of environmental aerosols coming from a variety of sources (natural and anthropogenic). Therefore, an unknown fraction of these compounds could be of exogenous origin. Additionally, some compounds (predominately dicarboxylic acids) can also be generated by other metabolic pathways (i.e., glutaric acid as an intermediate in lysine and tryptophan metabolism.

Figure 1c shows the normalized intensities of 11-hydroxyundecanoic acid, 11-oxoundecanoic acid, and undecanedioic acid plotted against each other. Pearson correlation coefficients of 0.91 (11-hydroxyundecanoic acid vs 11-oxoundecanoic acid), 0.85 (11-hydroxyundecanoic acid vs undecanedioic acid), and 0.86 (11-oxoundecanoic acid vs undecanedioic acid) underline the strong connection between these compounds. The abundance of dicarboxylic acids and ω-oxoacids in environmental aerosols correlates negatively with their chain length. Therefore, a stronger confounding effect on the correlation is expected for compounds of lower molecular weight, due to their higher abundance in environmental air.

Offline Identification of ω-Oxidation Metabolites in Exhaled Breath Condensate

Since online analysis lacks a separation step, compound identification is difficult, and isomers cannot be distinguished. We therefore used UHPLC-MS analysis of EBC as a complementary method to confirm the identity of the compounds. Due to confounding dilution effects by water in EBC, UHPLC-MS experiments were used only to obtain qualitative information about the structures. **Table 1** gives an overview of all compounds detected in this work. We were able to observe chromatographic peaks for all diacids (C5–15), ω-oxoalkanoic acids (C5–15), and ω-hydroxyalkanoic acids (C5–15). To confirm the identity of the compounds, retention times and fragment spectra were compared to commercial standards where available. In total, seven alkanedioic acids, one ω-oxoalkanoic acid, and three ω-hydroxyalkanoic acids were confirmed in this manner, and 23 compounds showed sufficient signal intensity to obtain MS2 fragmentation spectra. The full data (chromatograms and MS2 spectra with assigned neutral losses) are presented in the Supporting Information in original publication. **Figure 2** shows the extracted ion chromatograms of the $[M\text{-}H]^-$ ions of the linear alkanedioic acids in a UHPLC-MS run of EBC (a) as well as of the measured commercial standards (b). Figure 2c shows the head-to-tail plot of the MS2 spectra of octanedioic acid from EBC and the standard. To gain further evidence that this compound is also detected in the real-time analysis, we compared the fragments in an online MS2 spectrum of exhaled breath to the standard (Figure 2d). Due to the lack of chromatographic separation in online analysis, multiple isobaric precursors are selected (precursor selection: 173.1 ± 0.35 Da), leading to a very complex fragment spectrum. Because of the high mass resolution of the TOF analyser ($R \approx 30\,000$ at $m/z = 173$), we were nonetheless able to assign the corresponding real-time fragments to the fragments of the standard. While the alkanedioic acids showed strong fragmentation at the conditions used, the ω-hydroxyalkanoic acids and ω-oxoalkanoic acids showed only minor fragments. The most prominent neutral losses of the alkanedioic acids are $[M\text{-}H_2O]^-$ for C10–C15, $[M\text{-}CO_2]^-$ for C5–C7, and $[M\text{-}H_2O\text{-}CO_2]^-$ for all compounds, which is consistent with their chemical structure. While ω-hydroxyalkanoic acids and ω-oxoalkanoic acids both show neutral losses of 46.005 and 18.012 Da, probably corresponding to $[M\text{-}CH_2O_2]^-$ and $[M\text{-}H_2O]^-$, only in the case of the ω-oxoalkanoic acids could a $[M\text{-}CO_2]^-$ peak be detected.

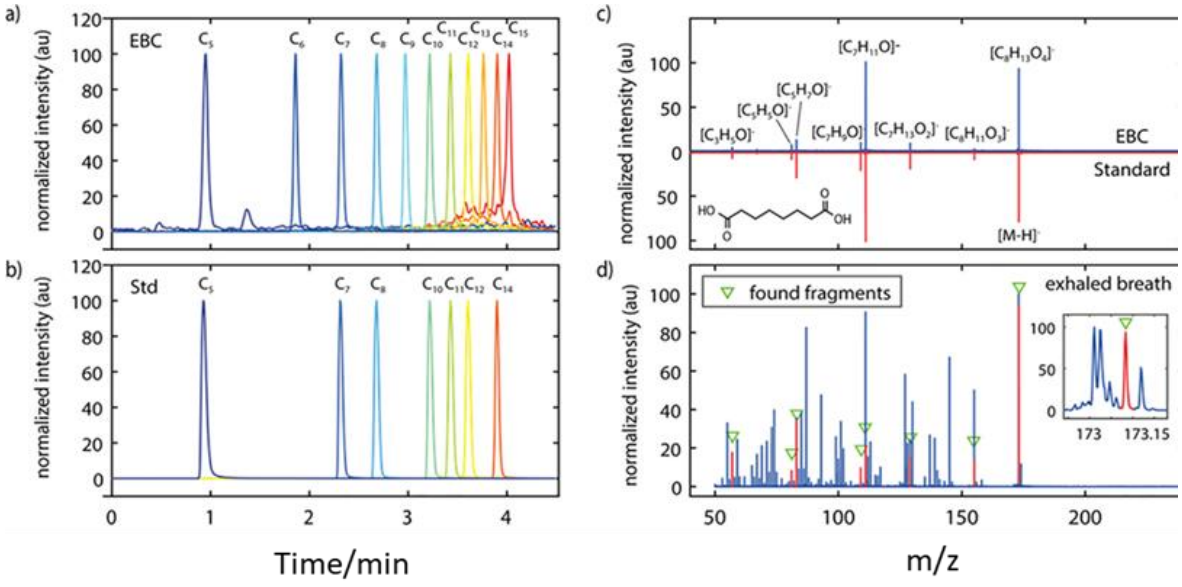

Figure 2. Extracted ion chromatograms of the [M-H]− ions of linear, saturated dicarboxylic acids with chain lengths of 5–15 in an UHPLC-MS run of exhaled breath condensate (EBC; a) as well as commercial standards (Std; b). The compounds were confirmed by matching retention time and MS2 fragments with the standards. (c) Head-to-tail plot of the base-peak normalized MS2 spectrum of octanedioic acid in EBC (top) and standard (bottom). (d) Base-peak normalized online MS2 spectrum of exhaled breath (precursor: 173.1 ± 0.35 Da). The inset shows a zoom of the precursor mass. As no chromatographic separation is performed, multiple parent masses are selected, explaining the abundance of other fragments. Nonetheless, the high resolution TOF analyser makes it possible to clearly identify all fragments of octanedioic acid, supporting the presence of this compound in breath.

Table 1. Families of ω-hydroxyalkanoic acids, ω-oxoalkanoic Acids and Alkanedioic Acids detected as the [M-H]⁻ ion in EBC with UHPLC-MS and on-line with SESI-MSa

	ω-hydroxyalkanoic acids ($C_xH_{2x}O_3$)				ω-oxoalkanoic acids ($C_xH_{2x-2}O_3$)				alkanedioic acids ($C_xH_{2x-2}O_4$)			
# C	t_r /min	m/z	ppm	NIST match	t_r /min	m/z	ppm	NIST match	t_r /min	m/z	ppm	NIST match
5	0.97	117.0559	5.1		0.95	115.0404	3.7		0.96	**131.0354**[b,c]	5.1	947
6	1.90	131.0721	5.6		1.81	**129.0562**	3.5		1.87	**145.0513**	5.6	
7	2.38	145.0875	5.1		2.34	**143.0717**	2.1		2.32	**159.0672**[b,c]	5.1	979
8	2.74	**159.1030**[b,c]	3.5	900	2.70	**157.0876**	3.7		2.68	**173.0828**[b,c]	3.5	962
9	3.03	**173.1183**	3.4		3.00	**171.1032**	3.3		2.98	**187.0983**	3.4	
10	3.28	**187.1341**[b,c]	3.9	911	3.24	**185.1189**[b,c]	3.4	851	3.22	**201.1139**[b,c]	3.9	973
11	3.49	**201.1500**[b,c]	6.0	908	3.45	**199.1347**	3.4		3.43	**215.1297**[b,c]	6.0	968
12	3.68	**215.1661**	3.6		3.64	**213.1502**	2.9		3.61	**229.1459**[b,c]	3.6	977
13	3.82	229.1818	5.4		3.79	227.1662	4		3.77	**243.1611**	5.4	
14	3.94	243.1974	6.1		3.92	241.1820	4.6		3.90	**257.1772**[b,c]	6.1	955
15	4.06	257.2123	5.1		4.03	255.1972	2.3		4.02	**271.1932**	5.1	

a For compounds in bold, signal intensity was sufficient to obtain UHPLC-MS2 spectra. The NIST matching scores were computed using the software NIST MS Search 2.0, where 800–900 indicates a good and 900–999 an excellent match.

b Compound confirmed by a match of retention time and MS2 spectrum with the standard.

c Fragments of standard also detected in an online MS2 experiment.

All measured standards showed a good match in retention time and MS2 spectra when compared to EBC. We used the NIST MS Search 2.0 software to compute matching scores for the fragment spectra, which ranged from 851–979. It is based on a modified cosine of the angle between the spectral vectors, where 800–900 indicates a good and 900–999 an excellent match. The retention time and fragments of 10-oxodecanoic acid matched well with the synthesized standard (NIST matching score = 851), and the fragmentation patterns of 6-oxohexanoic acid, 7-oxoheptanoic acid, 8-oxooctanoic acid, and 9-oxononanoic acid in EBC all showed a similar fragmentation behaviour to that of the 10-oxodecanoic standard (small neutral losses of H_2O, CO_2, and CH_2O_2).

Although ω-oxidation is considered a minor pathway of fatty acid degradation, this work clearly shows that metabolites produced via ω-oxidation are crucial components of exhaled breath. While alkanedioic acids show only one main chromatographic peak (except for pentanedioic acid), hydroxyalkanoic acids show multiple retention times, probably representing different positions of the hydroxyl group. For hydroxyoctanoic acid ($C_8H_{16}O_3$), we could observe three main chromatographic peaks in EBC, with 8-hydroxyoctanoic acid eluting first and 2-hydroxyoctanoic acid last (both confirmed by standard measurements, see **Figure 3**). The middle peak most probably corresponds to 3-hydroxyoctanoic acid as it shows the fragment ion $[C_2H_3O_2]^-$, resulting from a McLafferty-type rearrangement that leads to cleavage of the C(2)–C(3) bond and the formation of a stable enolate anion.

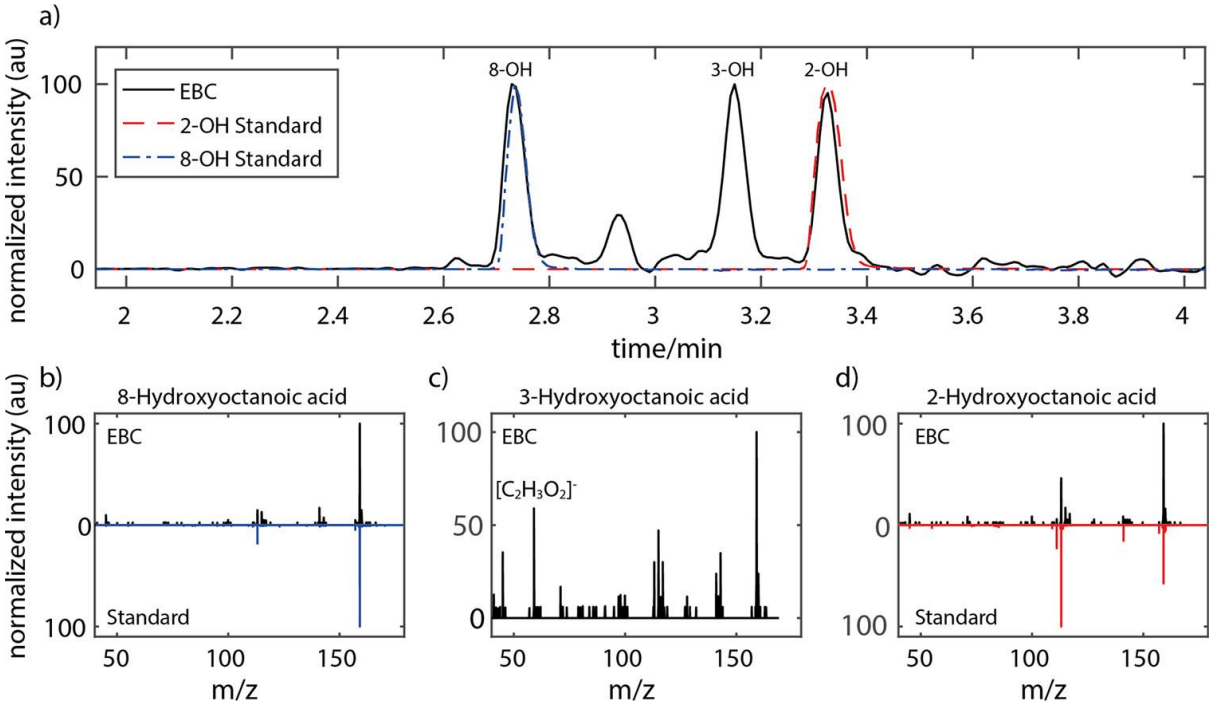

Figure 3. Top: Extracted ion chromatograms of the mass at 159.1027 Da corresponding to the molecular formula $[C_8H_{19}O_3]^-$ in a UHPLC-MS run of EBC after blank subtraction (a). The three main peaks were identified as the $[M\text{-}H]^-$ ions of 8-hydroxyoctanoic acid, 3-hydroxyoctanoic acid, and 2-hydroxyoctanoic acid. Bottom: Head-to-tail plot of the base-peak normalized MS2 spectra of 8-hydroxyoctanoic acid (b) and 2-hydroxyoctanoic acid (d) in EBC and the standards, as well as the MS2 spectrum of putative 3-hydroxyoctanoic acid (c). The fragment $[C_2H_3O_2]^-$ is formed by a McLafferty-type rearrangement, supporting the position of the hydroxyl group.