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RESEARCH ARTICLE

Stepwise extraction, chemical modification, GC–MS separation, and determination of amino acids in human plasma[#]

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[#]This is part 3 from the series "Analytical derivatives in mass spectrometry", parts 1, and 2 see [1,2].

A method for isolation of enriched fractions of amino acids from human plasma followed by derivatization, gas chromatography separation and mass spectrometry identification is described. The method involves a stepwise extraction of plasma constituents with the use of two solvents: (a) extraction with methanol yields a concentrate of polyols, urea, carbohydrates and most long-chain saturated and unsaturated aliphatic acids, and (b) further solubilization with water produces mainly a concentrate of amino acids. Chemical modification of amino acids with methyl chloroformate/methanol gives rise to methyl esters of methoxycarbonyl derivatives. The derivatization products are stable and quite suitable for gas chromatography with mass spectrometry analysis. The electron ionization mass spectra of the derivatization products reveal specific fragmentation patterns applicable for structure elucidation. The efficiency of the method is demonstrated by gas chromatography with mass spectrometry identification of 19 amino acids as their methyl esters of methoxycarbonyl derivatives in Standard Reference Material 1950 Metabolites in Frozen Human Plasma.

KEYWORDS
amino acids, derivatization, extraction, gas chromatography, human plasma

1 | INTRODUCTION

The presence of amino acids (AA) as end products, byproducts and intermediates of metabolism processes in biological objects is well described [3–6]. Assessment of AA profiles in biological systems and the exploration of systematic alteration in metabolic profiles can assist disease diagnosis, characterization of metabolic pathways and revelation of genetic defects. GC–MS is a reliable and effective method for such measurements. This technology has proved to be an effective and dependable instrumental tool for the comprehensive qualitative and quantitative analysis of complex mixtures of the metabolome based on its robustness, speed, high sensitivity, specificity and reproducibility [7–27]. The non-volatile nature and the zwitterionic structure of AA due to their polar (such as amino and carboxyl) groups preclude direct GC–MS experiments, and their conversion to volatile derivatives before GC–MS experiments is required [7–30].

Refinement and testing of a rapid and dependable analytical method for AA determination in biological substances by GC–MS is the goal of the present study since the conventional GC–MS remains one of the most effective methods for metabolite identification. The method was required to include the following: (1) selective extraction of AA from the complex biological matrix, (2) chemical modification of AA with suitable derivatization groups, (3) acquisition of GC–MS data for derivatized AA, and (4) automated data analysis with the use of reference GC retention index (GCRI) values and standard mass spectral data [31].

Abbreviations: AA, amino acid; AMDIS, the automated mass spectral deconvolution and identification system; CDC, center for Disease Control and Prevention; EIC, extracted ion chromatogram; EPA, Environmental Protection Agency; GCRI, gas chromatography retention index; ME, methyl ester; MOC, methoxycarbonyl; MOC/ME, methyl esters of methoxycarbonyl; MSTFA, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; NIDDK, National Institute of Diabetes and Digestive and Kidney Diseases; NIH, National Institute of Health; NIST, National Institute of Standards and Technology; SRM®, Standard Reference Material®; TIC, total ion chromatogram; TMS, trimethylsilyl; UCM, unresolved complex mixture

Conflict of interest: The authors have declared no conflict of interest.

The extraction of AA can be achieved by the fractionation of a plasma sample and separation of different classes of metabolic constituents with various solvents. The method selected here involves a stepwise methanol extraction/protein precipitation followed by additional water extraction. This choice was made from the consideration of a wide variety of known extraction methods ranging from organic solvents to acids [31–35].

For the selection of a derivatization method, reactions leading to various derivatives, such as trialkylsilyl-AA, alkyl esters of N-alkyl, N-acyl, N-perfluoroacyl and N-methyl-N-trifluoroacetyl AA, have been considered. All six major criteria for chemical modification reactions must be satisfied for successful application of the derivatization of amino acids: (1) the reactions should be fast, efficient and easily performed, (2) reactions should proceed quantitatively, (3) reactions should yield predominantly one product for each compound, (4) the reaction products should be volatile and stable under GC conditions, (5) reaction products should exhibit good GC properties, and (6) the mass spectra of derivatization products should contain characteristic peaks, and be readily distinguishable. Per the requirements, a reaction of AA with chloroformates was preferable mainly because several functional groups are derivatized in a single step, similar to trialkylsilylation, and additionally the reaction is performed in aqueous media (essential for the analysis of biological samples), and mass spectra of the derivatization products contain peaks of diagnostically important ions usable for structure elucidation. Reaction with methanol/methyl chloroformate [36] leading to a quantitative and selective chemical modification of most AA was selected for derivatization of the extracted AA.

Reference GCRI values of derivatization products were used for the location of compounds of interest in complex GC–MS data files, and standard electron ionization (EI) mass spectra played a key role in reliable identification of components in these mixtures. The publicly available NIST/NIH/EPA mass spectral library [31] can be used for the identifications of various chemicals including amino acids.

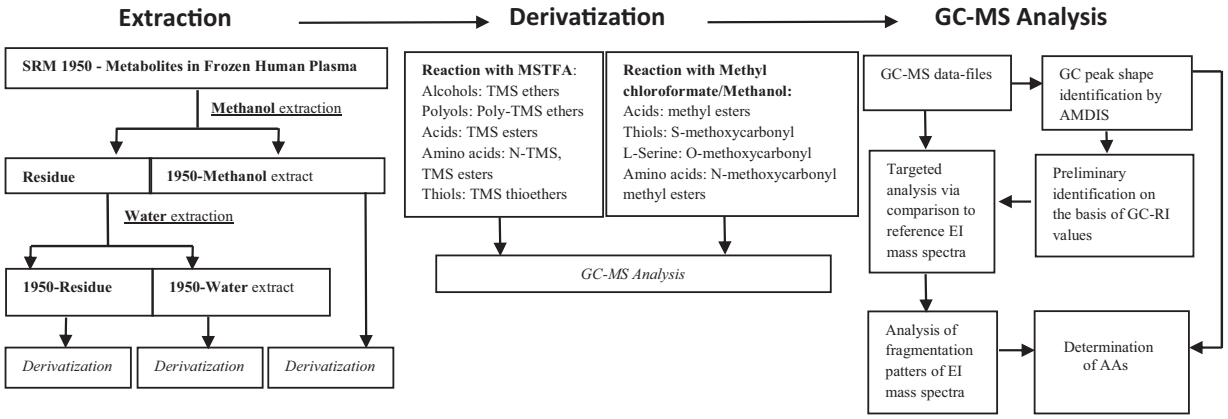
2 | MATERIALS AND METHODS

2.1 | Materials[1]

Standard Reference Material® (SRM) 1950 Metabolites in Frozen Human Plasma, a certified reference material intended for validation of methods for determining metabolites including amino acids in human plasma and similar materials, is well characterized [37,38] and available from the National Institute of Standards and Technology (NIST) at https://www-s.nist.gov/srmors/view_detail.cfm?srm=1950. SRM 1950 was handled at Biosafety Level 2 [39] as recommended in the certificate of analysis. GC derivatization synthesis grade *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), 99% purity methyl chloroformate, biotech. grade pyridine, 0.01 mol/L hydrochloric acid solution, certified reference material grade chloroform, HPLC grade ethyl acetate and B&J Brand® Multipurpose ACS, HPLC water were commercially available. A certified reference material C_7–C_{30} saturated alkanes (1000 µg/mL each component in hexane) for calibration of GCRIs was purchased from Sigma–Aldrich.

2.2 | Extraction

The extraction process is illustrated in Scheme 1. Five Ampules of SRM 1950, originally stored at −80°C, were thawed at room temperature for 30 min. To 20 tubes, each containing 200 µL of SRM 1950, 800 µL of methanol was added and vortexed for 30 s. The mixture was placed in a freezer at −20°C for 2 h, vortexed again for 180 s and placed in a freezer at −20°C for 1 h. Then the mixture was centrifuged at 14 000 × *g* for 10 min at 4°C. The supernatant (*1950-Methanol*) from 20 tubes was transferred to 11 1.5 mL glass vials and placed in a freezer at −80°C.



SCHEME 1 Graphic overview of sample preparation procedures and GC–MS analysis for the determination of amino acids in SRM 1950

The residues were combined into five tubes followed by the addition of 200 µL of water. The mixture was vortexed for 60 s and placed in a freezer at −20°C for 19 h, then centrifuged at 14 000 × *g* for 20 min at 4°C. The water layers (*1950-Water*) were transferred to three 1.5 mL glass vials and placed in a freezer at −80°C.

The residues from the previous step (*1950-Residue*) were combined into one vial and placed in a freezer at −80°C. As a result, three fractions (*1950-Methanol, 1950-Water and 1950-Residue)* were collected.

The supernatants from samples *1950-Methanol* and *1950-Water were* evaporated to dryness under a nitrogen stream just before the chemical modification reactions; water suspension of *1950-Residue* sample was directly derivatized.

2.3 | Chemical modification

2.3.1 | Silylation

The components of *1950-Methanol* sample were converted to trimethylsilyl (TMS) derivatives by directly treating the dry residue with MSTFA for 3 h at room temperature, according to a published protocol [19].

2.3.2 | Methylation/methoxycarbonylation

1950-Methanol and *1950-Water.* 170 µL solution containing 25 mmol/L aqueous hydrochloric acid, methanol and pyridine in a volume ratio 8:4:1 was added to dry samples of *1950-Methanol* and *1950-Water* followed by a slow addition of 5 µL of methyl chloroformate during 90 s at 20°C. The solution was vortexed for 5 s, and then 100 µL of chloroform containing 1% methyl chloroformate was added followed by further vortexing for 10 s. After 15 min, an aliquot was taken from the chloroform layer containing methyl esters of methoxycarbonyl (MOC/ME) derivatives and analyzed by GC–MS.

1950-Residue. 510 µL solution containing 25 mmol/L aqueous hydrochloric acid, methanol and pyridine in a volume ratio 8:4:1 was added to dry samples of *1950-Residue.* Then 15 µL of methyl chloroformate was added slowly during 180 s at 20°C. The solution was vortexed for 30 s, then 300 µL of chloroform containing 1% methyl chloroformate was added, followed by further vortexing for 30 s. After 15 min, an aliquot was taken from the chloroform layer, 75% of solvent was evaporated under a nitrogen stream; and the remaining solution was analyzed by GC–MS.

2.4 | GC–MS analysis

EI mass spectra and GCRI values were measured on the Agilent 5977A GC–MS system (Agilent Technologies, Santa Clara, CA, USA) at an ionization energy of 70 eV and ion source temperature of 230°C. Separation of components within the mixtures was achieved on a fused-silica capillary column (60 m, 0.25 mm i.d.; non-polar stationary phase: polymethylsiloxane + 5% phenyl groups) with programmed oven temperature from 60 to 270°C at a rate of 10°C min^{-1} then held at the final temperature for 10 min; the final temperature was held for 90 min only for case-control study. The injection port temperature was 270°C.

2.4.1 | Software

AMDIS. The Automated Mass Spectral Deconvolution and Identification System [40] was used for deconvolution of GC–MS data files, extraction of mass spectra of "pure" components and calculation of GCRIs.

Databases. Two libraries were used for comparison of experimental and reference data: NIST/NIH/EPA mass spectral library (NIST 17) and a special library containing EI spectra and GCRI data for TMS and methyl esters of methoxycarbonyl (MOC/ME) derivatives of AAs and small peptides (Target Library). The target library contains reference GCRI data and EI mass spectra of derivatives of AAs; they are obtained as a result of a study of the reaction for a large number of individual amino acids and small peptides with various alkyl chloroformates/alkanols including methyl analogs. All new data are incorporated into the 2017 release of the NIST Library [31].

3 | RESULTS

3.1 | Extraction

The qualitative analysis of AAs includes a stepwise extraction of metabolome constituents and subsequent chemical modification of extracted analytes as presented in Scheme 1. Three samples (a) *1950-Methanol*, (b) *1950-Water* and (c) *1950-Residue* are collected as a result of the extraction procedure presented in Scheme 1. The methanol extracts mostly contain fatty acids, hydroxyl acids, urea, glycerol and carbohydrates with small amounts of AA (less than 2%). The majority of AA remains in the Residue (Scheme 1), and it is subsequently extracted with water. After extraction of the residue with water, it was found that most AA were isolated in the *1950-Water* extract, and only trace levels of AA remain in the *1950-Residue*.

3.1.1 | Chemical modification. Silylation

Data interpretation and structure determination of trimethylsilyl derivatives of compounds presented in Figure 3 were carried by comparison to the reference data available in publications [30,41,42] and GC RI values and mass spectra of trimethylsilyl derivatives of amino acids obtained during the present study but not discussed in this publication.

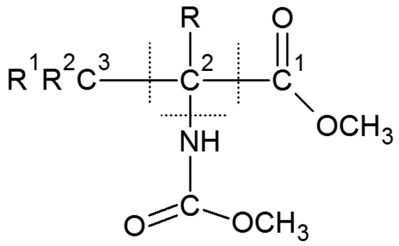


FIGURE 1 Major fragmentation pathways of α -amino acids due to bond cleavages at $C_{(2)}$

3.1.2 | Chemical modification. Methylation/methoxycarbonylation

Application of various alkyl(perfluoroalkyl) chloroformates as derivatization agents for the study of amino, oxo and hydroxy acids as their chemical modification products by GC–MS is well utilized [43,45,54–56]. The mechanisms of this reaction have been studied [43,44], and optimal conditions for complete metoxycarbonylation of most amino acids, and di- and tricarbonic acids have been established, and GC retention times and detection limits for the reaction products are provided [45]. However, mass spectra have been reported only for few ethoxycarbonyl derivatives of AA [46–49], and a systematic study of general and specific fragmentation patterns have been carried out for ethyl esters of ethoxycarbonyl AA [50]. Characteristic peaks in the spectra of methoxycarbonyl derivatives of perfluorobutyl esters of amino acids are also reported [43]. We carried out a preliminary study of reactions of methyl, ethyl, propyl and isopropyl chloroformates with selected, mainly proteinogenic, amino acids. The use of a reaction of AA with methanol/methyl chloroformate has been selected for the present study since the electron ionization mass spectra of corresponding derivatization products do not contain additional peaks due to fragmentation of longer alkyl groups, such as ethyl and propyl. General decomposition pathways due to cleavages at α-carbon for methyl esters of methoxycarbonyl AA is presented in Figure 1. Figure 2 depicts EI spectra of methoxycarbonyl and *n*-propyloxycarbonyl derivatives for L-valine, a simple representative of AA. It demonstrates that an "additional" fragmentation direction giving rise to a high intensity peak at 116 Da is becoming competitive (Figure 2B). This peak at 116 Da in the spectrum of *n*-propyl ester of *N*-*n*-propyloxycarbonyl-L-alanine is mainly due to the *N*-*n*-propyloxycarbonyl moiety. Reliable structure determination is mainly achieved the presence of peaks of diagnostically important ions in the spectra, and that is true especially for spectra extracted from a complex data-files; the absence of high intensity peaks characterizing only the derivatizing groups is always good.

The results of our study of the reaction of methyl chloroformate/methanol with amino acids in the presence of dodecane as an internal standard are presented in Tables 1 and 2. The 34 amino acids presented in Table 1 undergo quantitative and selective derivatization reactions and 33 of them produce the traditional derivatization products; one of them, L-asparagine, undergoes additional dehydration with the formation of methyl ester of *N*-methoxycaronyl-3-cyano-L-alanine.

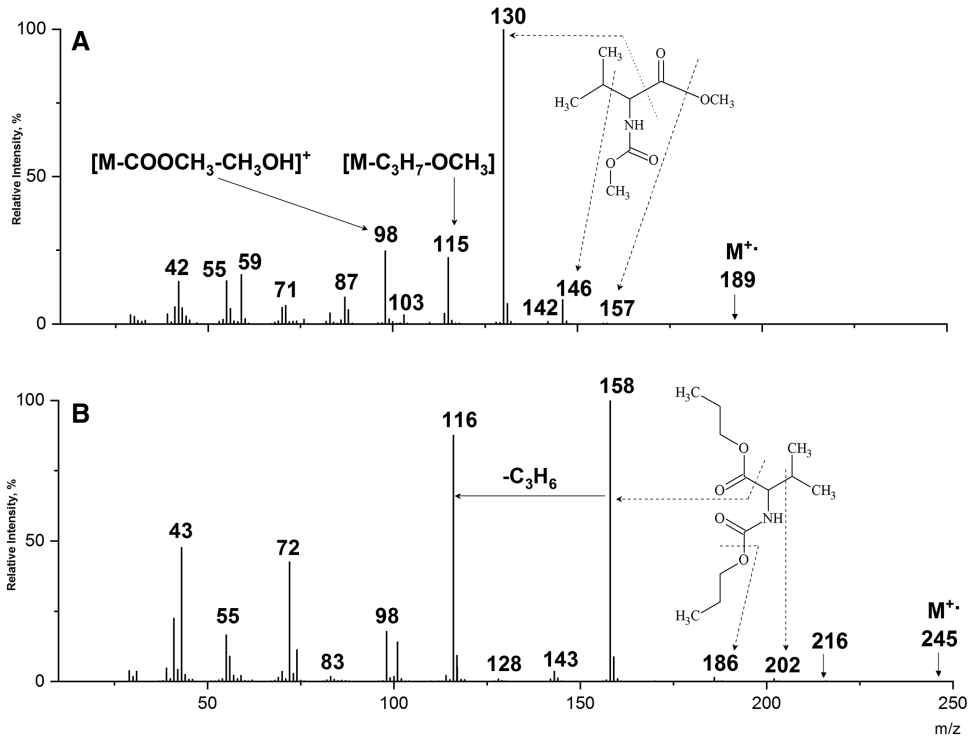


FIGURE 2 Mass spectra of methyl ester of *N*-methoxycarbonyl-L-valine (A) and *n*-propyl ester of *N*-propyloxycarbonyl-L-valine (B)

TABLE 1 Amino acids and amino amides undergoing complete and selective derivatization at 20°C in 2 min and AA: methyl chloroformate ratio 1:5. GCRI value follows the name of a derivatization product. Dodecane is used as an internal standard

Initial amino acid	Derivative/GCRI	Initial amino acid	Derivative/GCRI
L-Alanine	N-MOC, ME/1133.0	Glycinamide	N-MOC/1362.7
β-Alanine	N-MOC, ME/1217.7	L-Glutamic acid	N-MOC, di- ME/1576.3
Alanylamide-3-cyclohexyl-	N-MOC/1925.9	L-Isoleucine	N-MOC, ME/1346.6
L-Alanine-3-cyano	N-MOC, ME/1382.9	L-Isoserine	N,O-di(MOC), ME/1564.2
L-2-Aminobutyryc acid (Ethylglycine)	N-MOC, ME/1215.7	L-Leucine	N-MOC, ME/1335.0
(S)-3-Aminobutyric acid	N-MOC, ME/1245.3	Leucinamide	N-MOC/1548.3
Aminocaproic acid	N-MOC, ME/1575.3	L-Lysine	N,N'-Di(MOC), ME/2012.7
3-Aminohexanoic acid	N-MOC, ME/1403.4	L-Methionine	N-MOC, ME/1596.8
L-3-Aminoisobutyric acid	N-MOC, ME/1258.0	L-O-Methylthreonine	N-MOC, ME/1328.6
Aminomalonic acid	N-MOC, di-ME/1375.7	Norleucinamide	N-MOC/1592.8
(R)-3-Amino-4-methyl-pentanoic acid	N-MOC, ME/1378.2	L-Norvaline	N-MOC, ME/1302.4
3-Aminopentanoic acid	N-MOC, ME/1323.6	L-Phenylalanine	N-MOC, ME/1715.3
5-Aminovaleric acid	N-MOC, ME/1470.1	L-Tyrosine	N,O-Di(MOC), ME/2177.4
L-Asparagine	N-MOC-3-Cyano-L-alanine, ME/1382.9	Tyrosinamide	N,O-di(MOC)/2409.1
L-Aspartic acid	N-MOC, di- ME /1450.8	3-Ureidopropionic acid	N-MOC, ME/1546.2
L-Cysteine	N,S-Di(MOC), ME/1688.7	L-Valine	N-MOC, ME/1254.6
Glycine	N-MOC, ME/1130.2	Valinamide	N-MOC/1451.5

Table 2 contains amino acids with up to 99% selectivity. MOC/ME derivatization can be applied with caution when analyzing some AA listed in Table 2, such as *trans*-4-hydroxyproline, L-ornithine, L-proline, L-serine, L-threonine, L-tryptophan and tryptophan amide, and remember that 4-amino-4-methylpentanoic acid almost quantitatively undergoes dehydration/cyclization, and only traces of the traditional derivatization product are observed. While primary hydroxy-substituent in L-serine undergoes almost quantitative methoxycarbonylation, only up to 7% of the secondary hydroxy-group transforms to the MOC-derivative in the case of L-threonine. L-Tryptophan mono-methoxycarbonyl derivative is used as a chemical modification product (97%) since the yield of the *N*,*N*'-dimethoxycarbonyl derivative does not exceed 3% (Table 2). The data obtained in the study of methoxycarbonylation reaction and in the analysis of the EI spectra of derivatization products have been used in the study of the extraction products.

3.2 | Analysis

MOC/ME derivatives were analyzed for the *1950-Water* extract; the partial chromatogram of the MOC/ME derivatives is depicted in Figure 3A. TMS and MOC/ME derivatives were analyzed for *1950-Methanol*; partial chromatograms of the derivatization products are depicted in Figures 3B-C. During the reaction of AA with MSTFA all functional groups are silylated, and TMS esters of O-, N- and S-TMS derivatives are obtained. However, interaction of AA with methyl chloroformate/methanol derivatization reagent mainly produces methyl esters of methoxycarbonyl derivatives for amino and mercapto functional groups, and hydroxyl groups in some AA, such as L-serine and L-tyrosine. In the case of L-threonine, methoxycarbonylation of the secondary alcoholic hydroxyl group was incomplete; the methyl ester of N-MOC-L-threonine appears the major reaction product. Similarly, methoxycarbonylation was incomplete for the 4-hydroxyl group of stereoisomeric 4-hydroxyprolines.

The detection, deconvolution and identification of the components in the GC–MS data files are made by extracting a spectrum of a single compound by AMDIS, analyzing the spectrum, and comparing experimental mass spectrum and GCRI value to the reference data. The recognition of composite peaks is achieved by comparison of the total ion chromatogram (TIC) and the extracted ion chromatogram (EIC) for selected *m/z* values. Subsequently the content of major and minor components in a composite GC peak is determined by establishing peak starting (a), peak top (b), and peak ending points (c). All steps of data evaluation process are presented in Scheme 1. The chromatograms of derivatization products are depicted in Figures 3A through 3C, and the main components present in SRM 1950 are listed in Table 3.

A general overview of Figures 3A through 3C and Table 3 clearly demonstrates an acceptable fractionation of metabolome constituents when methanol and water are used sequentially as extraction solvents. The chromatogram of MOC/ME derivatives of 1950 Water extract (Figure 3A) does not show GC peaks of methyl alkanoates clearly indicating that the alkanoic acids are entirely extracted with methanol. Amino acid derivatives along with cholesterol

TABLE 2 Target and byproducts of amino acid derivatization reactions at 20°C in 2 min and at AA:methyl chloroformate ratio 1:5. GCRI values follow the name of a reaction product. Dodecane is used as an internal standard

Amino acid	Conversion	Derivatization product "A"	"A" Yield	By product "B"	"B" Yield
4-Amino-4-methylpentanoic acid	100%	N-MOC- 4-Amino-4-methylpentanoic acid, ME/1398.8	0.3%	5,5-Dimethylproline-2-one/1135.8	>99%
L-Arginine	100%	N-MOC-L-Arginine, ME	0%	Decomposition products	100%
Cystine	100%	N,N'-Di(MOC)-Cystine, di-ME/2589.6	40%	Decomposition products	60%
L-Glutamine	100%	N-MOC-L-Glutamine, ME/1821.2	15%	5-Oxo-L-proline, ME/1438.7 Other decomposition products	85%
L-Histidine	100%	N,N'-Di(MOC)-L-histidine, ME/2016.3	69%	Decomposition products	31%
cis-4-Hydroxyproline	100%	N-MOC-cis-4-Hydroxyproline, ME/1547.0	52%	N,O-Di(MOC)-cis-4-Hydroxyproline, ME/1787.3	48%
trans-4-Hydroxyproline	100%	N-MOC-trans-4-Hydroxyproline, ME/1602.0	85%	N,O-Di(MOC)-trans-4-Hydroxyproline, ME/1768.8	15%
L-Ornithine	100%	N,N'-Di(MOC)-L-ornithine, ME/1901.2	97%	N-MOC-3-Amino-2-oxopiperidine/1654.9	3%
L-Proline	100%	N-MOC-L-Proline, ME/1382.3	82%	N-MOC-L-Proline//1472.8	18%
L-Serine	100%	N,O-Di(MOC)-L-serine, ME/1519.4	≥98%	N-MOC-Serine, ME/ 1338.2	<2%
L-Threonine	100%	N-MOC-L-Threonine, ME/1355.3	≥93%	N,O-Di(MOC)-L-threonine, ME/1517.3	<7%
L-Tryptophan	100%	N-MOC-L-Tryptophan, ME/2375.1	97%	N,N'-Di(MOC)-L-tryptophan, ME/2400.5	3%
Tryptophan amide	100%	N-MOC-Tryptophanamide/2579.9	≥91%	Decomposition products	<9%

and its decomposition products become major components; cholesterol and its degradation products elute later than all major amino acid components, and corresponding GC peaks are not shown in Figure 3. This example confirms that stepwise extraction with several solvents can be employed for separation and concentration of various classes of chemicals.

Fractionation of a complex mixture can also be achieved by various chemical modification reactions. This statement can be illustrated by comparison of Figures 3B and 3C. A chromatogram of trimethylsilylation products of *1950-Methanol* extract reveals the following major components: TMS derivatives of lactic acid, hydroxy acid, urea, glycerol, glucofuranoses and glucopyranoses along with comparatively small quantities of TMS esters of alkan(en, dien, trien)oic acids (Figure 3C). However, a chromatogram of MOC/ME derivatives of the same extract (Figure 3B) does not indicate the presence of the above-listed major components of the metabolome, such as carbohydrates and polyols; these compounds under methylation/methoxycarbonylation conditions do not form derivatives; they remain intact or partially decompose giving rise to an unresolved complex mixture (UCM). This baseline signal hump does not interfere because of the ability of AMDIS [40] for reliable determination of peak shapes within composite GC peaks, and the extraction of spectra for each single component. As a result, small amounts of AAs can be detected (Figure 3B) even though in the case of *1950-Methanol* extract they elute in the region where alkanoic acids and their unsaturated analogs are dominant. The relative content of specific AA presented in Figures 3A and B are similar, and they are in agreement with the data provided in the Certificate [51].

In the search of di- and tripeptides, the *1950-Residue* sample was derivatized with methyl chloroformate/methanol

TABLE 3 Nineteen identified amino acid derivatives along with derivatives of major constituents of plasma (Figures 3A-C) and their semi-standard non-polar GCRI data

GC peak	Compound	GCRI
1	Glycine, N-MOC-, methyl ester	1128.9
2	L-Alanine, N-MOC-, methyl ester	1131.3
3	L-Valine, N-MOC-, methyl ester	1261.1
4	L-Leucine, N-MOC-, methyl ester	1340.5
5	L-Isoleucine, N-MOC-, methyl ester	1355.1
6	L-Threonine, N-MOC-, methyl ester	1359.3
7	L-Proline, N-MOC-, methyl ester	1393.2
8	3-cyano-L-alanine, N-MOC-, methyl ester	1395.3
9	L-Aspartic acid, N-MOC-, dimethyl ester	1462.8
10	L-Serine, N,O-di(MOC)-, methyl ester	1531.6
11	L-Glutamic acid, N-MOC-, dimethyl ester	1587.2
12	L-Methionine, N-MOC-, methyl ester	1605.5
13	L-Cysteine, N,S-di(MOC)-, methyl ester	1702.6
14	L-Phenylalanine, N-MOC-, methyl ester	1726.4
15	L-Ornithine, N,N'-di(MOC)-, methyl ester	1916.8
16	L-Lysine, N,N'-di(MOC)-, methyl ester	2024.4
17	L-Tyrosine, N,O-di(MOC)-, methyl ester	2199.2
18	L-Tryptophan, N-MOC-, methyl ester	2399.3
19	L-Cystine, N,N'-di(MOC), dimethyl ester	2578.3
20	Palmitic acid, methyl ester, $C_{17}H_{34}O_2$	1923.1
21	Linoleic acid, methyl ester, $C_{19}H_{34}O_2$	2095.7
22	Oleic acid, methyl ester, $C_{19}H_{36}O_2$	2100.0
23	Stearic acid, methyl ester, $C_{19}H_{38}O_2$	2122.7
24	Arachidonic acid methyl ester, $C_{21}H_{34}O_2$	2267.1
25	Eicosatrienoic acid, methyl ester, $C_{21}H_{36}O_2$	2282.6
26	Malic acid, O-MOC, dimethyl ester	1378.5
27	Z-Aconitic acid, trimethyl ester	1437.3
28	E-Aconitic acid, trimethyl ester	1451.2
29	Citric acid, O-MOC, trimethyl ester	1469.6
30	Salicylic acid	
	(a) Methyl ester	1205.9
	(b) O-MOC, methyl ester	1519.3
31	Ibuprofen, methyl ester	1549.1
32	Lactic acid, di-TMS	1062.6
33	2-Hydroxybutyric acid, di-TMS	1160.9
34	Urea, di-TMS	1242.6
35	Glycerol, tris-TMS	1275.7
36–39	Glucofuranoses and Glucopyranoses, penta -TMS	1810.1–1995.4

followed by chloroform extraction and GC–MS analysis. The resulting data identified trace levels of amino acid derivatives and no di- or tripeptides, while the dominant components were cholesterol and its degradation products.

4 | DISCUSSION

The method was developed with the use of the SRM 1950 Metabolites in Frozen Human Plasma. SRM 1950 is a

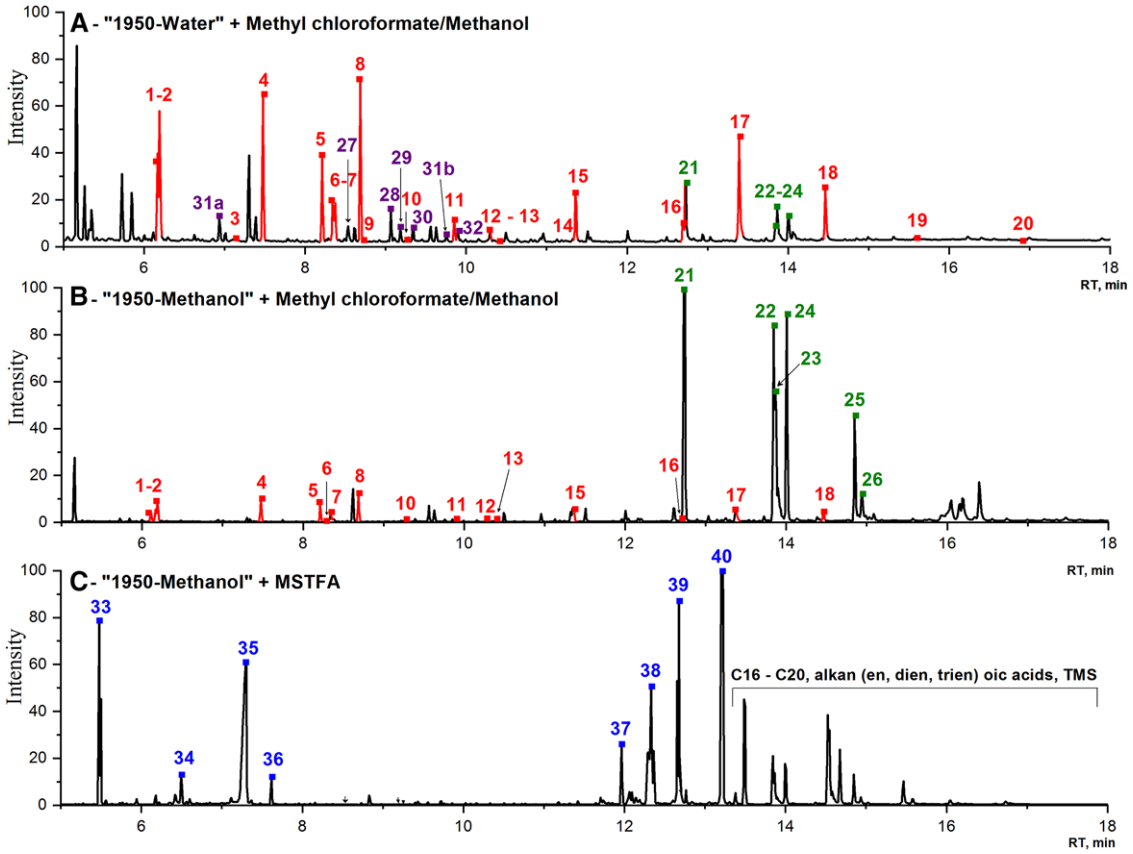


FIGURE 3 Partial chromatograms (GCRI 1016.9–2700.1 region) of methoxycarbonyl derivatives of "water extract" (A) and "methanol extract" (B), and TMS derivatives of "methanol extract" (C). The numbered peaks are identified in Table 3

product of NIST and the National Institute of Health (NIH) National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Qualitative and quantitative analysis of SRM 1950 were performed earlier by NIST and the Center for Disease Control and Prevention (CDC), Atlanta, GA with the use of various analytical methods [51]; trialkylsilyl and propyl/propyloxycarbonyl derivatives were used for structure determination and quantitation of AA by GC–MS [51].

As illustrated above in the Results section, we recorded GC–MS data for TMS and MOC/ME derivatives. While data analyses for TMS derivatives were helpful for the identification of most components present in the extracts, detailed examination of data-files of MOC/ME derivatives was selective for structure determination of amino acids. In particular, the derivatization reaction with the use of methyl chloroformate/methanol reagent is (a) soft, fast and efficient, (b) selective for most AAs, (c) proceeds close to completion, and (d) the reaction proceeds in an aqueous solution that is suitable for amino acids.

The first step in the preliminary assessment of GC–MS data-files was the identification of components giving rise to dominant GC peaks in the chromatograms. This step includes a transfer of EI spectra that was deconvoluted by AMDIS along with GCRI values to the NIST Library and the Target Library search programs. After evaluation of the mass spectra and GCRI data, preliminary identifications were made. The same kind of data processing was conducted for the minor GC peaks; EI spectra were examined, and corresponding compounds were identified.

The next step involved detailed examination of each GC peak aimed (a) to validate data associated to a specific GC peak, or (b) to detect components producing unresolved peaks and demarcate corresponding peak shapes within an unresolved GC peak; these analyses included comparative examination of TIC and EIC for various mass fragments present in a spectrum. Then automated or manual deconvolution of corresponding mass spectra was performed, and the extracted EI spectra were searched against the NIST Library and the Target Library.

Finally, detailed analysis of experimental mass spectral data was performed manually using known ion fragmentation rules to avoid false identifications. This step includes evaluation of the relative content of each amino acid when required.

The application of the procedures described above resulted in the characterization of nineteen AA as methyl esters of MOC derivatives in the SRM 1950. The identified derivatives and their GCRI values are listed in Table 3. The GCRI data

TABLE 4 Nineteen amino acids identified as alkyl esters of alkoxycarbonyl derivatives in some biological and food samples as methyl esters of methoxycarbonyl derivatives [55], as ethyl esters of ethoxycarbonyl derivatives [7,17,20,54], as propyl esters of propyloxycarbonyl derivatives [14,51], as *tert*-butyldimethylsilyl esters of ethoxycarbonyl derivatives [52], and as heptafluorobutyl esters of heptafluorobutyloxycarbonyl derivatives [53]

						Human urine						
GC peak	Amino Acid	SRM 1950 [23]	Plasma [24]	Human uremia [26]	Plasma and Human Urine [6]	[25]	[11]	[8]	[4]	Hair [4]	Soybean [4]	Plant tissue [27]
1	Glycine	x	x	x	x	x	x		x	x	x	x
2	L-Alanine	x	x	x	x	x	x		x	x	x	x
3	L-Valine	x	x		x	x	x		x	x	x	x
4	L-Leucine	x	x	x	x	x	x	x	x	x	x	x
5	L-Isoleucine	x	x	x	x	x	x	x	x	x	x	x
6	L-Threonine	x	x	x		x			x	x	x	x
7	L-Proline	x	x	x	x	x	x	x	x	x	x	x
8	Asparagine as 3-cyano-L-alanine				x	x		x	x			x
9	L-Aspartic acid		x	x	x	x	x	x	x	x	x	x
10	L-Serine	x	x			x						x
11	L-Glutamic acid	x	x	x	x	x	x	x	x	x	x	x
12	L-Methionine	x		x	x	x	x	x	x	x	x	x
13	L-Cysteine			x		x	x	x	x	x	x	x
14	L-Phenylalanine	x	x	x	x	x	x	x	x	x	x	x
15	L-Ornithine		x		x	x						
16	L-Lysine	x	x	x	x	x	x		x	x	x	x
17	L-Tyrosine	x	x	x	x	x	x	x	x	x	x	x
18	L-Tryptophan		x	x	x	x	x		x	x	x	x
19	L-Cystine				x	x						

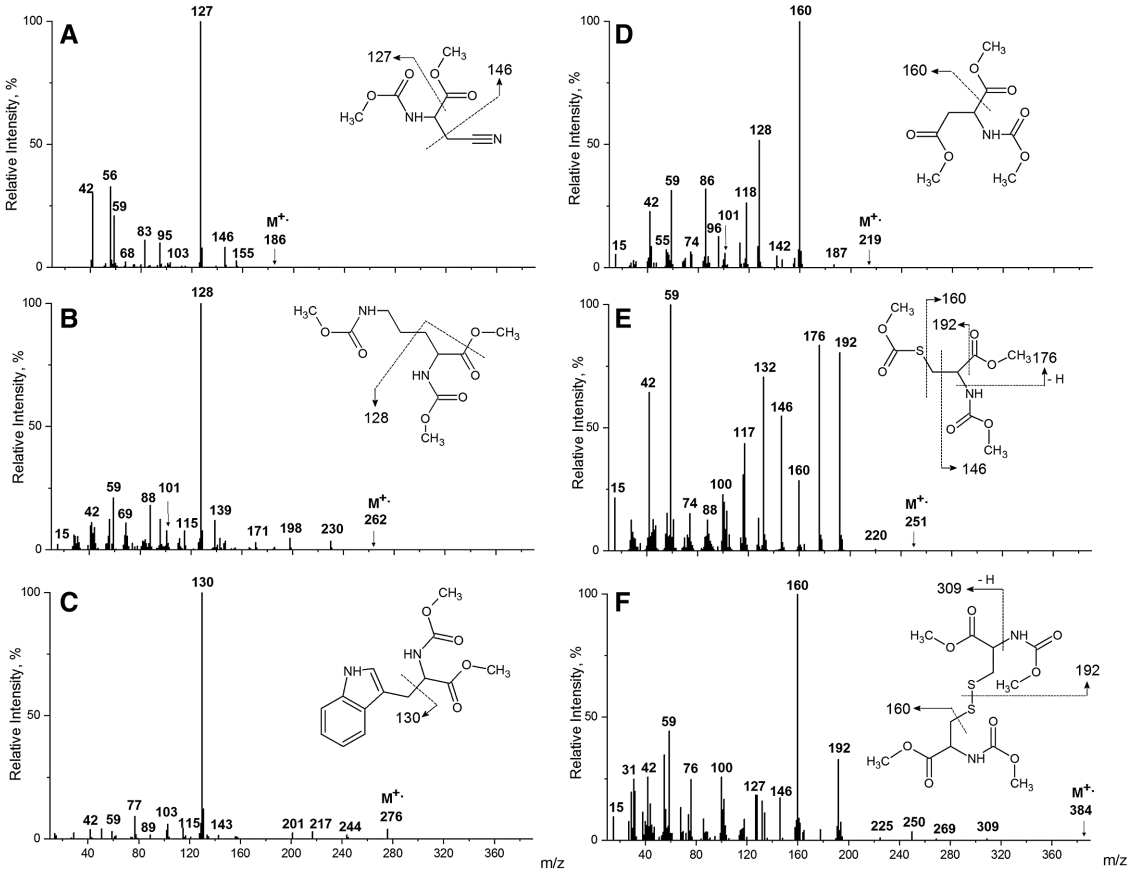


FIGURE 4 Mass spectra of methyl esters of N-MOC-3-cyano-L-alanine (A), *N*,*N*'-di(MOC)-L-ornithine (B), N-MOC-L-tryptophan (C), dimethyl esters of N-MOC-L-aspartic acid (D), *N*,*S*-di(MOC)-L-cysteine (E), and *N*,*N*'-di(MOC)-L-cystine (F)

have been successfully applied for the targeted analysis of AA during this study.

Thirteen of nineteen AAs were earlier identified and quantified in SRM 1950 by GC–MS as propyl esters of propyloxycarbonyl derivatives (Table 4), and their certified and reference values are provided in the Certificate of Analysis [51]. The remaining six AA determined in the present study were also identified earlier by GC–MS as alkoxycarbonyl derivatives in the samples of human plasma [14,52], human urine [7,14,17,20,53], uremia [54], human hair [7], and food samples [7,55] (Table 4). EI mass spectra for the derivatives of the six AA are depicted in Figure 4.

L-Asparagine in the SRM 1950 is identified as a derivative of its dehydration product—the methyl ester of MOC derivative of 3-cyanoalanine (Figure 4A). The formation of homologous ethyl and propyl esters of ethoxy- and propyloxycarbonyl-3-cyanoalanines as major products of L-asparagine derivatization with corresponding alkanol/alkyl chloroformates was demonstrated earlier, and the molecular structure of the *n*-propyl analog was confirmed by NMR spectroscopy [14]. The spectrum of N-MOC-3-cyanoalanine ME in Figure 4A parallels its ethyl and propyl analogs' fragmentation patterns. Figure 4A contains peaks of diagnostically important ions of $[M\text{-}COOCH_3]^+$ at 127 Da and $[M\text{-}CH_3CN]^+$ at 146 Da that are the products of β-cleavages.

The compound producing GC peak 15 in the chromatogram (Figure 3) is identified as the methyl ester of N,N'-di(MOC)ornithine based on its GCRI value and EI mass spectrum (Figure 4B). Peaks of $M^{+\cdot}$ (262 Da), $[M\text{-}CH_3OH]^{+\cdot}$ (230 Da), $[M\text{-}2CH_3OH]^{+\cdot}$ (198 Da), $[M\text{-}CH_3OH\text{-}COOCH_3]^+$ (171 Da) and $[M\text{-}2CH_3OH\text{-}COOCH_3]^+$ (139 Da) ions along with the base peak of ions $[M\text{-}CH_3OH\text{-}CH_2CHNHCOOCH_3]^+$ at 128 Da were identified as diagnostically important ions for structure elucidation of the ornithine derivatization product.

The methylindolyl cation peak at 130 Da is distinctive for MOC/ME-L-tryptophan; it is the key ion for structure determination, and the corresponding peak is dominant in its spectrum (Figure 4C). Note that the cleavage of $C_{(2)}$–$C_{(3)}$ bond is the most favorable dissociation process due to (a) branching at $C_{(2)}$, (b) the β-position of $C_{(2)}$–$C_{(3)}$ bond toward aromatic moiety, (c) the β-position of amino function, and (d) the stability of the resulting 3-methylindolyl cation.

Fragmentation of the dimethyl ester of N-MOC-aspartic acid is ruled by parallel and successive decomposition of all three functional groups (Figure 4D). As expected, the MOC/di-ME derivatives of homologous aspartic and

glutamic acids show similar fragmentation patterns. However, different ions have higher intensities depending on competing processes of dissociation, and accordingly their mass spectra exhibit very different fingerprints. Thus, their assignment is simplified when accompanied by GCRI data.

General fragmentation pathways of the di-MOC/ME derivative of L-cysteine are associated with the loss of each functional group giving rise to high intensity peaks at 146, 160, 176, and 192 Da (Figure 4E). Similar dissociation has been observed earlier for the corresponding ethoxycarbonyl derivative [47,56]. The highest intensity peaks at 160 and 192 Da in the spectrum of L-cysteine derivative (Figure 4F) corresponds to ions due to the cleavage of S–S disulfide bond; the low mass region of the spectrum is similar to that of the L-cysteine derivative (Figure 4E).

5 | CONCLUDING REMARKS

A GC–MS method was refined and tested for identification and detection of amino acids in human plasma. It includes separation of amino acids from polyols, carbohydrates and aliphatic acids using a stepwise extraction of human plasma constituents with methanol and water. Extracts are derivatized with a mixture of methanol and methyl chloroformate. The formation of methyl esters of methoxycarbonylamino acids facilitates further discrimination of amino acids for GC–MS analysis. Experimental conditions for the analysis of amino acids as their methyl esters of methoxycarbonyl derivatives by GC–MS is established. Successful application of specific EI fragmentation pathways for corresponding derivatives and analysis of GC–MS data files by comparing to reference mass spectral and GCRI data leads to dependable characterization of amino acids in human plasma. We demonstrate the practical application of this method by detecting trace quantities of L-aspartic acid, L-methionine, L-cysteine, L-cystine, L-tryptophan and L-asparagine along with 13 major components as their MOC/ME derivatives in SRM 1950 by GC–MS. The ultimate goal is the development of a precise and reliable quantitative method for determination of amino acids in human plasma and other biological objects.

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[1] Certain commercial materials and instruments are identified in this paper in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the identified materials are necessarily the best available for the purpose.

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