Received: 6 February 2022 | Revised: 19 June 2022 | Accepted: 26 June 2022

DOI: 10.1002/jms.4873

FEATURED ARTICLE

Journal of MASS SPECTROMETRY WILEY

Whole blood analysis for medical diagnostics by GC-MS with Cold EI

Benny Neumark[1] | Oneg Elkabets[1] | Gabi Shefer[2] | Assaf Buch[2] | Naftali Stern[2,3,4] | Aviv Amirav[1,5]

[1]School of Chemistry, Tel Aviv University, Tel Aviv, Israel

[2]The Institute of Endocrinology, Metabolism and Hypertension, Tel-Aviv Sourasky Medical Center, Tel Aviv, Israel

[3]The Sagol Center for Epigenetics of Aging and Metabolism, Tel-Aviv Sourasky Medical Center, Tel Aviv, Israel

[4]Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

[5]Aviv Analytical, Hod Hasharon, Israel

Correspondence
Aviv Amirav, School of Chemistry, Tel Aviv University, Tel Aviv 6997801, Israel.
Email: amirav@tau.ac.il

Abstract

This study covers a new method and related instrumentation for whole blood analysis for medical diagnostics. Two-μL whole blood samples were collected using "minimal invasive" diabetes lancet and placed on a thin glass rod mounted on a newly designed BloodProbe. The BloodProbe with the whole blood sample was inserted directly into a ChromatoProbe mounted on the GC inlet, and thus, no sample preparation was involved. The analysis was performed within 10 min using a GC-MS with Cold EI that is based on interfacing GC and MS with supersonic molecular beams (SMB) along with electron ionization of vibrationally cold sample compounds in the SMB (hence the name Cold EI). Our blood analysis revealed several observations: (1) Detailed mass chromatograms were generated with full range of all the nonpolar lipids in blood including fatty acids, cholesterol, cholesteryl esters, vitamin E, monoglycerides, diglycerides, and triglycerides. (2) The analysis of whole blood was found to be as informative as the conventional clinical analysis of blood serum. (3) Cholesteryl esters were more sensitive than free cholesterol alone to the effect of diet of obese people. (4) Major enhancement of several fatty acid methyl esters was found in the blood of a cancer patient with liver dysfunction. (5) Vitamin E as both α- and β-tocopherol was found with person-dependent ratio of these two compounds. (6) Elemental sulfur S_8 was identified in blood. (7) Several drugs and other compounds were found and need further study of their correlation to medical issues.

KEYWORDS
GC-MS with Cold EI, lipids in blood analysis, medical diagnostics, whole blood analysis

1 | INTRODUCTION

The analysis of cholesterol and triglycerides in human blood is one of the most important and widely used medical diagnostic tests.[1–4] These tests are vital for the prevention, control, and monitoring of cardiovascular risk and disease and metabolic state.[2,5,6] Adults should have blood lipids analysis at least every 5 years, whereas older people and those with high cardiovascular risk or unstable metabolic state are often analyzed at least every 3 months.[7] Current methods of analysis require vein puncture with collection of several milliliters of blood and require variable time for analysis. Furthermore, the results provide information that is limited to total

cholesterol, high-density protein-bound cholesterol (HDL), and total glycerides that are referred to as "triglycerides" but actually reflect the sum of all glycerides. Of critical importance is the fact that low-density cholesterol (LDLc) is actually calculated, not measured. No information is provided on the circulating free cholesterol, various cholesteryl esters, or the type of glycerides (monoglycerides, diglycerides, and triglycerides) or their fatty acid compositions at the molecular level. Naturally, if such information will be obtained, it will expand the horizons of metabolic knowledge, which will vastly enrich current medical diagnostics and potentially contribute to improved personalized diagnostics and treatment.

Hence, current clinical methods of blood lipid analysis face several challenges, and improvements could be achieved as follows:

A. Using minimally invasive blood collection to replace the current procedure of vein puncture.
B. Reducing the needed drawn volume of blood such to a few microliters (e.g., 1–2 μL), which is of critical importance for per-term babies and newborns.
C. Using whole blood, without any sample preparation, to eliminate time and cost of blood sample preparation.
D. Shorten turnaround time due to fast analysis (maximum of 10 min).
E. Enlarge the spectrum of information on blood lipids to include more complete composition of measured lipids and expanded information on other compound classes. This will allow personalized assessment of the ratios between the different blood lipids/vitamins, for example.
F. The analysis should include all currently provided information and thus be compatible with serum analysis for direct comparison.
G. The instrumentation used should be reliable, robust, and available at reasonable cost.

Currently, mass spectrometry (MS) and particularly LC-MS are establishing itself in lipidomics analysis[8–10] and with advances in dried blood spot analysis.[11]

The central technology used for this research of whole blood analysis for medical diagnostics is gas chromatography–mass spectrometry (GC-MS) with Cold EI.[12–16] GC-MS with Cold EI is based on interfacing the GC and MS with supersonic molecular beams (SMB) along with electron ionization (EI) of vibrationally cold sample compounds in the SMB in a fly-through ion source (hence the name Cold EI).[14–16] Cold EI was initially developed by Amirav and his group in 1990,[12,13] and it is reviewed in literature[14,15] as well as in a book on GC-MS with Cold EI that was recently published.[16] GC-MS with Cold EI improves all the central performance aspects of GC-MS and uniquely provides enhanced molecular ions, improved sample identification, significantly extended range of compounds amenable for analysis,[17] uniform response to all analytes (internal quantitation), faster analysis, greater selectivity, and lower limits of detection.[14–16,18] The aim of this study is to describe the application of GC-MS with Cold EI for the analysis of full range of nonpolar lipids in whole blood, from free fatty acids all the way to triglycerides.

We also developed for this study a new type of a "BloodProbe" device for sampling whole blood and its introduction to GC-MS with Cold EI. The BloodProbe is based on a modified ChromatoProbe for the sampling of whole blood as described below. The ChromatoProbe was developed by Amirav and Dagan[19] and was commercialized by Varian, Bruker, and Agilent who sells it under the name Thermal Separation Probe.[20] The ChromatoProbe serves as both an MS probe when a 1-m microbore capillary (with 100 μ ID) connects the injector and MS ion source and as a thermal desorption tool for the analysis of dirty samples (such as blended food items[21,22]) when standard 30-m column is being used. The ChromatoProbe uses 15-mm-long vials with 3 mm OD and 2.4 mm ID that carries any solid liquid or dirty sample in it. However, whole blood introduction into such small vials is not easy, and thermal extraction from the bottom of such narrow vials is not effective and could require too high temperatures. Thus, the BloodProbe was designed based on the conversion of ChromatoProbe vial holder into carrying a thin melting point vial or a thin glass rod the same as used in the Open Probe Fast GC-MS[23,24] (Agilent name QuickProbe[25]). Accordingly, the thin glass rod (100 mm length × 1.56 mm diameter) carries the blood droplet near its edge, and due to its exposed nature and minimal heat capacity, thermal vaporization from it is very effective. We tested blood analysis for its small molecules content with the Open Probe Fast GC-MS as described in[24] and found the step of thermal extraction to be effective and quantitative. However, with the Open Probe fast GC-MS, we are unable to increase the column flow rate, and thus, we need the BloodProbe in the sealed ChromatoProbe injector that can produce high pressure behind the column for the analysis of the low-volatility diglycerides and triglycerides.

2 | EXPERIMENTAL

We used the 5977-SMB GC-MS with Cold EI system that is based on the combination of an Agilent 7890A GC + 5977 MSD (Agilent Technologies, Santa Clara, CA, USA) with the Aviv Analytical SMB interface and its dual-cage fly-through ion source (Aviv Analytical Ltd, Hod Hasharon, Israel). The technology of GC-MS with Cold EI is reviewed in literature[14–16] and further described in refs.[26,27] In this system, the GC column output is mixed with helium make-up gas (~50 ml/min typical total column and make-up flow rate combined), in front of a supersonic nozzle that is located at the end of a heated and temperature-controlled transfer line. The helium makeup gas flow can be mixed (via the opening of one valve) with perfluorotributylamine (PFTBA) for periodic system tuning and mass calibration. The sample compounds seeded in the helium gas expand from a 100-μm-diameter supersonic nozzle into an SMB nozzle vacuum chamber that is differentially pumped by a Varian Navigator 301 turbo molecular pump (Varian Inc., Torino, Italy) with 250 L/s pumping speed. The helium pressure at this vacuum chamber is about 6×10^{-3} mbar. The supersonic expansion vibrationally cools the sample compounds, and the expanded supersonic free jet is skimmed by a 0.8-mm skimmer and collimated in a second differentially pumped vacuum chamber, where

an SMB is formed. The second vacuum chamber is pumped by the Agilent 5977 system "Performance" turbo molecular pump that pumps the dual-cage fly-through ion source and MS (Pfeiffer 250 L/s pump). The SMB seeded with vibrationally cold sample compounds pass a fly-through dual-cage EI ion source[28] where these beam species are ionized by 70-eV electrons with 6-mA emission current. It is worthwhile to note that the Cold EI ion source was used for over 6 years without any service while maintaining almost the same performance. The ions are focused by an ion lens system, deflected 90° by an ion mirror, and enter the Agilent 5977 MS for their mass analysis. The 90° ion mirror is separately heated and serves to keep the mass analyzer clean from possible sample-induced contaminations. The ions that exit the Agilent quadrupole mass analyzer are detected by the Agilent triple axis ion detector, and the data are processed by the Agilent ChemStation software. GC separation was performed with a 15-m column with 0.32 mm ID, 0.1 μ DB1HT films, and 8 mL/min column flow rate with flow program at the late stage of the run from 8 to 30 mL/min at the rate of 8 mL/min.min after 6 min. The GC oven was ramped from 50 to 350°C at 40°C/min and maintained at 350°C for 3.5 min. The BloodProbe that we used (shown in Figure 1) is based on a modified ChromatoProbe[19] for the sampling of whole blood. The BloodProbe is based on the conversion of the ChromatoProbe vial holder into carrying a thin glass rod the same as used in the Open Probe Fast GC-MS[23,24] (Agilent name QuickProbe[25]). Accordingly, the thin glass rod (100 mm length × 1.56 mm diameter) carries the blood droplet near its edge, and due to its exposed nature and minimal heat capacity, thermal vaporization is very effective from it. We used the standard Agilent split splitless injector at 280°C injector temperature and split ratio of 5. The ChromatoProbe external portion was air-cooled to prevent the premature vaporization of volatile compounds during sample insertion, and the split ratio was selected to avoid saturation by cholesterol. The choice of 280°C injector temperature was made to retain the triglycerides yet to minimize background from protein thermal decomposition.

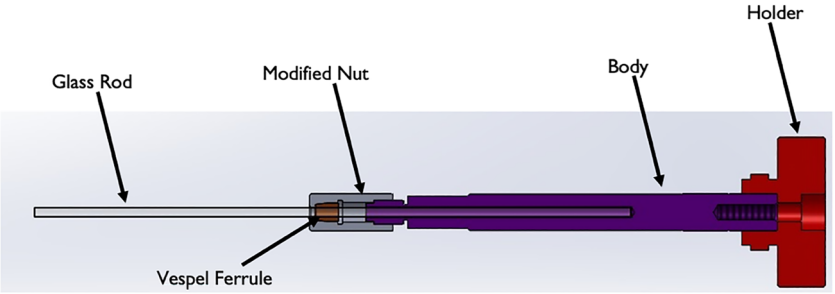

FIGURE 1 BloodProbe device for the sampling of whole blood deposited on its glass rod edge and later introduced via the ChromatoProbe into the GC injector of a GC-MS with Cold EI for blood content thermal desorption and analysis

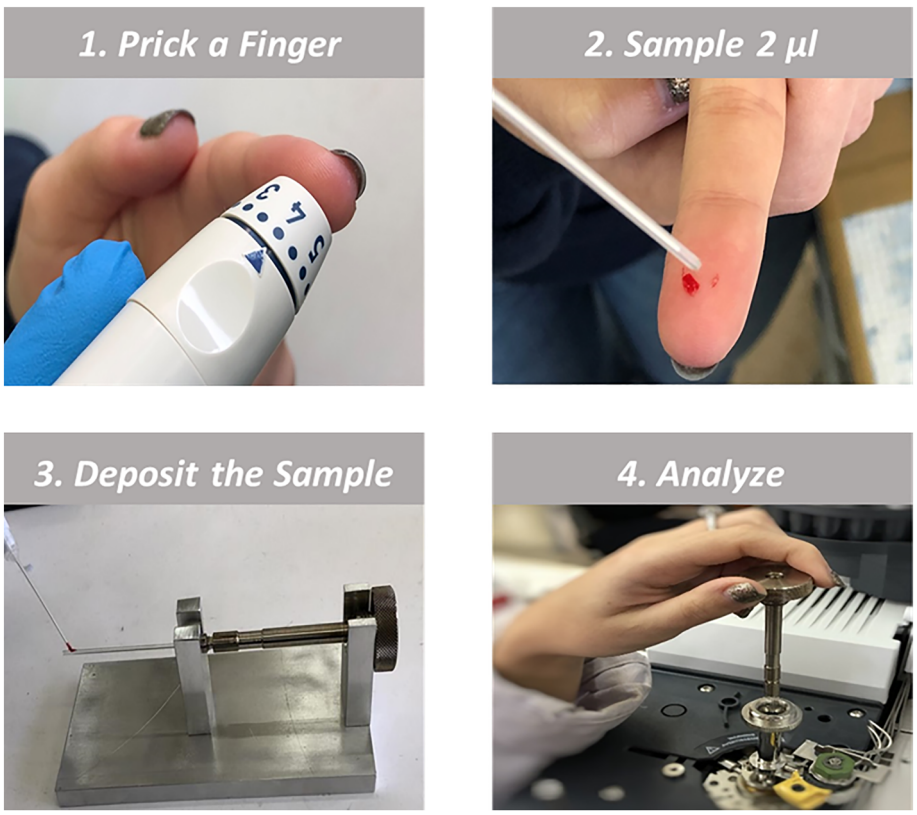

FIGURE 2 Whole blood sample handling procedure for preparations for its analysis as shown in the indicated steps

In Figure 1, we show the design of the BloodProbe.

We also developed whole blood sample handling procedure as shown in Figure 2. We used a diabetes lancet to prick the finger (Figure 2, upper left). Before the finger pricking, we replaced for each blood donor the sharp needle inside the lancet to avoid the touch of old blood residues with the new blood. The next step was to press by a hand on the finger with exposed blood to form an exposed blood droplet on that finger (as practiced for blood glucose tests). We then used a positive displacement micro pipette to draw 2 μL as shown in Figure 2 (upper right). The entire 2 μL volume of whole blood was then deposited on the BloodProbe glass rod at its left (outer) side edge as shown in Figure 2 (bottom left). In order to properly clean the glass rods to eliminate all contaminations, each glass rod was heated for 1 min to 450°C using a heating fan, prior to its use for sampling whole blood. The next step was that the BloodProbe with its dried whole blood spot (dried in a few seconds) was inserted into the ChromatoProbe for intra-injector blood content thermal desorption vaporization (Figure 2, bottom right) followed by GC-MS with Cold EI analysis.

3 | RESULTS AND DISCUSSION

In Figure 3, we show mass chromatograms of lipids in whole bloods of two people, one from a healthy person (upper trace) and one from a person with Stage 4 colorectal cancer (bottom trace) possibly also with liver dysfunction. As shown, highly detailed mass chromatograms were obtained with full nonpolar lipid information. The names of the main various compounds and compound families are indicated in the upper mass chromatogram and include free fatty acids, monoglycerides, cholesterol, vitamin E, diglycerides, cholesteryl esters, and triglycerides, and all exhibited molecular ions. Cold EI mass spectra of selected triglycerides are shown at the inserts. Of note are some major differences between the two human samples. For example, the blood of the cancer patient had over 30 times more triglycerides, whereas the relative amount of the free cholesterol was similar. Furthermore, the blood sample of the cancer patient has more saturated triglycerides as shown from the masses of the molecular ions. We note that Cold EI is characterized by approximately uniform compound independent response,[16,29] and thus, from Figure 3, we can also conclude and estimate the relative abundance of each analyzed compound without additional calibration analysis.

An emerging initial question about the medical diagnostics value of GC-MS with Cold EI analysis of whole blood is how well its generated data correlate with currently available medical diagnostics methods such as total cholesterol and triglycerides in blood analysis. Total cholesterol is currently analyzed after its hydrolysis,[30] and thus, it provides mostly the sum of free cholesterol and cholesteryl esters. We found that human blood includes a few cholesteryl esters

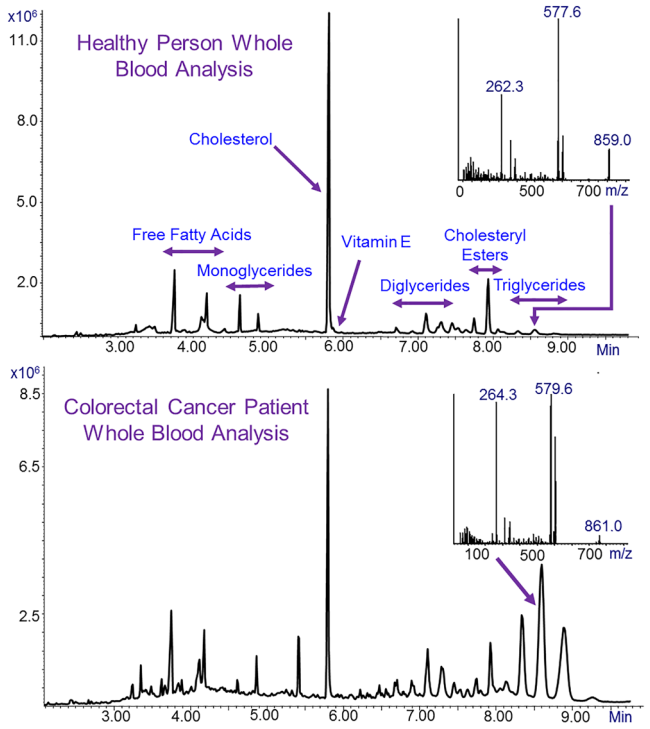

FIGURE 3 GC-MS with Cold EI total ion mass chromatograms and Cold EI mass spectra of two humans whole blood samples obtained with a diabetes lancet, deposited on a BloodProbe device and inserted into a ChromatoProbe mounted on a GC-MS with Cold EI. The names of the various compounds and compound families are indicated and all with molecular ions. Cold EI mass spectra of selected triglycerides (indicated by the arrows) are shown at the inserts

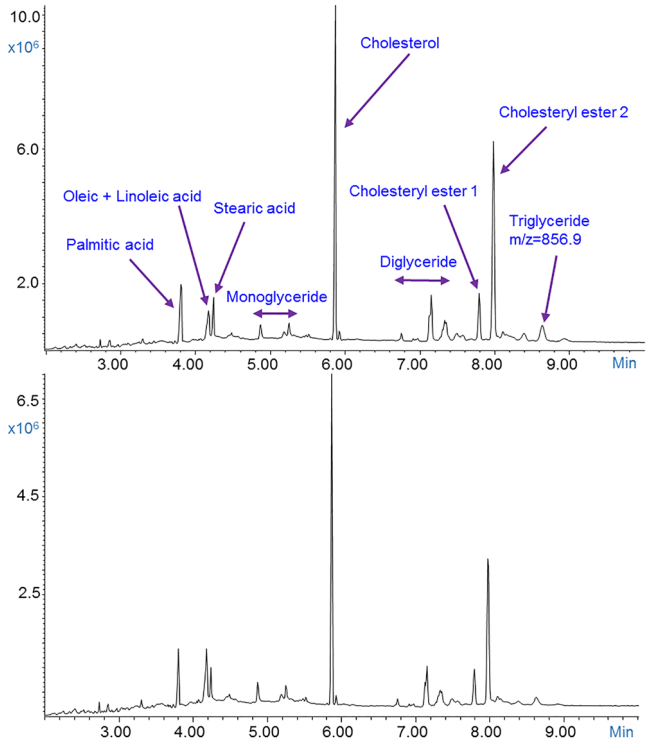

FIGURE 4 The effect of 10-week diet on an obese subject with Type 2 diabetes on the blood serum mass chromatograms. Analysis of 2 μL of blood serums is shown, before the beginning of the diet (upper mass chromatogram) and 10 weeks after the start of the diet (bottom mass chromatogram)

although one of them is by far the most abundant, and its molecular weight is m/z = 648.7, which seems to indicate the ester of cholesterol with an acid with molecular weight of 280.3 such as linoleic. We do not know why the esterification is with an unsaturated acid and not with the much more abundant stearic acid and why is the ester with palmitic acid so much weaker in its abundance. Next, we analyzed serum samples of several subjects with obesity and Type 2 diabetes who were placed on a Mediterranean vegetarian diet for 10 weeks.[31] These samples allowed us to compare cholesterol in blood serum prior to and at the end of the intervention, and results were compared to the results obtained from a standard hospital clinical laboratory. We found that serum analysis and whole blood analysis were similar in their procedures (serum replaced whole blood on the BloodProbe) and generated data.

In Figure 4, we show the generated GC-MS with Cold EI data of the same person serum analysis before and after 10 weeks of diet. The mass chromatograms are normalized to the highest peak of cholesterol, and thus, the difference in cholesterol amount is found in the Y axis numbers, which show that the cholesterol concentration was reduced by the diet by a factor of about 1.41. However, an important additional observation is that the cholesteryl ester concentrations were more prominently reduced, by a factor of about 1.92 (for the most abundant cholesteryl ester #2). We found this trend in several other subjects' serum. Therefore, this might represent a valuable finding that is not thus far accessible by current standard clinical methodology. Accordingly, it is possible that the measurement of cholesteryl esters can provide a more sensitive medical diagnostic information than free or bound cholesterol. Furthermore, our method of analysis had reproducibility (RSD) of about 8%–10%, mostly due to the variability in the micro pipette blood sampling (we made 10 analyses for the RSD measurements). The measurement of cholesteryl esters can be normalized to that of free cholesterol, and thus, their ratio could be very accurate and provide additional valuable medical diagnostics value. Our method allows us to perform on the generated data a reconstructed single ion monitoring (RSIM) on m/z = 368.3 and obtain a clean mass chromatogram of cholesterol and the various cholesteryl esters alone at high sensitivity and accuracy with very little baseline noise.

In Figure 5, we show the comparison of changes after 10 weeks of diet in the measurement of total cholesterol as measured by the

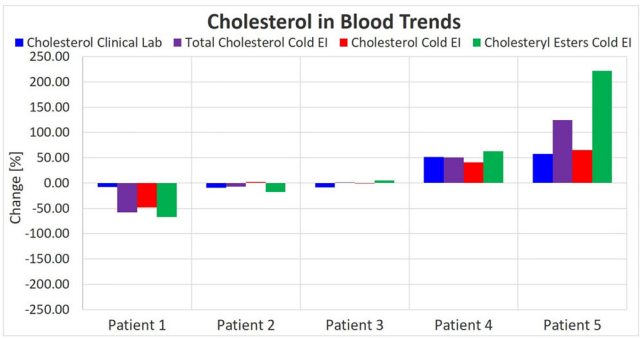

FIGURE 5 Changes in cholesterol and cholesteryl esters after 10 weeks of diet in the serum of five obese people with Type 2 diabetes. For each person, we show in blue the % change in cholesterol as measured by current standard method, whereas changes detected by GC-MS with Cold EI are shown in purple. The change in free cholesterol is shown in red, and the change in cholesteryl esters alone, in green. Note that the per-person trends are about the same for both measurement methods, whereas the magnitude of the change is relatively higher for cholesteryl esters

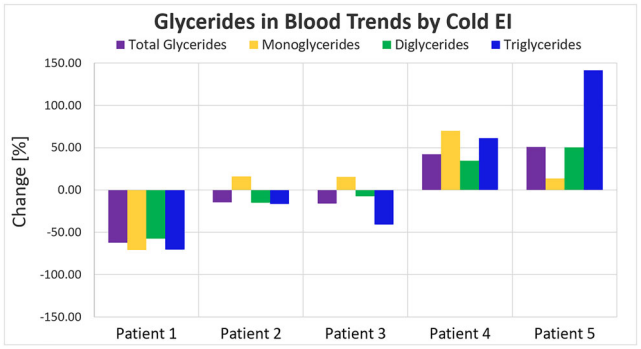

FIGURE 6 Changed ratios in the total glycerides as measured by GC-MS with Cold EI (purple) and monoglycerides (yellow), diglycerides (green), and triglycerides (blue) as separately measured by GC-MS with Cold EI in the serum of five people after 10 weeks of diet

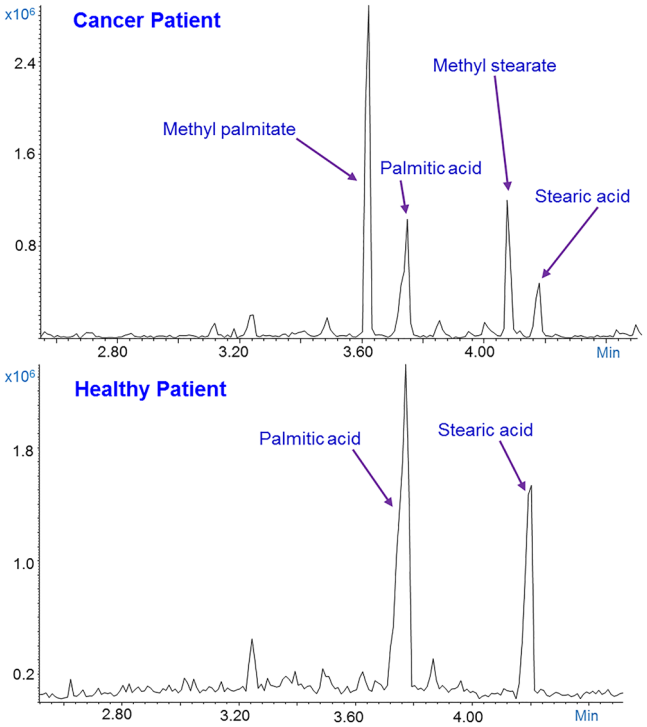

FIGURE 7 Fatty acid methyl esters (FAMEs) analysis in the blood of a healthy person (bottom mass chromatogram) and a Stage 4 colorectal cancer patient (upper mass chromatogram). The mass chromatograms show the extracted ion m/z = 74.1, which is found in all the FAMEs of saturated fatty acids

standard method and the measurement of free cholesterol, cholesteryl esters and their sum as measured by GC-MS with Cold EI in the same serum of five people that had 10 weeks of diet. As shown, the relative changes in total cholesterol and free cholesterol are similar although measured via totally different methods, whereas cholesteryl esters were found to exhibit greater changes after the diet. Thus, we conclude that GC-MS with Cold EI can provide valuable diagnostics information via its separate free cholesterol and cholesteryl ester measurements. Whereas free cholesterol is the most abundant vaporizable compound in human blood, cholesteryl esters comprise another abundant group of compounds. However, additional related compounds are thus captured by this method such as cholestadiene, which is cholesterol with loss of water. Its relative abundance is below or about 1% of the cholesterol peak, but it is easily identified and quantified via RSIM on its molecular ion m/z = 368.3, which is also a fragment ion in cholesterol mass spectrum. The medical diagnostics value of cholestadiene concentration and its relative amount to cholesterol remain to be explored.

We similarly tried to correlate the measurement of triglycerides, but although the measurements exhibited some correlation, it was not as good as for cholesterol. We suggest that the current standard measurement of triglycerides is not optimal because it is based on initial hydrolysis of all the glycerides followed by the measurement of resulting glycerol, which is the sum of monoglycerides, diglycerides, and triglycerides.[32] As exemplified in Figure 4, diglycerides are usually the most abundant glycerides. Furthermore, they include an OH group that is chemically reactive, and thus, we suspect that among the glycerides the diglycerides (and similarly the monoglycerides) are the most reactive and potentially detrimental to blood vessels.[33] As shown in Figures 3 and 4, GC-MS with Cold EI enables the separate determination of each glyceride group, and the future attempted correlation of their concentrations for improved medical diagnostics value.

In Figure 6, we show the changes in total triglycerides and separately, monoglycerides, diglycerides, and triglycerides as measured by GC-MS with Cold EI in the serum of five people after 10 weeks of diet. Note that the changes are relatively larger for triglycerides as measured by GC-MS with Cold EI. However, as mentioned previously, we suggest that due to chemical activity considerations, the currently unmeasured diglycerides are potentially highly valuable for medical diagnostics as due to their unesterified OH group they are assumed to be more chemically reactive. Much more research can and should be devoted to this medical diagnostic aspect once GC-MS with Cold EI will be commercially available.

GC-MS with Cold EI provides a wealth of information on various nonpolar lipids in blood. Accordingly, we aimed to evaluate its value for various medical diagnostics scenarios and explored differences in blood content between various people. One group of compounds, usually at low concentrations, is fatty acid methyl esters (FAMEs).

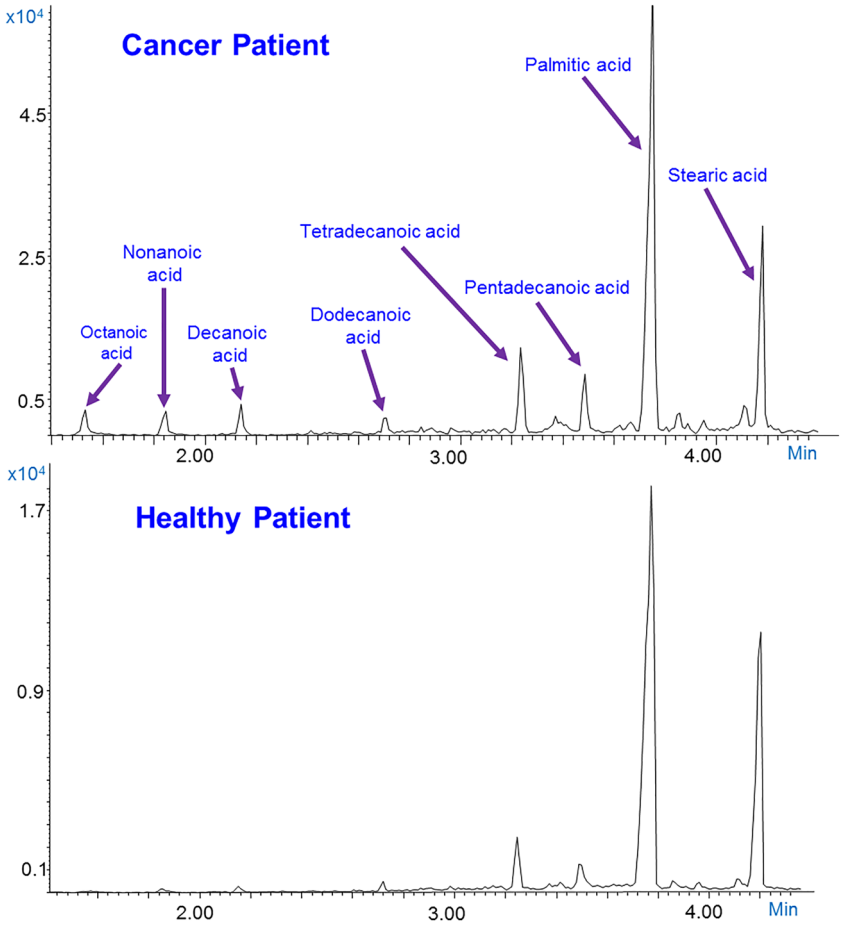

FIGURE 8 Saturated fatty acid analysis in a cancer patient blood (upper RSIM) versus in a healthy person blood (bottom RSIM). The m/z = 73 ion is used to monitor saturated fatty acids as indicated. Note that the smaller free fatty acids with less than 16 carbon atoms are much weaker in the healthy blood

However, as shown in Figure 7, the analysis of FAMEs in the blood of a healthy person was significantly different from that of a cancer patient that exhibited over 30 times higher FAMEs concentrations (mostly methyl stearate and methyl palmitate) in the colorectal cancer patient blood. We used for convenience the extracted ion m/z = 74.1 in the mass chromatogram because it is found in all the FAMES of saturated fatty acids. This ion is also found in saturated fatty acids as the isotopologue of the abundant m/z = 73 ion, which as a result brings also free fatty acids into the RSIM mass chromatogram as observed in Figure 7 in which the fatty acids peaks are fronted as they saturated the GC column due to their high polarity. It is documented in the literature[34] that people with liver dysfunction exhibit higher FAMES levels, and indeed, the cancer patient (upper mass chromatogram in Figure 7) also suffered from liver dysfunction. It is described that FAMEs can be enzymatically synthesized by carboxyl group alkylation.[35] We do not claim that we established here a new type of medical diagnostics capability, but we showed that GC-MS with Cold EI can provide, in the same run, information on saturated FAMEs concentrations and on their ratio to the saturated free fatty acids. Such analysis can noticeably increase the medical diagnostics value of lipids in blood. We note that, if desirable, much higher signal-to-noise ratio can be obtained for the FAMEs analysis via RSIMs on their molecular ions.

GC-MS with Cold EI can also serve for the analysis of free fatty acids without any need for derivatization due to its contact-free fly-through ion source (unlike standard EI ion sources). Currently, fatty acids are not monitored in blood although we suggest that they contain vast amount of potentially useful diagnostics information. As shown in Figures 3 and 4, the most abundant fatty acids in human blood are the C16 saturated palmitic acid, the C18 saturated stearic acid, and the C18 monounsaturated oleic acid and diunsaturated linoleic acid. As shown in Figures 3 and 4 and as we found in several other blood and serum samples, the concentrations of these acids vary from person to person, and interestingly, the ratio of the saturated stearic acid to the unsaturated oleic and linoleic can significantly change from person to person and may depend on the diet and other parameters. Furthermore, the free fatty acids also include minor amounts of acids with higher than C18 carbon atoms and small amount of acids with less than C16 carbon atoms. Whereas the C18 acids include various acids with several degrees of unsaturation such as 1, 2, 3, and more the C16 acids are predominantly the saturated palmitic acid with very small amount of the m/z = 254.3 of the monounsaturated C16 acid.

The saturated acids are all characterized by a major fragment ion with m/z = 73, and thus, they can be easily monitored together via RSIM on m/z = 73 as shown in Figure 8. Accordingly, the fatty acids in blood analysis can provide information on the concentration and

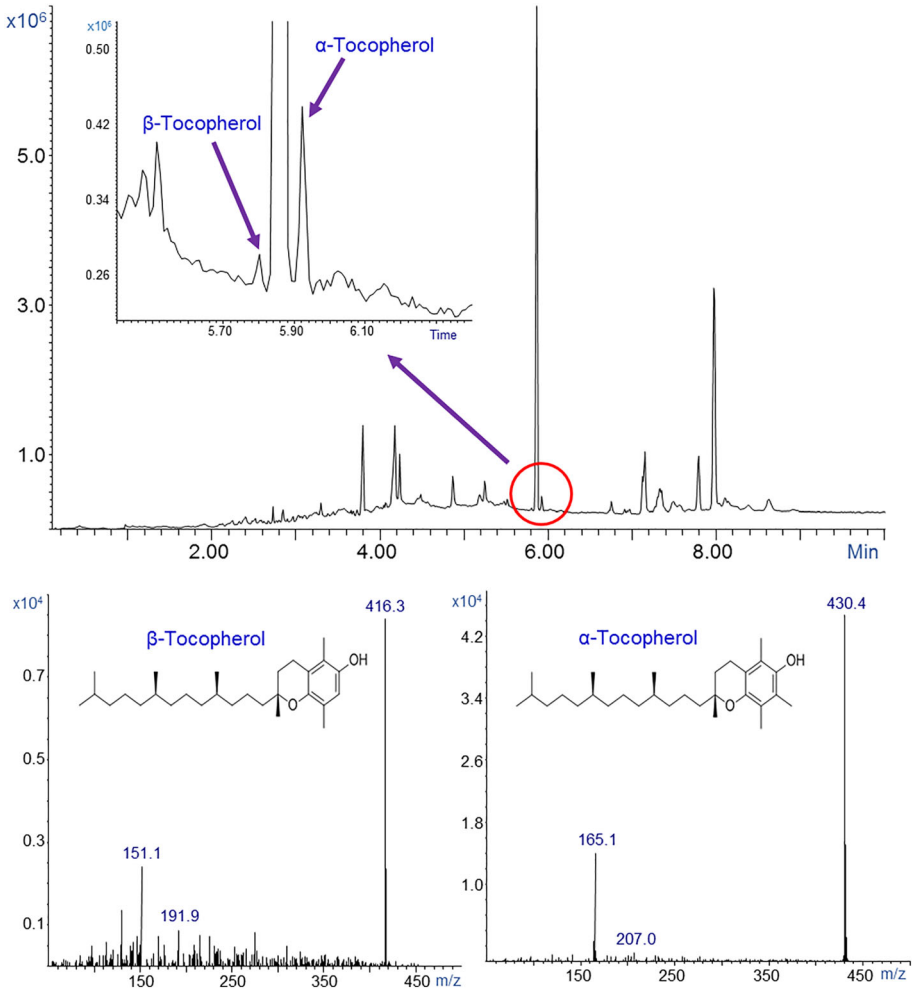

FIGURE 9 Vitamin E in blood analysis. A mass chromatogram of 2 μL of whole blood is shown using the BloodProbe and GC-MS with Cold EI. The upper trace is zoomed near the elution of vitamin E, and it is marked with a red circle. The two arrows in the zoomed mass chromatogram indicate the peaks of β-tocopherol and α-tocopherol correspondingly. The bottom mass spectra are the Cold EI mass spectra of α-tocopherol (right trace) and beta-tocopherol (left trace)

ratio of the smaller fatty acids and the rare fatty acids with odd number of carbon atoms such as C15. In Figure 8, we show saturated fatty acid analysis in a colorectal cancer patient (upper RSIM) versus of a healthy person (bottom RSIM). Note that the smaller free fatty acids with less than 16 carbon atoms are less abundant in blood of healthy persons. Although the use of m/z = 73 provide a picture of all the saturated fatty acids (including the rare C15), we can use the molecular ions for a cleaner and more sensitive separate monitoring of each fatty acid.

Another group of medically important compounds in blood are vitamins. Only rare medical laboratories monitor vitamin D in blood by LC-MS, in its 25-hydroxyvitamin D3 form.[36,37] We found in GC-MS with Cold EI mass chromatograms of blood and serum vitamin E in the form of two of its isomers. In Figure 9, we show the analysis of vitamin E in blood. The upper trace is the Cold EI mass chromatogram of whole human blood. The mass chromatogram also includes a zoomed trace near the elution time of vitamin E, which is also near the elution time of cholesterol, and it is marked with a red circle. The two arrows in the zoomed mass chromatogram indicate the peaks of β-tocopherol and α-tocopherol correspondingly. The bottom mass spectra are the background subtracted Cold EI mass spectra of α-tocopherol (right trace) and β-tocopherol (left trace), and as shown, they are characterized by enhanced molecular ions. Thus, with GC-MS with Cold EI, vitamin E can be effectively monitored, and its RSIM traces on the molecular ions are clean and can serve for its accurate quantification.

In Figure 10, we show the ratio of β- to α-tocopherol in the blood of two persons and as demonstrated this ratio can greatly change between blood samples. In general, α-tocopherol is about 10 times more abundant than β-tocopherol, but this ratio changes between individuals. We found that in general, the blood samples of cancer patients (unknown types) show higher ratio of β- to α-tocopherol. Vitamin E deficiency is rare in healthy people. With the exclusion of malnutrition, vitamin E deficiency is most commonly caused by a condition where nutrients are not properly digested or absorbed. These include liver disease, cystic fibrosis, and some rare genetic disorders.[38] Vitamin E deficiency may also be caused by a very low-fat diet. As demonstrated the use of GC-MS with Cold EI can also serve for vitamin E analysis in both of its α- and β-tocopherol forms.

We also found in blood several new and/or unidentified compounds as well as known yet unexplored compounds in terms of their medical diagnostics value. Among these compounds, we show here for the first time free elemental sulfur S_8 in blood and serum samples. The concentration of S_8 varied greatly from person to

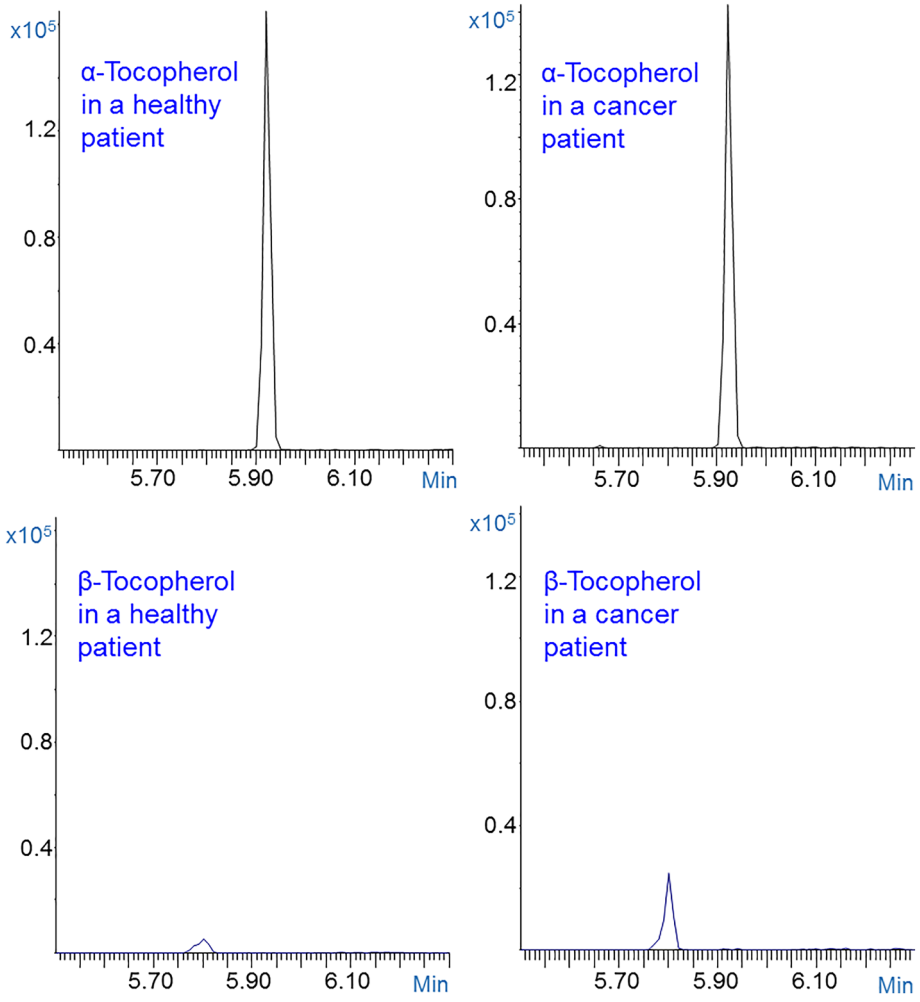

FIGURE 10 Vitamin E as α-tocopherol and β-tocopherol comparison in cancer and healthy patients. The mass chromatograms were obtained via RSIM on the molecular ions

person. In future research, this should be correlated with health conditions. In Figure 11, we show the finding of S_8 in human blood. The upper trace shows GC-MS with Cold EI mass chromatogram zoomed around the elution time of elemental sulfur and palmitic acid. The background subtracted Cold EI mass spectrum of S_8 is shown at the bottom trace with abundant molecular ion at m/z = 255.8 and distinct isotope abundance pattern. The highly negative mass defect and its identification by the NIST library unambiguously prove that this is S_8. This finding is further supported by our TAMI software[39,40] that identifies elemental formulae via the isotope abundance patterns in combination with the quadrupole 0.14 u mass accuracy.

In Figure 12, we demonstrate the ability to obtain high signal-to-noise ratio for the S_8 peak. We employed RSIM in the complex mass chromatograms of human blood while using m/z = 255.8 with ±0.2 u mass window. We used the fact that S_8 is unique in having very negative mass defect to filter out noise and including the elimination of the nearby eluting palmitic acid with m/z = 256.3. The obtained signal-to-noise ratio is about 700 in peak to peak, leading to a clean mass chromatogram of only S_8 and thus showing the ability to accurately measure small concentrations of S_8 even in full scan in the complex matrix of human blood.

In Figure 13, we show the S_8 concentrations and relative abundances to palmitic acid in four human blood samples. Four RSIM traces are plotted with m/z = 256.0 zoomed around the elution time of S_8 that include both S_8 and palmitic acid. We note that the ratio change can be significant (over a factor of 10) and in a few blood samples the S_8 signal was near the detection limit. In Figure 13, the sulfur S_8 peak areas in counts were 238,222 in Serum A, 867569 in Serum B, 558782 in Serum C and 315,882 in Serum D, as extracted from the Agilent ChemStation software.

We are aware of possible criticism that this S_8 compound can originate from sulfur containing amino acids in proteins owing their thermal degradation at the heated GC injector. However, we unambiguously found the sulfur as S_8, and it seems unlikely to be formed from atomic or dimeric S_2 in the fast vaporization at the GC injector. In addition, the finding that S_8 concentration in blood largely varies between people further suggests that it is an independent circulating element in the human blood. Admittedly, further experiments are needed to validate this finding. To our knowledge, sulfur was not analyzed for medical diagnostics, and only few articles discuss it in blood.[41,42]

Another interesting compound that we were able to detect in blood is dioctyl phthalate in the isomeric form of diethylhexyl

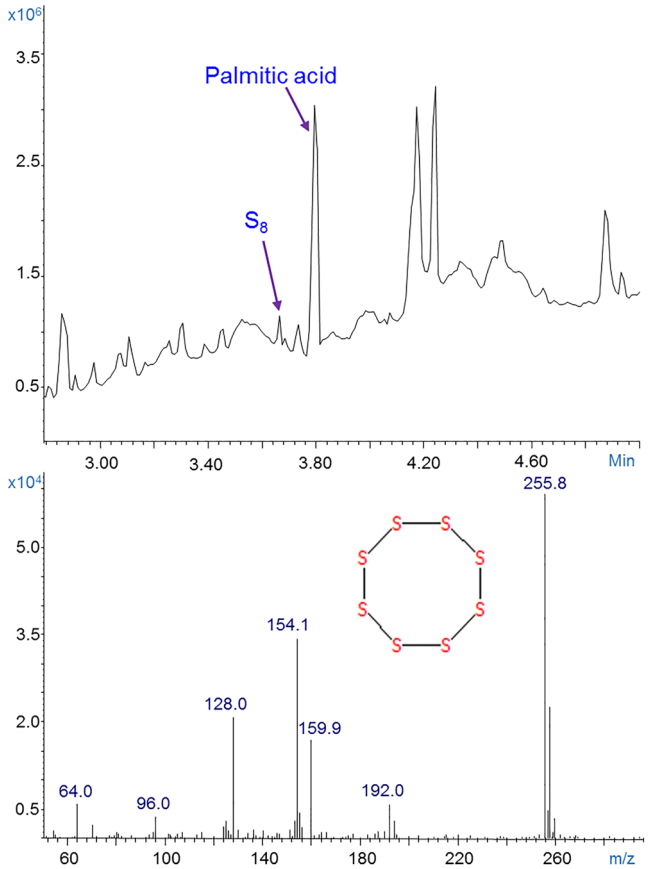

FIGURE 11 The finding of S_8 in human blood. The upper trace shows GC-MS with Cold EI mass chromatogram zoomed around the elution time of palmitic acid and elemental sulfur. The background subtracted Cold EI mass spectrum of S_8 is shown at the bottom trace with abundant molecular ion at m/z = 255.8 and distinct isotope abundance pattern

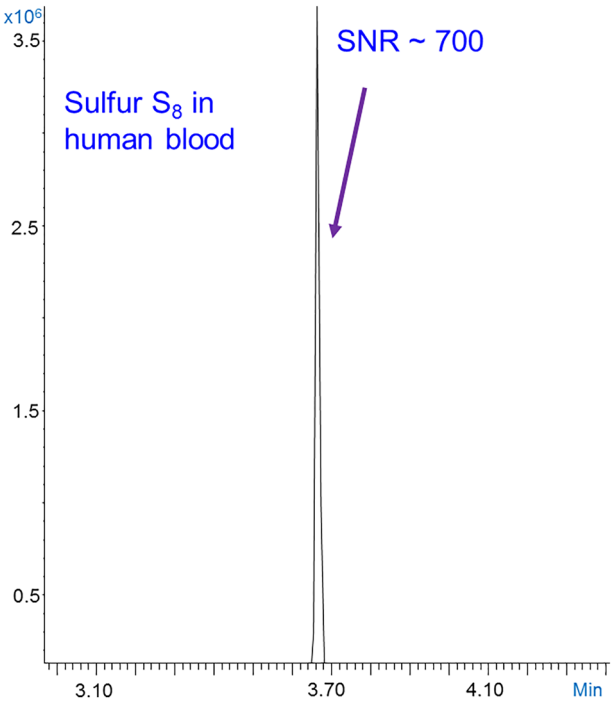

FIGURE 12 High signal-to-noise ratio in S_8 analysis in blood. Reconstructed single ion monitoring of human blood with m/z = 255.8 with ±0.2 u mass window. The obtained signal-to-noise ratio is about 700, and the elimination of the palmitic acid peak is due to the unique negative mass defect of the elemental sulfur that is leading to a clean mass chromatogram of only S_8

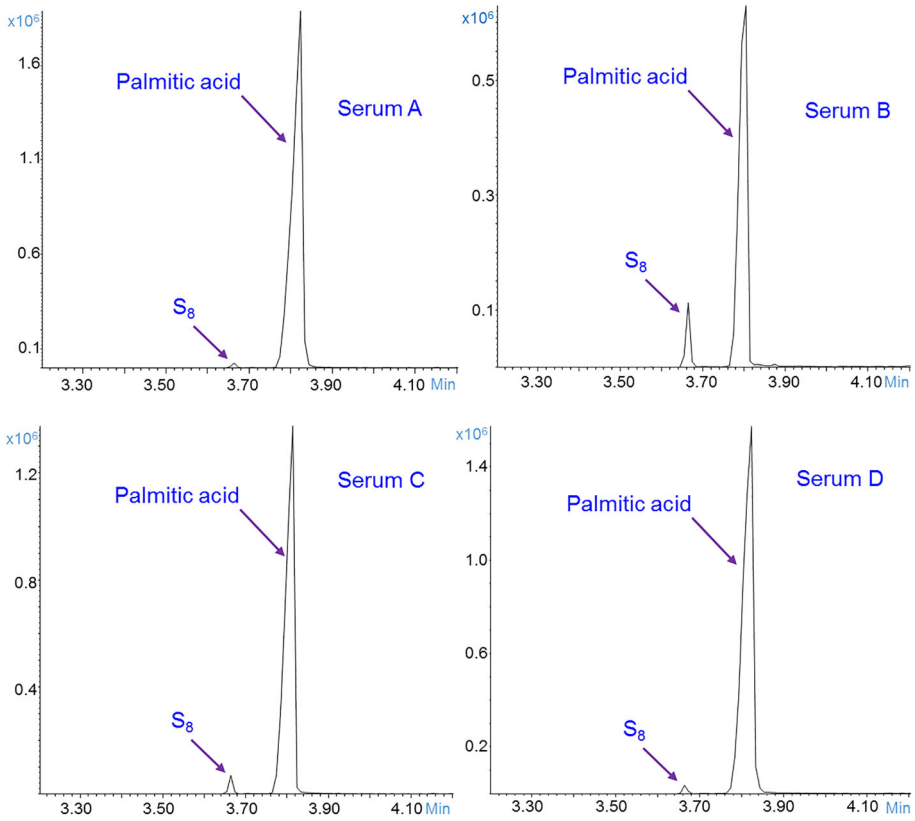

FIGURE 13 S_8 concentrations and relative abundances to palmitic acid in four human blood samples. Four RSIM plots at m/z = 256.0 + −0.3 u are shown, zoomed around the elution time of S_8 that include both S_8 and palmitic acid

phthalate. This compound belongs to the group of potentially endocrine disruptor compounds. Phthalate acid esters are widely used as plasticizers in the formulation of plastics. In particular, certain members of this chemical class, such as di-(2-ethylhexyl)phthalate (DEHP), have been shown to cause reproductive and developmental toxicity and are suspected to be endocrine disruptors.[43] Although we report this finding, we do not show it in a figure because it is difficult to assure the origin of the DEHP to be in the blood and not from our tools and analysis process. Thus, we report that this technique has the potential to detect these components, yet further investigation is required.

3.1 | Fast drugs in blood analysis for hospital emergency rooms

In the last decade, drug overdose-related death became an epidemic in the United States with over 70,000 deaths in 2020,[44] which was about 200/day, and this trend continues to climb with over 93,000 deaths (over 250/day) in 2020.[45] This is greater than the combined rate of death from car accidents and homicides. Among the drugs, fentanyl and its derivatives are the most dangerous and deadly. Thus, in many cases, people arrive into hospital emergency rooms with certain severe drug poisoning or other symptoms without knowing which drugs they took or how it could interfere with their optimal treatment. As a result, a method and instrument that could provide fast analysis of drugs in blood can help doctors to quickly provide the best and most suitable treatment and save lives in cases of toxic drug consumption. We note that although GC-MS with Cold EI improves all the central performance aspects of GC-MS, its significantly extended range of compounds amenable for analysis is of particular importance for drugs analysis to effectively compete with LC-MS.

Accordingly, we also explored the analysis of drugs in whole blood in order to provide fast drugs in blood medical diagnostics information. In Figure 14, we show drugs identification in human blood using 2-μL whole blood samples with BloodProbe sampling and GC-MS with Cold EI analysis. The right side shows the obtained blood mass chromatogram (healthy person) zoomed around the elution time of ibuprofen (indicted with an arrow) and the background subtraction Cold EI mass spectrum shown at the bottom right trace. As shown, the ibuprofen drug exhibits noticeable peak and it was easily identified by the NIST library. The left side of Figure 14 shows the obtained blood (taken from a cancer patient) mass chromatogram zoomed around the elution time of a cancer drug metabolite (indicted with an arrow) and its background subtraction Cold EI mass spectrum (bottom left). Again, the 4-chlorobenzoic acid 4 chlorophenyl ester drug (or drug metabolite) exhibits noticeable peak, and it was easily identified by the NIST library. It should be mentioned that compounds with chlorine atoms in blood must originate from drugs or another external source and they are absent in blood of healthy people. We further note that caffeine was found in most blood and/or serum samples. This offers an opportunity to address and possibly monitor, in terms of nutritional recommendations for coffee consumption, the achieved actual blood levels of caffeine.

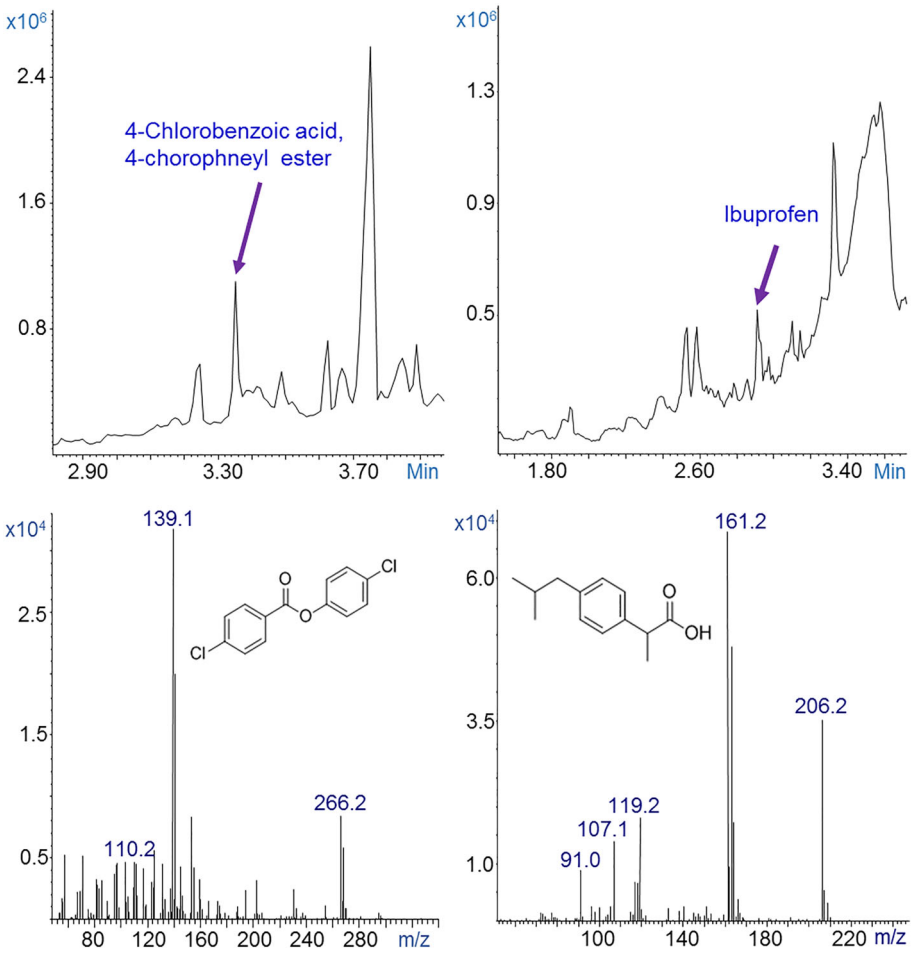

FIGURE 14 Drugs identification in human blood using 2-μL whole blood with BloodProbe sampling and GC-MS with Cold EI analysis. The right side shows the obtained blood mass chromatogram (healthy person) zoomed around the elution time of ibuprofen (indicted with an arrow) and the background subtraction Cold EI mass spectrum shown at the bottom right trace. The left side shows the obtained blood (taken from a cancer patient) mass chromatogram zoomed around the elution time of a cancer drug metabolite (indicted with an arrow) and its background subtraction Cold EI mass spectrum (bottom left)

As shown earlier, GC-MS with Cold EI can serve for the analysis of various lipids and other compounds in blood together with the provision of information on drugs in the blood samples. Our demonstrated blood analysis takes a few minutes for the blood collection, whereas the analysis takes 10 min, and cooling back and ready for next analysis may take another 5 min, whereas the next blood sample can be collected during the previous sample analysis thus it does not add time. Balog et al developed the iKnife for intraoperative tissue identification using rapid evaporative ionization MS,[46] which provides online chemical information during surgery. In case of need for such much faster real-time analysis, we also developed an Open Probe[47] and Low Thermal Mass (LTM) Fast GC[48] and combined them into our Open Probe Fast GC-MS[23,24] for the provision of real-time analysis with separation and library based sample identification including at the isomer level. Sample introduction into the Open Probe is similar to our use of BloodProbe, and the fast GC separation takes about 30 s. The separation of the shorter Open Probe Fast GC column (Agilent name QuickProbe[25]) is a little poorer than with our 15-m column, and in view of its limited column flow rate, it cannot be used for triglycerides analysis and thus not optimal for blood analysis as described in this paper, but it can serve for ultrafast drugs in blood analysis.

4 | CONCLUSIONS

Blood tests for cholesterol and triglycerides are widely used for medical diagnostics at hospitals worldwide. Blood samples are commonly obtained directly from the vein in relatively large volumes (approx. 3–30 ml), which can be painful and challenging when the veins are damaged or small as in newborns. Currently blood samples must undergo some sample preparation, such as centrifugation and separation of the serum prior to analysis, and the time to results is around an hour. Moreover, results provided by currently used methods deliver limited information only regarding the total cholesterol, cholesterol bound to proteins, and total glycerides.

Here, we described a new approach for blood analysis for medical diagnostics based on GC-MS with Cold EI, which provide information beyond the boundaries of currently targeted serum lipids. We developed for this project a BloodProbe device based on a modified ChromatoProbe[19] vial holder to accommodate a 10-cm-thin glass rod. Using a diabetes lancet and a positive displacement micropipette, 2-μL blood samples were taken directly from donor fingers, and the blood was deposited on the glass rod, which is mounted into the BloodProbe. Then, samples were analyzed using GC-MS with Cold EI equipped with a ChromatoProbe in which the whole blood was thermally extracted for its analysis.

We showed that blood analysis by GC-MS with Cold EI provide mass chromatograms with full nonpolar lipids in blood information including the full range of free fatty acids, FAMEs, cholesterol, vitamin E, cholesteryl esters, monoglycerides, diglycerides, triglycerides, drugs, and other compounds. Each of them was detected at the molecular level so that currently detected triglycerides, for example, are given as several monoglycerides, diglycerides, and triglycerides, each with information on its fatty acid content. It should be mentioned that Cold EI uniquely provides enhanced molecular ions together with lower mass fragment ions. Thus, the Cold EI mass spectra are fully compatible with NIST library identification which is even improved compared with standard EI.[27,49] Our whole blood analysis revealed:

A. Detailed mass chromatograms were generated with full range of all the nonpolar lipids in blood.
B. The analysis of 2-μL whole blood was compared with serum extracts analysis using the same BloodProbe and GC-MS with Cold EI. We show that the analysis of whole blood is as informative as the analysis of blood serum. However, the use of BloodProbe and whole blood was far simpler and faster.
C. We found that cholesteryl esters were more sensitive than free cholesterol alone to the effect of at least one form of diet of obese people. Separate monitoring of cholesteryl esters may be potentially highly valuable for medical diagnostics.
D. We found major enhancement of several fatty acid methyl esters in the blood of a cancer patient with liver dysfunction compared with the blood of healthy patients.
E. We discovered elemental sulfur as S_8 in whole blood, which varied in its concentration among people but not yet in a clear fashion.
F. We found vitamin E in the forms of both α-tocopherol and β-tocopherol with person-dependent ratio.
G. We also found several drugs and other known and unknown compounds that need further study of their correlation to medical status.

Finally, we note that the feature of our instrument and method of using only 2-μL blood sample that is taken in a minimally invasive way from fingers (as being done on a daily basis by diabetes patients) is very important. Although we could use up to 10-μL blood sample for analysis, we think that the smaller the amount of blood the easier it is to get. This means that blood analysis for medical diagnostics by GC-MS with Cold EI can be easily performed due to the simplicity of access to blood, reminiscent of self-glucose monitoring. This way, the evolution of various medical conditions can be better monitored, followed, and personalized. Taken together, GC-MS with Cold EI offers a unique opportunity for improved medical diagnostics that should be further explored, established, and validated in a range of medical scenarios.

DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restrictions.

ORCID

Aviv Amirav https://orcid.org/0000-0003-4982-7984

REFERENCES

1. Doll H, Shine B, Kay J, James T, Glasziou P. The rise of cholesterol testing: how much is unnecessary? *Br J Gen Pract*. 2011;61:81-88.
2. Cox RA, Garcia-Palmieri M. Cholesterol, triglycerides, and associated lipoproteins. In: Walker HK, Hall WD, Hurst JW, eds. *Clinical Methods: The History, Physical, and Laboratory Examinations*. 4rd ed. Boston: Butterworth; 1990 ISBN-10: 0-409-90077-X.
3. Li LH, Dutkiewicz EP, Huang YC, Zhou HB, Hsu CC. Analytical methods for cholesterol quantification. *Analysis*. 2019;27:375-386.
4. Laufs U, Parhofer KG, Ginsberg HN, Hegele RA. Clinical review on triglycerides. *Eur Heart J*. 2020;41(1):99-109c. doi:10.1093/eurheartj/ehz785
5. Bonow RO, Mann D, Tomaselli G (Eds). Risk markers and the primary prevention of cardiovascular disease. In: *Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine*. 11th ed. Philadelphia, PA: Elsevier; 2019.
6. Duncan MS, Vasan RS, Xanthakis V. Trajectories of blood lipid concentrations over the adult life course and risk of cardiovascular disease and all-cause mortality: observations from the Framingham study over 35 years. *J Am Heart Assoc*. 2019;8:e011433.
7. Grundy SM, Stone NJ, Bailey AL, et al. Guideline on the management of blood cholesterol: A report of the American College of Cardiology/American Heart Association task force on clinical practice guidelines. *Circulation*. 2019;139(25):e1082-e1143. doi:10.1161/CIR.0000000000000698
8. Sethi S, Brietzke E. Recent advances in lipidomics: analytical and clinical perspectives. *Prostaglandins Other Lipid Mediat*. 2017;128-129:8-16. doi:10.1016/j.prostaglandins.2016.12.002
9. Bird SS, Marur VR, Sniatynski MJ, Greenberg HK, Kristal BS. Serum lipidomics using LC-MS and high energy collisional dissociation fragmentation: focus on triglycerides detection and characterization. *Anal Chem*. 2011;83(17):6648-6657. doi:10.1021/ac201195d
10. Pati S, Nie B, Arnold RD, Cummings BS. Extraction, chromatographic and mass spectrometric methods for lipids analysis. *Biomed Chromatogr*. 2016;30(5):695-709. doi:10.1002/bmc.3683
11. Freeman JD, Rosman LM, Ratcliff JD, Strickland PT, Graham DR, Silbergeld EK. State of the science in dried blood spots. *Clin Chem*. 2018;64(4):656-679. doi:10.1373/clinchem.2017.275966
12. Amirav A, Danon A. Electron impact mass spectrometry in supersonic molecular beams. *Int J Mass Spectrom and Ion Proc*. 1990;97(1):107-113. doi:10.1016/0168-1176(90)85042-Z
13. Amirav A. Electron impact mass spectrometry of cholesterol in supersonic molecular beams. *J Phys Chem*. 1990;94(13):5200-5202. doi:10.1021/j100376a002
14. Amirav A, Gordin A, Poliak M, Fialkov AB. Gas chromatography mass spectrometry with supersonic molecular beams. *J Mass Spectrom*. 2008;43(2):141-163. doi:10.1002/jms.1380
15. Amirav A, Fialkov AB, Margolin Eren KJ, et al. Gas chromatography–mass spectrometry (GC–MS) with cold electron ionization (EI): bridging the gap between GC–MS and LC–MS. *Curr Trend Mass Spectrom Suppl LCGC North Am*. 2020;18:5-15.
16. Amirav A. *Gas Chromatography - Mass spectrometry with cold EI: Leading the way to the future of GC-MS*. USA: Scientific Research Publishing Inc; 2021 ISBN: 978-1-64997-142-5.
17. Fialkov AB, Gordin A, Amirav A. Extending the range of compounds amenable for gas chromatography mass spectrometry analysis. *J Chromatogr A*. 2003;991(2):217-240. doi:10.1016/S0021-9673(03)00247-4
18. Fialkov AB, Steiner U, Lehotay SJ, Amirav A. Sensitivity and noise in GC-MS: achieving low limits of detection for difficult analytes. *Int J Mass Spectrom*. 2007;260(1):31-48. doi:10.1016/j.ijms.2006.07.002
19. Amirav A, Dagan S. A direct sample introduction device for mass spectrometry studies and GC-MS analysis. *Europ Mass Spectrom*. 1997;3(1):105-111. doi:10.1255/ejms.27

20. https://www.agilent.com/en/products/gas-chromatography/sample-preparation-introduction/thermal-separation-probe
21. Amirav A, Jing H. Simultaneous PFPD and MS detection for enhanced pesticide analysis capabilities. *J Chromatogr A*. 1998;814(1-2):133-150. doi:10.1016/S0021-9673(98)00415-4
22. Lehotay SJ. Analysis of pesticide residues in mixed fruit and vegetable extracts by direct sample introduction/gas chromatography/tandem mass spectrometry. *J AOAC Int*. 2000;83(3):680-697. doi:10.1093/jaoac/83.3.680
23. Amirav A, Keshet U, Alon T, Fialkov AB. open probe fast GC-MS – real time analysis with separation. *Int J Mass Spectrom*. 2014;371:47-53. doi:10.1016/j.ijms.2014.08.002
24. Keshet U, Alon T, Fialkov AB, Amirav A. Open probe fast GC-MS - combining ambient sampling ultra-fast separation and in-vacuum ionization for real time analysis. *J Mass Spectrom*. 2017;52(7):417-426. doi:10.1002/jms.3941
25. https://www.agilent.com/en/products/gas-chromatography-mass-spectrometry-gc-ms/gc-ms-ion-sources-inlets/quickprobe-gc-ms-system
26. Fialkov AB, Steiner U, Jones L, Amirav A. A new type of GC-MS with advanced capabilities. *Int J Mass Spectrom*. 2006;251(1):47-58. doi:10.1016/j.ijms.2005.12.045
27. Margolin Eren KJ, Elkabets O, Amirav A. A comparison of electron ionization mass spectra obtained at 70 eV, low electron energies and with cold EI and their NIST library identification probabilities. *J Mass Spectrom*. 2020;55(12):e4646. doi:10.1002/jms.4646
28. Amirav A, Fialkov AB, Gordin A. Improved electron ionization ion source for the detection of supersonic molecular beams. *Rev Sci Instrum*. 2002;73(8):2872-2876. doi:10.1063/1.1488683
29. Amirav A, Gordin A, Hagooly Y, et al. Measurement and optimization of organic chemical reaction yields by GC-MS with supersonic molecular beams. *Tetrahedron*. 2012;68(29):5793-5799. doi:10.1016/j.tet.2012.05.031
30. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. *Clin Chem*. 1974;20(4):470-475. doi:10.1093/clinchem/20.4.470
31. Buch A, Eldor R, Kis O, et al. The effect of circuit resistance training, empagliflozin or "vegeterranean diet" on physical and metabolic function in older subjects with type 2 diabetes: a study protocol for a randomized control trial (CEV-65 trial). *BMC Geriatr*. 2019;19(1):228. doi:10.1186/s12877-019-1219-7
32. Fossati P, Prencipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin Chem*. 1982;28(10):2077-2080. doi:10.1093/clinchem/28.10.2077
33. Shiba T, Inoguchi T, Sportsman JR, Heath WF, Bursell S, King GL. Correlation of diacylglycerol level and protein kinase C activity in rat retina to retinal circulation. *Am J Physiol*. 1993;265(5 Pt 1):E783-E793.
34. Aleryani SL, Cluette-Brown JE, Khan ZA, Hasaba H, de Heredia LL, Laposata M. Fatty acid methyl esters are detectable in the plasma and their presence correlates with liver dysfunction. *Clin Chim Acta*. 2005;359(1-2):141-149. doi:10.1016/j.cccn.2005.03.038
35. Akamatsu Y, Law JH. The enzymatic synthesis of fatty acid methyl esters by carboxyl group alkylation. *J Biol Chem*. 1970;245(4):709-713. doi:10.1016/S0021-9258(18)63320-4
36. Maunsell Z, Wright DJ, Rainbow SJ. Routine isotope-dilution liquid chromatography-tandem mass spectrometry assay for simultaneous measurement of the 25-hydroxy metabolites of vitamins D2 and D3. *Clin Chem*. 2005;51(9):1683-1690. doi:10.1373/clinchem.2005.052936
37. Gao C, Bergagnini-Kolev MC, Liao MZ, et al. Simultaneous quantification of 25-hydroxyvitamin D3-3-sulfate and 25-hydroxyvitamin D3-3-glucuronide in human serum and plasma using liquid chromatography–tandem mass spectrometry coupled with DAPTAD-derivatization. *J Chromatogr B*. 2017;1060:158-165. doi:10.1016/j.jchromb.2017.06.017
38. Traber MG. Vitamin E inadequacy in humans: causes and consequences. *Adv Nutr*. 2014;5(5):503-514. doi:10.3945/an.114.006254
39. Alon T, Amirav A. Isotope abundance analysis method and software for improved sample identification with the supersonic GC-MS. *Rapid Commun Mass Spectrom*. 2006;20:2579-2588.
40. Alon T, Amirav A. A comparison of isotope abundance analysis and accurate mass analysis in their ability to provide elemental formula information. *J Am Soc Mass Spectrom*. 2021;32(4):929-935. doi:10.1021/jasms.0c00419
41. Kovacs L, Zamboni C, Lourenco T, Madeco D. Sulfur status in long distance runners. *J Physics: Conference Series*. 2015;630:1-7. doi:10.1088/1742-6596/630/1/012009
42. Brown BH, Lewis HB. The metabolism of sulfur XXVII. The distribution of sulfur in the ultra-filtrates of blood plasma. *J Biol Chem*. 1941;138(2):705-716. doi:10.1016/S0021-9258(18)51395-8
43. Dorman DC, Chiu W, Hales BF, et al. Systematic reviews and meta-analyses of human and animal evidence of prenatal diethylhexyl phthalate exposure and changes in male anogenital distance. *J Toxicol Environ Health B Crit Rev*. 2018;21(4):207-226. doi:10.1080/10937404.2018.1505354
44. https://www.drugabuse.gov/related-topics/trends-statistics/overdose-death-rates; https://www.nytimes.com/interactive/2020/07/15/upshot/drug-overdose-deaths.html
45. https://www.washingtonpost.com/health/2021/07/14/drug-overdoses-pandemic-2020/
46. Balog J, Sasi-Szabo L, Kinross J, et al. Intraoperative tissue identification using rapid evaporative ionization mass spectrometry. *Sci Transl Med*. 2013;5(194):194ra93. doi:10.1126/scitranslmed.3005623
47. Poliak M, Gordin A, Amirav A. Open Probe – a novel method and device for ultra fast electron ionization mass spectrometry analysis. *Anal Chem*. 2010;82(13):5777-5782. doi:10.1021/ac100834h
48. Fialkov AB, Morag M, Amirav A. A low thermal mass fast gas chromatograph and its implementation in fast gas chromatography mass spectrometry with supersonic molecular beams. *J Chromtogr A*. 2011;1218(52):9375-9383. doi:10.1016/j.chroma.2011.10.053
49. Alon T, Amirav A. How enhanced molecular ions in cold EI improve compound identification by the NIST library. *Rapid Commun Mass Spectrom*. 2015;29(23):2287-2292. doi:10.1002/rcm.7392

How to cite this article: Neumark B, Elkabets O, Shefer G, Buch A, Stern N, Amirav A. Whole blood analysis for medical diagnostics by GC-MS with Cold EI. *J Mass Spectrom*. 2022;57(7):e4873. doi:10.1002/jms.4873