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Two-Dimensional Gas Chromatographic and Mass Spectrometric Characterization of Lipid-Rich Biological Matrices—Application to Human Cerumen (Earwax)

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Cite This: *ACS Omega* 2022, 7, 230−239

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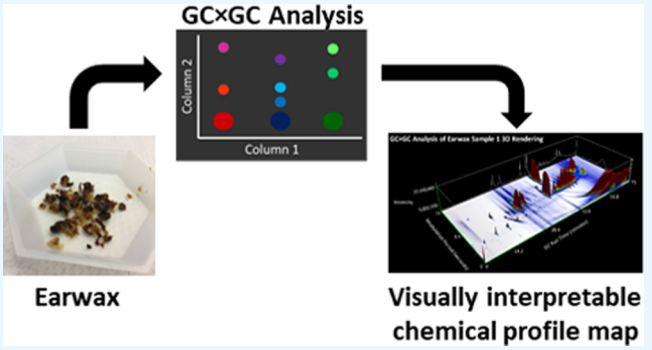
ABSTRACT: Earwax is a readily accessible biological matrix that has the potential to be used in disease diagnostics. However, its semisolid nature and high chemical complexity have hampered efforts to investigate its potential to reveal disease markers. This is because more conventional methods of analysis such as gas chromatography−mass spectrometry (GC−MS) and liquid chromatography−mass spectrometry yield unsatisfactory results due to the presence of many nonvolatile and/or coeluting compounds, which in some cases have very similar mass spectrometric profiles. In addition, these routine methods often require the sample to be saponified, which dramatically increases the complexity of the analysis and makes it difficult to determine which compounds are actually present versus those that are produced by saponification. In this study, two-dimensional GC mass spectrometry (GC × GC−MS) was successfully applied for the characterization of the chemical components of earwax from healthy donors using nonpolar (primary) and midpolar (secondary) columns without saponification. Over 35 of the compounds that were identified are reported for the first time to be detected in unsaponified earwax. The resulting GC × GC−MS contour plots revealed visually recognizable compound class clusters of previously reported groups including alkanes, alkenes, fatty acids, esters, triglycerides, and cholesterol esters, as well as cholesterol and squalene. The application of GC × GC−MS revealed results that provide a foundation upon which future studies aimed at comparing healthy donor earwax to that from individuals exhibiting various disease states can be accomplished.



INTRODUCTION

Blood and tissue constitute the primary matrices that are analyzed for the detection of disease in humans and animals. However, due to the pain, discomfort, time, and human resource costs associated with sample acquisition, an increasing trend in medical diagnostics is the development of less invasive approaches utilizing biological fluids such as urine[1] or nontraditional matrices including saliva[2] and sweat.[3] One such matrix that is receiving greater attention is cerumen, also known as earwax. This readily accessible material has been shown to contain biomarkers indicative of underlying disease states. For example, the compound 4,5-dimethyl-3-hydroxy-2[5*H*]-furanone (sotolone),[4] which is responsible for the characteristic odor of maple syrup urine disorder, has been found to be present in the earwax of infants afflicted with this in-born error of metabolism.[5] It has also been shown that the profiles within earwax of volatile organic compounds, including acetone, methoxyacetone, hydroxyurea, ethanol, isobutyraldehyde, and acetic acid, can be used to differentiate healthy patients from those with type 1 and type 2 diabetes.[6] The profile of volatile compounds in earwax has also been used to differentiate between healthy individuals and patients with cancer.[7] As its common name implies, earwax contains a lipid-rich mixture of waxes as well as fatty acids, and it serves as a source of the greatest diversity of fats that are accessible from the surface of the body. This attribute implies its possible usefulness as a reporter of the presence of disorders of lipid metabolism, although this has yet to be fully investigated.

The potential of earwax to serve as a viable biological matrix that can be used for disease diagnostics hinges not only on the development of methods for its routine analysis but also on the determination of the profile of constituents that characterize "normal" samples. However, a systematic approach to the documentation of the range and profile of compounds that appear in this matrix has not been reported. If earwax is to be used as a tool for diagnosis, a baseline of constituents in earwax from healthy donors must be established. The characterization

Received: August 19, 2021
Accepted: November 4, 2021
Published: December 27, 2021

work that has been done on cerumen constituents includes the identification of large classes of molecules such as organic acids,[8−12] amino acids,[9,13] carbohydrates,[14] lipids,[15,16] alcohols,[10,11] hydrocarbons,[11,17] and esters.[11] The techniques employed in these studies included paper chromatography,[8,9] column chromatography,[14] thin-layer chromatography,[11,15,16] gas chromatography (GC),[10,11] GC−mass spectrometry (GC−MS),[10−12] and pyrolysis GC−MS.[17] In these studies, analyses were performed either immediately following solubilization or after significant processing by treatments such as saponification. Because of the presence of triglycerides and a variety of other esters in earwax, as well as amides and other compounds that are susceptible to cleavage by hydrolysis, the saponification reaction results in a dramatic increase in the number of compounds contained within the sample such that the profile of molecules observed does not reflect that of the original earwax sample. For example, Stránský et al. detected almost 1000 compounds in earwax that was analyzed following saponification.[11] Many of these were not identified, and although in some cases, the molecular formulas were determined and compound class information could be inferred (e.g., alkanes or alkenes), their precise structures, including the positioning of double bonds within alkenes, were not determined. Even in the absence of saponification, one prominent feature of earwax is the presence of homologous series of molecules such as long-chain alkane congeners. When such closely related compounds are detected, the similarities of their fragmentation patterns and absence of the molecular ion in electron ionization (EI) GC−MS analysis can complicate data interpretation and compound identification,[10−12,17] particularly for coeluting compounds. Furthermore, structural similarities between several constituents, the presence of many isobaric compounds, and the nonvolatility of many of its components make earwax a challenging matrix for routine analysis by commonly used conventional methods. This reduces its potential utility as a disease diagnosis tool.

Analysis by two-dimensional GC (GC × GC) coupled with high-resolution mass spectrometry (HRMS) is an approach that has the potential to address some of the aforementioned challenges to cerumen analysis. It involves the use of two sequentially oriented chromatographic columns that have different stationary phase selectivity properties.[18,19] The terminus of the first longer primary column is connected to a temperature-controlled cryogenically cooled unit termed a thermal modulator, which rapidly condenses and focuses the effluent from the first column. It then periodically injects fractions of the effluent onto the second (secondary) column via heat-promoted release through a modulator loop. The modulator loop consists of a short (1 m) length of deactivated fused silica coiled into loops that pass through the thermal modulator gas jets. The samples are thermally released onto the first coil of the modulator loop and are cryofocused by the hot and cold gas jets on the second pass as they move onto the secondary column. Since these columns are connected in tandem, elutions from the first column, which are typically released onto the second column on the millisecond timescale, can be further partitioned,[19] thereby providing an additional dimension of separation.[20] Parameters such as the duration and frequency of the cryotrapping and injection pulses are variable and allow for the precise tuning of the instrument parameters according to the requirements of the analysis.[21] As a result, GC × GC can accommodate more complex mixtures, exhibit greater sensitivity and peak capacity, and provide data not only with increased separation efficiency but which is also more readily interpretable through the use of two retention times (RTs), as compared with the traditional one-dimensional (1-D) GC.[18,19] By convention, the resulting two-dimensional (2-D) GC × GC chromatogram, also known as a contour plot, shows the RT of the primary column on the *x*-axis and that of the secondary column on the *y*-axis. Chromatographic peaks representing each detected compound appear as arbitrarily colored spots or bands whose color intensity reflects the magnitude of the peak area. In a three-dimensional (3-D) rendering of the plot, the peak intensity is projected in the *z*-dimension. One of the advantages of viewing the results within the 2- or 3-D space is that related members of a chemical class often appear clustered within the same region, which reveals spot patterns and enables the presence of compound families to be readily detected at a glance.

To date, GC × GC and GC × GC−mass spectrometry (GC × GC−MS) have been most widely employed for the analysis of fossil fuels and their related products.[22−31] Recently however, the technique is receiving increased attention as a tool that can be applied to address the separation and compound identification challenges associated with inherently complex mixtures which, by 1-D GC analysis, are characterized by the presence of numerous coeluting compounds whose concentration ratios may differ by several orders of magnitude. Examples include the metabolomes of plants[32] and insects,[21,33] the small-molecule profiles of foods,[34−37] and biological materials such as urine,[38−42] blood,[42,43] and tissues.[42] This approach has also been applied to the analysis of emission volatiles from various types of samples[20,28,29,34,35,43−45] and environmental contaminants.[46]

The attributes of GC × GC, coupled with HRMS, make it a potentially powerful tool for the routine characterization of earwax. We report here the application of this approach for the analysis of earwax samples acquired from healthy donors in the course of its routine removal in a clinical setting in order to assess the general profile of the compounds it contains. Consistent with previous observations, the classes of molecules observed included alkenes, alkanes, fatty acids, esters, cholesterol esters, and triglycerides. The high-resolution time-of-flight (TOF) MS analysis enabled compound attributions to be made for the observed peaks, and the GC × GC contour plots allowed the salient represented compound class features within the samples to be readily visualized.

RESULTS AND DISCUSSION

Rationale for Performing the Analysis without Sample Saponification. The earwax analyzed was collected from healthy individuals in the United States representing all genders and races during routine ear cleanings conducted in a clinical setting. The reported results were acquired from the analysis of samples that are representative and were collected from over 1000 earwax plugs acquired from >100 donors.

Published reports describe two general approaches to the analysis of earwax: (1) direct analysis following solubilization and (2) analysis after treatment under acid- or base-catalyzed hydrolysis conditions (i.e., saponification). Saponification, when performed, is conducted for a variety of reasons. One is to reduce the complexity of molecules such as triglycerides through liberation of the fatty acids from glycerol. Once released, the structure of the fatty acid can in principle be more readily determined. A second is the generation of compounds that are more readily volatilized, which is important for GC

https://doi.org/10.1021/acsomega.1c04535
ACS Omega 2022, 7, 230−239

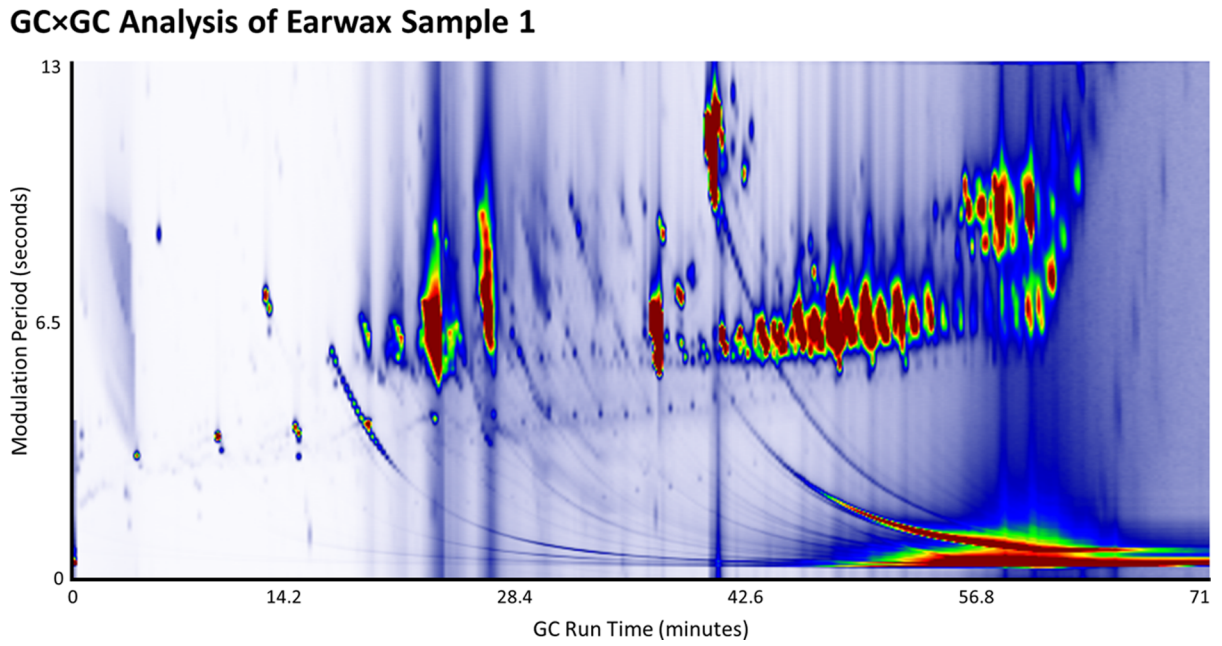


Figure 1. Representative 2-D GC contour plot of the analyzed earwax. The *x*-axis represents the total run time and the *y*-axis represents the modulation period. Each colored spot represents the detection of a compound and the more intense color corresponds to a higher concentration. The large band in the bottom right region is indicative of column bleed.

analysis. However, hydrolysis greatly increases the complexity of the mixture by amplifying the number of compounds within it. It also makes determination of which components are hydrolysis-derived, versus bona fide constituents of earwax, very difficult, if not impossible. Furthermore, the nonuniformity of this matrix from person to person, coupled with the diversity of hydrolyzable compounds it can contain, makes selection of suitable saponification reaction conditions very challenging. If the intent is to more accurately detect the actual profile of compounds in earwax, then direct analysis of solubilized samples is preferred. In this regard, it is essential that the selected solvent dissolves the broadest range of compounds. Previous studies have used both dimethyl sulfoxide (DMSO)[13] and *n*-hexane[15] to extract lipids. However, the number of compounds taken up and detected was extremely low. Using DMSO, Burkhart et al. reported the identification of nine unique compounds,[13] while Inaba et al. reported the identification of two unique compounds and four compound classes when using *n*-hexane.[15] We tested a variety of solvents including ethyl acetate, hexanes, glycerol, and so forth to determine which enabled the detection of the greatest number of compounds. These experiments were performed by extracting the cerumen with each solvent and analyzing the sample by direct analysis in real time-mass spectrometry. The results (data not shown) revealed that ethyl acetate extracted the broadest range of compounds. Therefore, ethyl acetate extracts were used in this study.

Rationale for the Utilization of GC × GC. One of the difficulties associated with GC analysis of earwax is that its constituents span the extremes of the dielectric constant spectrum, making it challenging to identify columns that enable separation of its components with minimal tailing. While some columns are optimal for detection and separation of hydrocarbons (i.e., alkenes, alkanes, and arenes), others are optimized for more polar compounds, such as carboxylic acids, amines, or alcohols. Since earwax contains compounds exhibiting all of these functional groups and others, we were confronted with the finding that columns that were better suited for hydrocarbon detection and separation resulted in severe tailing for the more polar compounds, while columns that excelled with separation of more polar compounds exhibited tailing for hydrocarbons. Ultimately, a nonpolar primary column coupled with a midpolar secondary column, using the GC × GC parameters described below, was found to enable detection of compounds containing a wide range of functional groups, with minimal or greatly reduced tailing of individual molecules.

GC × GC Analysis. GC × GC analyses of ethyl acetate extracts of earwax were performed. The representative results are shown in Figure 1, in which the observed 2-D chromatogram is rendered as a contour plot, where the *x*-axis displays the RT for the primary column and the *y*-axis displays the modulation period (13 s). Although somewhat longer than what is typically reported, this modulation period was found to be most optimal for separating the range and diversity of the compounds detected in cerumen while also avoiding wrap-around. Chromatographic peaks appear as colored spots or bands which extend from blue (low relative levels) to dark red (high relative levels) of the represented compounds. The large, intense band seen in the bottom right quadrant of the contour plot represents column bleed, which appears in a region entirely separate from that in which the earwax components are found. Overall, approximately 120 colored spots were observed in the 2-D contour plot.

One of the attributes of earwax that has made it challenging to analyze by a single method is the mass fraction differences between the compounds of which it is comprised. Among other things, this means that the sample preparation steps should enable detection of dominant compounds on the one hand and lower-level or trace compounds on the other, all while using instrument settings and a choice of columns that enables the peaks to be resolved. Through iterative determination of the parameters that enabled detection of compounds at both extremes, we found that balance in this

https://doi.org/10.1021/acsomega.1c04535
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Table 1. Compound Matches for the GC × GC Contour Plots[a]

first RT (min)	second RT (s)	compound	MF	measured RI	actual RI	molecular Ion	GC−MS	GC × GC−MS
7.683	3.080	1-dodecene	937			√	√	
7.900	2.800	dodecane	904			√	√	
12.450	3.600	1-tetradecene	947	1391	1392	√	√	
12.667	3.240	tetradecane	926	1400	1400	√	√	
17.000	3.800	1-hexadecene	935	1580	1590	√	√	
17.217	3.440	hexadecane	915	1590	1600	√	√	
21.333	3.920	1-octadecene	916	1789	1791	√	√	
21.333	6.120	tetradecanoic acid	891	1764	1752	√	√	
23.283	6.080	pentadecanoic acid	893	1845	1848	√	√	
24.367	6.040	hexadecanoic acid	809	1920	1954	√	√	
25.017	6.120	*cis*-9-hexadecenoic acid	900	1950	1953	√	√	
25.233	4.000	1-eicosene	868	1987	1993	√	√	
26.750	6.080	*cis*-10-heptadecenoic acid	848	2067	2073	√	√	
28.267	6.760	*cis*-9-octadecenoic acid	898	2144	2141	√	√	
28.700	4.120	1-docosene	863	2187	2194	√	√	
28.700	5.240	octadecanoic acid	824	2167	2172	√	√	
30.217	5.720	1-eicosanol	848	2275	2282	√	√	
31.300	7.440	*cis*-11-eicosenoic acid	850	2338	2339	√	√	
38.233	6.160	squalene	921	2780	2808	√	√	
38.667	8.680	cholest-3,5-diene	935	2826	2880	√	√	
39.750	7.120	1,6,10,14,18,22-tetracoshexaen-3-ol 2,4,6,10,15,19,23-hexamethyl	895	2920	3003	√		
39.967	5.600	tetradecanoic acid tetradecyl ester	843	2950	2947	√	√	
41.267	11.080	cholesterol	921	3014	3052	√	√	
42.133	11.080	lathosterol	923	3071	3170	√	√	
42.350	5.760	hexadecanoic acid tetradecyl ester	826	3160	3148	√		
42.783	5.600	$C_{31}H_{62}O_2$				√		
42.783	10.880	$C_{28}H_{48}O$				√		
42.783	12.200	cholest-4-en-3-one	931	3114	3236	√		√
43.433	10.240	lanost-8-en-3-ol (3β)	886	3260	3287	√		
43.867	11.280	lanosterol	912	3300	3293	√		√
44.517	6.120	hexadecenoic acid hexadecyl ester	843	3340	3329	√		
46.683	6.280	$C_{34}H_{66}O_2$				√		
50.583	6.520	$C_{34}H_{64}O_2$				√		
56.531	10.046	cholesteryl myristate	867			√		
58.688	9.492	cholesteryl palmitate	818			√		√
59.326	8.714	cholesteryl heptadecanoate	844			√		√
60.206	9.574	cholesteryl oleate	890			√		
60.421	9.430	cholesteryl stearate	800			√		
61.633	7.560	tripalmitin	725					√

[a]Listed are the first and second RT, compound, and MF. For identity confirmation, RIs were calculated for 14−33 carbons. Checkmarks indicate that the compound identity was confirmed based on GC−MS or GC × GC−MS analysis of a standard and/or detection of the molecular ion. An empty cell indicates that the listed technique was not used or that the indicated parameter was not determined.

work. However, this means that of necessity, in order to detect the presence of trace compounds in the contour plot, more abundant compounds appear as very intense bands. To have the samples diluted to levels that would have eliminated overloading of the column by more abundant compounds such as squalene and cholesterol would have resulted in a total loss of the peaks associated with lower-level components. It is for this reason that there is such a wide intensity difference for the detected peaks.

MS-Facilitated Compound Identification. The coupling of the 2-D gas chromatograph with a high-resolution TOF mass spectrometer that was operated in the positive-ion mode enabled determination of the EI mass spectral fragmentation patterns of the peaks observed in the 2-D contour plots. Of the 120 peaks observed in the chromatogram, 39 were tentatively identified through the molecular formula information furnished by the high-resolution mass spectra alongside NIST mass spectral library EI fragmentation pattern matching. To make confirmations, an alkane standard was analyzed by GC × GC−MS and retention indices (RI) were calculated. GC−MS analysis was performed on the earwax, and compound identities were confirmed through the use of RT comparison of standards. The same was also true for standards run using the GC × GC−MS system. The 120 peaks observed and 39 compounds identified are significantly greater than the 32 1-D GC−MS peaks with 9 identified compounds previously reported to be the maximum number of molecules detected in an unsaponified earwax sample.[13] Table 1 displays the first and second RTs, compound matches, match factors (MFs), and, when available, the RIs for the identified compounds. In addition, the checkmarks indicate how the identification was confirmed (i.e., whether it was through the presence of the molecular ion, GC−MS analysis of standards, and/or GC × GC−MS analysis of standards). The identified molecules fell

https://doi.org/10.1021/acsomega.1c04535
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into six classes: alkanes, alkenes, fatty acids, esters, triglycerides, and cholesterol esters.

The alkanes observed, all of which have previously been detected in saponified cerumen,[11,17] were dodecane, tetradecane, and hexadecane. Tetradecane has also been observed in earwax headspace volatiles.[6] The presence of these long-chain compounds is consistent with the physical attributes of earwax, which is often observed to be viscous or semisolid at room temperature. In the alkene group, formulas consistent with the presence of dodecene, tetradecene, hexadecene, octadecene, eicosene, and docosene were identified, although the precise location of their double bonds was not determined through this technique. However, based upon GC−MS RT comparison with the results obtained for authentic standards, dodecene, tetradecane, hexadecane, and octadecene were determined to have the double bond at C-1. 1-Eicosanol was also observed and confirmed through GC−MS analysis. Compounds with formulas consistent with the presence of these parent molecules have previously been reported in saponified earwax that was analyzed by pyrolysis GC−MS.[17]

The fatty acids tetradecanoic acid, pentadecanoic acid, hexadecanoic acid, hexadecenoic acid, heptadecenoic acid, octadecenoic acid, octadecanoic acid, and eicosenoic acid were all observed. Tetradecanoic, pentadecanoic, hexadecanoic, octadecanoic, and hexadecenoic acids are all previously reported components of earwax.[6,10,11] However, while molecules with formulas corresponding to those of octadecenoic and eicosenoic acids have been reported, the precise location of the double bond was not determined due to RT similarities of the different double bond configurations in the 1-D GC analysis.[11] From our GC−MS RT analyses, the unsaturated fatty acids were *cis*-9-hexadecenoic acid, *cis*-10-heptadecenoic acid, *cis*-9-octadecenoic acid, and *cis*-11-eicosenoic acid. These types of compounds are typically derived from carbohydrates in fatty acid synthesis cascades involving acetyl-CoA and NADPH. Hexadecanoic acid, which is a highly dominant component of the lipid profile of mammals, is typically the shortest-chain fatty acid formed in this process.[47] *cis*-9-Octadecenoic acid is the most common fatty acid in human adipose tissues.[48] The synthesized fatty acids are packaged as triglycerides for energy storage.[48,49] In addition, fatty acids are also components of phospholipids, which form cell membranes.[49]

The esters observed here were tetradecanoic acid tetradecyl ester, hexadecanoic acid tetradecyl ester, and hexadecenoic acid hexadecyl ester. Molecular ions were also observed corresponding to esters with the chemical formulas of $C_{31}H_{62}O_2$, $C_{34}H_{66}O_2$, and $C_{34}H_{64}O_2$. Esters have been detected in the volatiles of many matrices derived from the human body.[50] Doležal et al. reported the observation of tetradecanoic acid tetradecyl ester, hexadecanoic acid tetradecyl ester, hexadecenoic acid hexadecyl ester, and $C_{34}H_{66}O_2$ by GC × GC−MS analysis of human skin volatiles.[44] In previous work, molecules with formulas corresponding with tetradecanoic acid tetradecyl ester, hexadecanoic acid tetradecyl ester, and hexadecenoic acid hexadecyl ester have been reported to be in earwax, but their structures were not previously confirmed.[11] Our work also confirmed the detection of compounds with formulas $C_{31}H_{62}O_2$, $C_{34}H_{66}O_2$, and $C_{34}H_{64}O_2$ that were previously reported,[11] and their structural characterization will be the focus of our future work.

The presence of cholesterol esters in earwax has been reported previously,[16,51] although not all have been structurally identified. We report for the first time the detection of cholesteryl myristate, cholesteryl heptadecanoate, and cholesteryl stearate. Cholesteryl palmitate and cholesteryl oleate were also found and have been previously detected in earwax.[51] Cholesteryl heptadecanoate has been reported in goat serum[52] but not in earwax. In addition, several related compounds were observed. These included cholesterol, which has previously been reported,[9,15−17] as well as several cholesterol derivatives such as cholest-3,5-diene, lathosterol, cholest-4-en-3-one, lanost-8-en-3-ol (3β), and lanosterol. A cholesterol ester with the formula $C_{28}H_{48}O$ was also found. Cholesterol esters are typically present in tissues and blood. In many cases, they are introduced into the body through the diet and then subsequently converted into cholesterol and fatty acids after which they are absorbed, prior to being re-esterified and distributed through the bloodstream (where they can contribute to plaque buildup) and into the tissues.[53,54] The compounds squalene and 1,6,10,14,18,22-tetracoshexaen-3-ol 2,4,6,10,15,19,23-hexamethyl were also detected.

Triglycerides, a compound class that has previously been reported to be present in earwax,[15,16] were detected. These molecules are the primary energy storage units in animals and humans.[55] They are also a common component of skin oils.[56] Our work specifically detected tripalmitin, a compound whose presence in earwax has not been reported previously, although the formula associated with this triglyceride has been reported.[11] We observed several other peaks whose RTs and fragmentation patterns (as well as their location in the 2-D GC × GC contour plot) implied the likelihood that they were also triglycerides. However, due to their high molecular weights and low volatility, structure determination could not be accomplished using this method alone, and future studies will be focused on their separation and purification, followed by saponification to release structural components that can be further characterized by other spectroscopic and mass spectrometric techniques such as matrix-assisted laser desorption/ionization mass spectrometry.[57]

Consistency of the GC × GC Contour Plot Profiles. From the structural identifications of several of the detected compounds, it was apparent that members of the same compound family appeared clustered within the contour plots generated by GC × GC analysis of the ethyl acetate extracts. The regions in which these families appear are highlighted in Figure 2A. Alkenes, fatty acids, alkanes, esters, triglycerides, and cholesterol esters are encircled in green, dark blue, light blue, red, yellow, and pink, respectively. In addition, dominant peaks representing cholesterol and squalene are labeled. Because the utility of GC × GC−MS analysis of cerumen as a means for assessing its chemical profile lies in part in the consistency of the chemical signature among like samples (e.g., samples acquired from healthy donors vs those from individuals with a health condition), multiple samples of healthy donor earwax were subjected to GC × GC−MS analysis to assess the represented compound class similarities between samples. The GC × GC contour plots observed for the analysis of three ethyl acetate extracts are presented in Figure 2B−D. Comparison of the contour plots in Figure 2A−D shows that they exhibited similar profiles with compound families appearing in similar areas. This indicates that the earwax of healthy donors is consistent in terms of the classes of compounds that appear. MS analysis of the bands appearing in

https://doi.org/10.1021/acsomega.1c04535
ACS Omega 2022, 7, 230−239

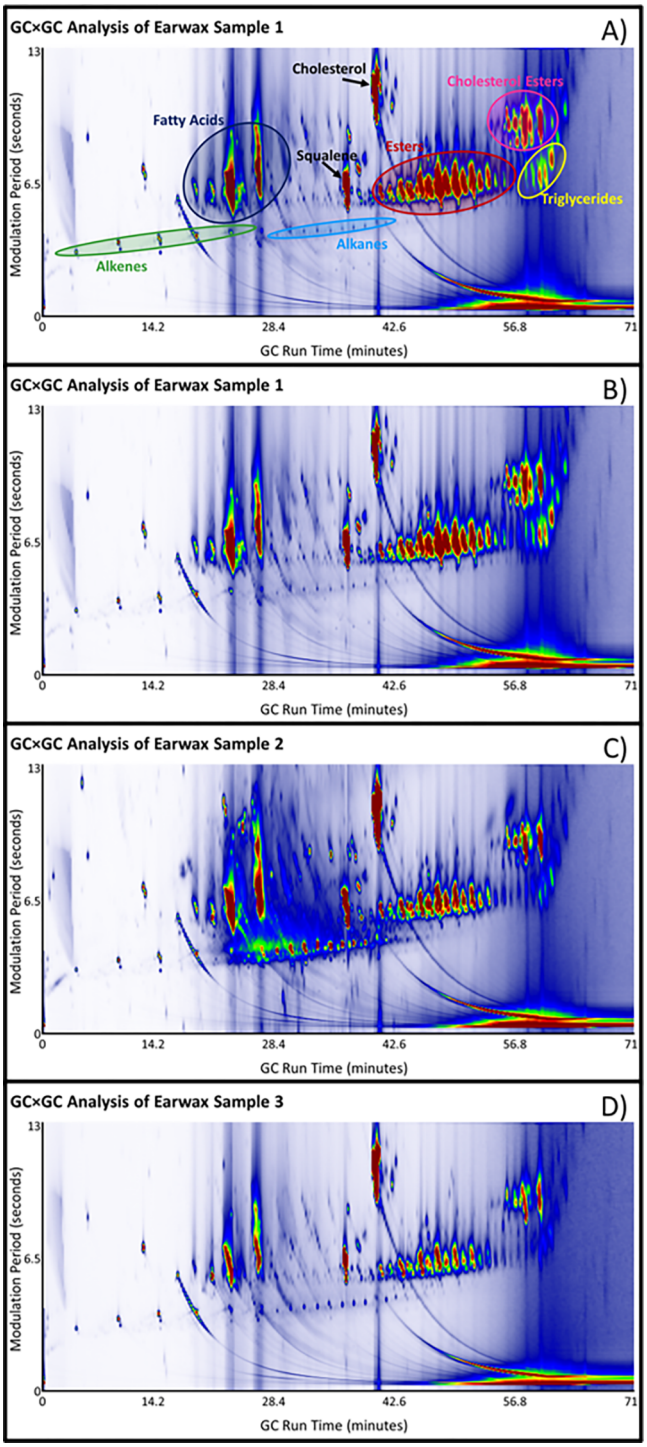

Figure 2. GC × GC contour plots observed for the analysis of ethyl acetate extracts of earwax. (A) Locations of compound classes in sample 1. The alkene region is shown in green, fatty acids in dark blue, alkanes in light blue, esters in red, triglycerides in yellow, and cholesterol esters in pink. The two major peaks cholesterol and squalene are also labeled. (B–D) Contour plots of the three samples analyzed.

these chromatograms confirms that their RT profiles and MS fragmentation patterns were similar in all cases. It should be noted that the mass spectral analysis could readily be performed on the data without interference from column bleed, solvent, or other extraneous synthetic chemicals because the bands were well-enough resolved.

Supporting Information Tables 1 through 39 display the high-resolution mass tables for each of the identified peaks. Even though not all of the peak identities within each of the highlighted regions in the contour plots were determined, the compound classes to which they belonged could still be inferred based upon the characteristics of their mass spectra. For example, in the triglyceride region, only tripalmitin was identified. However, the mass spectral fragmentation patterns of the other bands in the cluster aligned with those of known triglycerides. The absence of a molecular ion in their mass spectra, which resulted in low MFs, prevented tentative assignments from being made. Definitive identification of these molecules was not possible due to the relatively small amounts of the earwax material available, which precluded the isolation of large-enough amounts of purified compounds to perform structural characterization studies. However this, as well as an investigation of the extent to which chemical profile differences exist between the cerumen acquired from healthy donors, and the quantification of detected compounds, will be the subjects of our future studies.

The results of GC × GC analysis of earwax reveal that this technique has the ability to overcome the challenge of coelution of compounds when the experiment is performed by 1-D GC. Furthermore, the characteristics of the resulting 2-D GC contour plot make the general motifs of the earwax profile readily visually apparent, such that the presence of compound classes can be clearly detected. In principle, with the establishment of the earwax profile of healthy donors, the profiles of the earwax of patients presenting with diseases which have lipid dysregulation as a basis can be compared. If consistent differences that can be correlated to particular disease states are observed, 2-D GC has the potential to serve as a clinically useful tool in disease diagnostics. However, as a potential reporter of disease, earwax presents with some limitations. While blood is more homogeneous and reveals a real-time snapshot of the metabolic profile, earwax is not only heterogeneous, but it also accumulates more slowly over time. Therefore, it may not register the presence of a particular pathology in real time. Nevertheless, its gradual buildup over time could prove to be advantageous in studies of disease progression. Also, it may be possible that depending on the disease, matrices such as blood or urine provide little, if any information, in which case, clues about the presence of the disease from the examination of earwax profiles could be very valuable. It has also been noted that there are merits to analysis of earwax in comparison to other noninvasive biological matrices (sweat, saliva, hair, urine, and feces) including a reduction in the extent to which it is prone to external contamination since the ear canal is more protected from the external environment.[6] However, since the ear canal is exposed to the outside of the head, earwax could register the presence of nonbiological molecules derived from cosmetic products or other chemicals to which an individual is exposed (e.g., the components of cigarette smoke). These would be readily detectable by the 2-D GC method used here.

Interestingly, the consistency characteristics of earwax are influenced by genetics. The production of "wet" versus "dry" earwax is based on a single nucleotide polymorphism on the ATP-binding cassette C11 gene. The cerumen analyses described here were conducted using pooled earwax batches without patient identifiers, and samples were not separated by

https://doi.org/10.1021/acsomega.1c04535
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the wet or dry type. Thus, it remains unknown whether the profiles observed would remain consistent between the wet and dry forms. An investigation of this question is planned for future studies.

CONCLUSIONS

GC × GC−MS interrogation of human earwax from healthy donors furnishes 2-D GC contour plots that reveal a readily recognizable range of compound classes that appear within this complex lipid-rich matrix. The identities of 39 compounds, the greatest number ever reported in unsaponified earwax, were confirmed and found to be consistently present in earwax derived from multiple healthy subjects. The compound classes observed were alkanes, alkenes, fatty acids, esters, triglycerides, and cholesterol esters. Cholesterol and squalene were also prominent. For the first time, 1,6,10,14,18,22-tetracoshexaen-3-ol 2,4,6,10,15,19,23-hexamethyl, lathosterol, $C_{28}H_{48}O$, cholest-4-en-3-one, lanost-8-en-3-ol (3β), lanosterol, cholesteryl myristate, cholesteryl heptadecanoate, and cholesteryl stearate were detected in earwax. The compound class-based clustering that was observed in the contour plots for the multiple earwax samples analyzed appears highly similar, which on visual inspection shows the patterns associated with cerumen from healthy individuals. The characterization of these profiles sets the stage for comparative analysis investigations to be made using samples from patients as a function of disease state in order to determine whether the presence of various pathologies is registered in earwax in a manner that enables preliminary conclusions about the presence of a particular illness to be drawn from visual inspection of the contour plot.

EXPERIMENTAL SECTION

Materials. HPLC-grade ethyl acetate was purchased from Pharmco (Brookfield, CT, USA). Compound standards were purchased from Sigma-Aldrich (St. Louis, MO), Cayman Chemical (Ann Arbor, MI), Fisher Scientific (Waltham, MA), Oakwood Chemical (Eastill, SC), Acros (Waltham, MA), and TCI America (Philadelphia, PA). Earwax was obtained from patients at Albany ENT & Allergy Services (Albany, NY, USA) in accordance with the guidelines of the University at Albany-SUNY Institutional Review Board (IRB). As the earwax plugs represented discarded specimens acquired during the course of routine earwax removal and no patient identifiers were associated with them, the project was classified as "exempt". The plugs were transferred to sterile containers, combined, weighed, sealed with parafilm, and stored at −80 °C in 20 mL glass scintillation vials (Fisher Scientific, Waltham, MA, USA) until analysis.

Sample Preparation. Approximately 100 mg of earwax consisting of a minimum of 10 plugs from various individuals was transferred to a glass 1 dram vial (VWR, Radnor, PA, USA), and the sample was made more uniform by manual mixing using a metal spatula. To this, 1 mL of ethyl acetate was added. The suspension was first sonicated for 20 min using a FS30 sonic cleaner (Fisher Scientific, Waltham, MA, USA) and then filtered through a 2 mL Pasteur pipette (Fisher Scientific, Waltham, MA, USA) containing a small plug of cotton wool (Walmart, Albany, NY, USA). The cotton plug was then washed by application of an additional 500 μL of ethyl acetate, and the eluted solvent was added to the original eluate. This washing process was completed twice more, and all three eluates were combined. These three steps were repeated twice more using 100 mg of earwax each time to generate a total of three separate samples. For samples 1 and 2, the ethyl acetate was allowed to slowly evaporate under ambient conditions, leaving behind a residue. For sample 3, half of the ethyl acetate solution was stored for a separate, independent analysis, and the solvent was allowed to evaporate from the remainder under ambient conditions, leaving behind a residue. In addition, a small plug of the same cotton wool was washed with 500 μL of ethyl acetate, and the eluate was used as a blank in the GC × GC−MS analyses.

For analysis, 2 mL of ethyl acetate was added to the sample 1 and 2 residues and 1 mL of ethyl acetate was added to the sample 3 residue. All of the samples were sonicated for 15 min. A 1:2 dilution of each sample was then prepared by combining 50 μL of the residue solution with 50 μL of ethyl acetate in a GC vial with an insert (Agilent, Santa Clara, CA, USA) for subsequent analysis by 2-D GC (GC × GC).

To verify the compound identities, authenticated standards were analyzed. An earwax sample was prepared by suspending 500 mg (approximately 20 plugs) in 1.5 mL of ethyl acetate. This was sonicated for 20 min and centrifuged, and the supernatant was filtered through a cotton plug lodged within a Pasteur pipette. Authentic standards were prepared in ethyl acetate to a final concentration of 25 μg/mL. The solutions were vortexed and filtered through a Pasteur pipette with a cotton plug.

GC × GC−MS Instrument Parameters. Sample analysis was performed by using cool-on-column injections into a 7890B GC (Agilent, Santa Clara, CA, USA) equipped with a ZX2 closed-cycle refrigerator/heat exchanger thermal modulator (Zoex, Houston, TX, USA) in combination with a JMS-T200GC AccuTOF GCx plus (JEOL, Tokyo, Japan). Mass spectral measurements were performed using the positive EI mode at 70 eV. A general-purpose DB-5HT column (15 m, 0.25 mm I.D., 0.25 μm) (Agilent, Santa Clara, CA, USA) was used as the first-dimension column, and an Rxi-17Sil MS column (1 m, 0.15 mm I.D., 0.15 μm) (Restek Corporation, Bellefonte, PA, USA) was used as the second-dimension column. A deactivated capillary tube (1 m, 0.15 mm I.D.) (Restek Corporation, Bellefonte, PA, USA) was used for both the modulator loop and transfer line. The oven program had an initial temperature of 60 °C, where it was held for 1 min before increasing at a rate of 5 °C/min until reaching 360 °C, where it was held for 10 min for a total run time of 71 min. The modulator hot jet program had an initial temperature of 210 °C that was held for 1 min before increasing at a rate of 5 °C/min to 400 °C, where it was held for 32 min. The GC interface temperature was 350 °C, the modulation duration was 650 ms, and the helium flow rate was held constant at 1.7 mL/min. The modulation period for these experiments was found to be optimal at 13 s. A total of 0.5 μL of the sample was injected for each run.

The mass spectrometer ion source temperature was set to 280 °C. The spectrum recording interval for all analyses was 25 Hz (0.04 s). A 4 min solvent delay was used to prevent the detection of the solvent. Spectra were collected in the m/z range of 50−1000 and a single-point external mass drift compensation was performed for the whole mass calibration using the column bleed peak corresponding to $C_7H_{21}O_4Si_4^+$ (m/z 281.05114). An intensity of 30 was used as the peak detection threshold.

GC × GC−MS Data Processing. JEOL msAxel software (3.0.0.1) was used for the tuning of the mass spectrometer,

https://doi.org/10.1021/acsomega.1c04535
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chromatographic method configuration, acquisition of GC × GC−MS data, drift compensation, mass calibration, and data export (JEOL, Tokyo, Japan). For visualization and interpretation of GC × GC data, GC Image R2.9 software (GC Image, Lincoln, NE, USA) was used. To identify the compounds detected, the mass spectral fragmentation patterns were compared to those of compounds in the 2017 National Institute of Standards and Technology (NIST) Library Database (NIST MS Search 2.3). The RT indices, logical order of elution in the horizontal and vertical planes, and high-resolution masses were also considered. With the exception of one compound, only compounds with a library match score of >750 were assigned.

Compound Confirmations. To confirm the identities of the compounds detected, the earwax sample was analyzed by GC−MS using a 7890A gas chromatogram and a 5977B mass spectrometer (Agilent, Santa Clara, CA) coupled with a GERSTEL multipurpose sampler (MPS) (GERSTEL, Linthicum, MD). The oven had an initial temperature of 60 °C, which was held for 1 min before increasing at a rate of 10 °C/min to 100 °C. The temperature then increased at a rate of 5 °C/min until reaching 300 °C, where it was held for 15 min. The column used was a DB 5-MS UI (30 m, 0.25 mm I.D., 0.25 μm) (Agilent, Santa Clara, CA). The inlet temperature was 250 °C, the helium flow rate was 1 mL/min, and 1 μL was injected in the splitless mode. The mass spectrometer parameters were as follows: the ionization mode was EI, the ion source temperature was 230 °C, the m/z range was 35−1000, and the solvent delay was 4 min. Data processing was performed using MassHunter qualitative analysis software (Agilent, Santa Clara, CA). The authentic standard solutions were analyzed using the method described above. The RTs and fragmentation patterns of the standards were compared to the compounds detected in the earwax sample to confirm peak identities. For compounds that were not detected by GC−MS, the standards were analyzed by GC × GC−MS using the same method that was used to analyze the earwax (previously described). In addition, an alkane standard was analyzed by GC × GC−MS to determine the RI of the compounds.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c04535.

High-resolution mass spectrometry data (m/z values and the corresponding ion counts) for identified compounds (PDF)

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Funding

The support of the University at Albany—State University of New York is gratefully acknowledged.

Notes

The authors declare the following competing financial interest(s): Dr. A. John Dane and Dr. Robert B. Cody are employed by the manufacturer of the JEOL JMS-T200GC AccuTOF GCx plus instrument used in this study.

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