Send Orders for Reprints to reprints@benthamscience.net

Current Chromatography, **2019**, *6*, 3-14

RESEARCH ARTICLE

Unusual Regularity in GC Retention of Simple Amino Acid Derivatives

Igor G. Zenkevich[1,2,*], Nino G. Todua[2,3,*] and Anzor I. Mikaia[2,*]

[1]*Institute for Chemistry, St. Petersburg State University, Universtitetskii Prospect, 26, St. Petersburg 198504, Russia;* [2]*National Institute of Standards & Technology, Gaithersburg, 100 Bureau Drive, 221/A345, Gaithersburg, MD 20899-8362, USA;* [3]*Strativia, LLC, 1401 Mercantile Ln #501, Largo, MD 20774, USA*

Abstract: ***Background*:** Application of simple regularities and general principles along with direct use of reference gas chromatography retention index data for reliable structure determination of compounds can be enhanced by determination of new regularities that are specific to certain structural elements.

***Objective*:** Revelation and interpretation of an anomaly in the elution order of alkyl esters of alkoxycarbonyl derivatives of glycine and alanine on standard and semi-standard non-polar phases.

***Method*:** Preliminary derivatization of amino acids to alkyl esters of N-alkoxycarbonyl analogs and interpretation of their gas chromatographic characteristics.

***Results*:** Alkyl esters of N-alkoxycarbonyl derivatives of alanine (Alkyl = C_2H_5, *n*- and *iso*-C_3H_7) elute prior to the same derivatives of glycine, despite the presence of an additional methyl group at $C^{(2)}$ in the molecule. Elution order is reversed for methyl esters of N-methoxycarbonyl derivatives.

***Conclusion*:** It is established that the peculiar behavior of alkyl esters of N-alkoxycarbonyl derivatives of glycine and alanine agrees with the concepts of gas chromatography and the known retention index regularities of organic compounds. A decrease of retention index values is a result of an introduction of an additional methyl group to a carbon atom connected to two polar fragments in a molecule like CH_2XY. The dependence of the difference of retention index values for homologs of the types of CH_3-CHXY and CH_2XY *vs.* the total mass of fragments (X + Y) is similar to those for other sub-groups of analytes.

ARTICLE HISTORY

Received: April 11, 2019
Revised: June 04, 2019
Accepted: June 27, 2019

DOI:
10.2174/2213240606666190709100858

Keywords: Amino acid, glycine, alanine, alkyl ester, N-alkoxycarbonyl derivative, standard non-polar stationary phases, retention index, elution order anomaly, GC-MS.

1. INTRODUCTION

Conventional gas chromatography-mass spectrometry (GC-MS) remains one of the most informative instrumental methods for the determination of organic and bioorganic compounds in complex mixtures. It is the function of Gas Chromatography (GC) to separate individual components of mixtures and provide pure constituents for the analysis to Mass Spectrometry (MS). Additionally, GC is a unique asset for the differentiation of compounds with the same molecular mass and with very similar fragmentation patterns under mass spectrometry conditions. The advantages of GC-MS are utilized in an optimized way when gas chromatographic retention indices (GC-RI) [1] are analyzed along with the corresponding Electron Ionization Mass Spectra (EI-MS). This type of analysis is currently available with the use of a single database containing both reference GC-RI values and EI-MS data. The 2017 release of the NIST/NIH/EPA mass spectral library includes 306,622 EI-MS for 267,376 compounds and 404,045 GC-RI data for 99,400 compounds on standard non-polar (polydimethyl siloxanes) and polar (polyethylene glycols) stationary phases [2].

Similar to the analysis of EI-MS data, some regularities shall be taken into account in contemporary chromatographic practice in addition to the direct use of reference GC-RI values. The simplest regularities, along with the general princi-

*Address correspondence to these authors at the Institute for Chemistry, St. Petersburg State University, Universtitetskii Prospect, 26, St. Petersburg 198504, Russia; Tel: +7-812-428-4045; E-mail: izenkevich@yandex.ru
and Department of Material Measurement Laboratory, National Institute of Standards and Technology, 100 Bureau Drive, 221/A365, Gaithersburg, MD 20899-8362, USA; Tel: +1-301-975-2511; E-mail: nino.todua@nist.gov; ORCID: 0000-0001-5023-2916 and Department of Material Measurement Laboratory, National Institute of Standards and Technology, 100 Bureau Drive, 221/A345, Gaithersburg, MD 20899-8362, USA; Tel: +1-301-975-2521; E-mail: anzor.mikaia@nist.gov

2213-2406/19 $58.00+.00

Table 1. GC retention indices for selected "*iso*-structural" homologues on standard non-polar phases.

Homologue	RI(*n*)	Next Homologue	RI(*n*+1)	ΔRI[1] ***
2,2,4-Trimethylpentane	690 ± 2	2,2,4-Trimethylhexane	791 ± 2	101
2,2,4-Trimethylpentane	690 ± 2	2,2,5-Trimethylhexane	784 ± 1	94
n-Butylbenzene	1047 ± 6	*n*-Pentylbenzene	1145 ± 3	98
2-Methyl-1-propanol	614 ± 6	3-Methyl-1-butanol	719 ± 5	105
Methyl *tert.*-butyl ether	562 ± 5	Methyl *tert.*-pentyl ether	673 ± 2	111
Diethyl amine	548 ± 11	Dipropyl amine	750 ± 2	202*
N-Ethylaniline	1106 ± 6	N-Butylaniline	1300**	194*
Dipropyl sulfide	881 ± 5	Dibutyl sulfide	1075 ± 6	194*
Average ΔRI value				100 ± 5

*) Value corresponds to a structural difference in two carbon atoms.
**) A single reference RI values is presented without a MAD.
***) Here and below the superscript symbol corresponds to the Table number.

Table 2. GC retention indices for selected compounds and their branched isomers on standard non-polar phases.

Homologue	RI(*n*)	Branched Isomer	RI(*iso*)	ΔRI[2]
n-Hexane	600	2-Methylpentane	569 ± 2	-31
3-Methyloctane	872 ± 1	3,5-Dimethylheptane	838 ± 1	-34
Butylbenzene	1047 ± 6	Isobutylbenzene	995 ± 3	-52
1-Pentanol	753 ± 7	3-Methyl-1-butanol	719 ± 5	-34
1-Chloropentane	743 ± 3	3-Methyl-1-chlorobutane	706 ± 1	-37
Dibutyl ether	875 ± 2	Diisobutyl ether	811*	-64**
Dibutyl amine	949 ± 5	Diisobutyl amine	850 ± 1***	-99**
Dibutyl sulfide	1075 ± 6	Diisobutyl sulfide	983 ± 14	-92**
Average ΔRI value				-40 ± 8

*) A single reference RI value is presented without a MAD.
**) Value corresponds to a structural difference in two isomers when introducing additional branching.
***) RI value is for semi-standard non-polar phases [2].

ples, were formulated in the pioneering work of E. Kovats [3]. Interpretation and description of unexpected regularities, which are reflected via unusual tendencies of GC-RI, shall be performed with the use of well-established estimates designed to evaluate RI data for unavailable or not characterized compounds and/or for verification of experimental data. Some of these regularities reflect unusual tendencies of retention indices.

The postulated (100 × *n*) GC-RI value for the reference *n*-alkanes C_nH_{2n+2} leads to 100 i.u. (index units) increments between homologs differing in one carbon atom within the same series. Reasonably, the difference in two or more (*k*) carbon atoms will result in RI differences of about 200 or (100 × *k)* i.u. This type of estimation is often used in GC practice for rapid evaluations of GC-RI values for most of the homologs and can be illustrated by numerous examples. The GC-RI values for normal and branched chain homologs and the differences between them (ΔRI^1) are presented in Table **1**; the average difference between the homologs is 100 ± 5 i.u. Here and below, unless otherwise specified, all RI values for standard non-polar phases are taken from NIST-17 [2]. The retention index values given in NIST-17 are median values of multiple measurements (if available) and the associated uncertainty is the Median Absolute Deviation (MAD).

The GC-RI regularity for organic compounds with branched carbon skeletons is different when compared to their normal chain isomers. That is reasonable since the boiling points of branched isomers are lower compared to their isomers with normal linear carbon skeleton [4] because of the effect of sterically non-hindered positions. The data presented in Table **2** illustrates this type of GC-RI regularity.

Combination of the above regularities leads to the conclusion that the RI values increase when moving from a compound to the next homolog by formally replacing hydrogen with a methyl group at a "secondary carbon" atom in the

molecule. However, this rise (ΔRI^3) is less than 100 i.u. because this addition leads to the appearance of additional non-hindered branching (methyl group) of the carbon skeleton. Scheme **1** illustrates this type of structural transformation.

$$X^- \text{ ... } -CH_2^- \text{ ... } -CH_3 \xrightarrow{-H / +CH_3} X^- \text{ ... } -CH(CH_3)^- \text{ ... } -CH_3$$

Scheme 1. Introduction of an additional methyl group to n-alkane leads to a homolog with branched carbon chain.

The difference in value (ΔRI^3) for such a transformation can be roughly evaluated as an additive combination of both addends mentioned above (Tables **1** and Table **2**), namely $(100 \pm 5) + (-40 \pm 8) \approx 60 \pm 9$. So far as both ΔRI^1 and ΔRI^2 values are statistically independent, the measure of standard deviation of the result is the square root from the sum of the square errors of the addends. The validity of this type of approximation is well illustrated by the examples of several types of pairs depicted in Table **3**: *n*-alkyl/*iso*-alkyl hydrocarbons, *n*-alkyl-aryl/*iso*-alkyl-aryl compounds and *n*-alkyl/*iso*-alkyl pairs containing functional groups; the average ΔRI^3 value and its MAD (61 ± 10) correlates well with the data obtained from the evaluation of ΔRI^1 and ΔRI^2 values.

Steric hindrance in the molecules of organic compounds affect the values of boiling points and GC-RI parameters; any restrictions to the intramolecular rotation and vibration processes increase the values of both properties when compared to corresponding non-restricted structures. This type of regularity has not been sufficiently discussed in gas chromatography even though its importance has been acknowledged in chemistry. There have been just a few attempts to apply molecular dynamics modeling to explain the steric anomalies in the interpretation of GC-RI data [5-7].

The simplest example of a sterically hindered transformation of a structure is an introduction of a methyl group to an α-position of the neighboring methylene resulting in the formation of a vicinal dimethyl-homolog (also known as a "*vic*-dimethylated" compound); this process is illustrated in Scheme **2**.

$$X^- \text{ ... } -CH(CH_3)-CH_2^- \text{ ... } -CH_3 \xrightarrow{-H / +CH_3} X^- \text{ ... } -CH(CH_3)-CH(CH_3)^- \text{ ... } -CH_3$$

Scheme 2. Formation of a vicinal dimethylalkane.

In the case of isomers, the differences in GC-RI values (ΔRI^4) between single-branched compounds and their structural isomers with hindered double-branched carbon skeletons are negligible, as shown in Table **4**. Concurrently, the ΔRI^2 values for the pairs of isomeric compounds with normal linear *vs.* single-branched molecular carbon skeletons are significantly higher as depicted in Table **2**. Due to the importance of this phenomenon, a few examples are presented in Table **4** with additional structural information. Therefore, any statistical analysis of such ΔRI^4 values for the types of compounds presented in Table **4** is unreasonable.

Further consideration of the effect of branching on GC properties can be followed by the analysis of the differences in GC-RI values. For that purpose, the differences occurring when moving from a branched compound to the following homolog(s) by successive addition of extra carbons to the skeleton and increasing branching are given in Table **5**. The ΔRI^5 data presented in Table **5** for dimethyl-, trimethyl-, tetramethyl- and pentamethylpentanes, and some arylalkanes show that the replacement of hydrogen atom with methyl group ($H \rightarrow CH_3$) in sterically hindered position increases much more than 100 i.u. The latter has more value ($\Delta RI^3 \approx 61 \pm 10$) observed for the *n*-alkane/*iso*-alkane pairs presented in Table **3**.

Considering the well-established regularities that are typical and expected for the majority of compounds, it can be stated that "shifting" from a homolog to the next member in a series by ($H \rightarrow CH_3$)-replacement and leading to additional branching of carbon skeleton causes an increase of GC-RI values on standard non-polar phases. The ΔRI value

Table 3. GC retention indices of selected compounds and their homologs additional non-hindered branching (methyl group) on standard non-polar phases.

Homologue	RI(*n*)	Next Branched Homologue	RI[*iso*-(*n*+1)]	ΔRI^3
n-Hexane	600	2-Methylhexane	667 ± 1	67
Propylbenzene	945 ± 5	Isobutylbenzene	995 ± 3	50
2-Ethylnaphthalene	1381 ± 13	2-Isopropylnaphthalene	1435 ± 9	57
Propyl bromide	614 ± 2	Isobutyl bromide	677*	63
1-Butanol	650 ± 7	3-Methyl-1-butanol	719 ± 5	69
Propyl acetate	695 ± 2	Isobutyl acetate	755 ± 5	60
Dipropyl amine	750 ± 2	Diisobutyl amine	850 ±1***	100**
2-Ethylpyridine	883 ± 8	2-Isopropyl pyridine	961 ± 1	79
Average ΔRI value				61 ± 10

*) Single reference RI value is presented without a MAD.
**) Value corresponds to a structural difference in two carbon atoms.
***) RI value is for semi-standard non-polar phases [2].

Table 4. GC retention indices for simple organic compounds with single-branched carbon skeletons and their isomers with double-branched carbon skeletons containing two sterically hindered methyl groups on standard non-polar phases.

Branched Homologue	RI(*n*)	Doubly Branched Isomer	RI(*iso*)	ΔRI[4]
2-Methylpentane	569 ± 2	2,3-Dimethylbutane	566 ± 3	-3
2-Methylhexane	667 ± 1	2,3-Dimethylpentane	670 ± 2	+3
Dimethyl propyl amine	604± 8	Dimethyl isopropyl amine	601*	-3
Dimethyl butyl amine	697*	Dimethyl *tert.*-butyl amine**	693*,***	-4

*) Single reference RI value is presented without a MAD.
**) *tert.*-Butyl group formally contains a doubly branched carbon.
***) RI value is for semi-standard non-polar phases [2].

Table 5. GC retention indices of selected "branched" compounds and their next member homologs with additional hindered branching (methyl group) on standard non-polar phases.

Homologue	RI(*n*)	Next Branched Homologue	RI[*iso*-(*n*+1)]	ΔRI[5]
2,4-Dimethylpentane	631 ±1	2,3,4-Trimethylpentane	750 ±2	119
2,2,4-Trimethylpentane	690 ± 2	2,2,3,4-Tetramethylpentane	820 ± 2	130
2,2,4,4-Tetramethylpentane	773 ± 2	2,2,3,4,4-Pentamethylpentane	928 ± 7	155
2,2,3,4-Tetramethylpentane	820 ± 2	2,2,3,3,4-Pentamethylpentane	959 ± 8	139
2,2-Dimethylpropylbenzene	1048**	1,2,2-Trimethylpropylbenzene	1186*	138
Diphenylmethane	1412 ± 11	1,1-Diphenylethane	1565*	153

*) A single reference GC-RI value is presented without a MAD.
**) Neopentyl group formally contains a doubly branched carbon.

is approximately 60 i.u. when the structural transformation implies an appearance of non-hindered branching and this value may exceed 100 i.u. to 150 i.u when steric hindrances take place. It is worth noting that up to the present any examples showing a decrease of GC-RI values of homologs on standard non-polar phases in the result of H $\rightarrow$ CH_3 replacement have not been discussed in the literature.

These regularities can be applied for the rationalization of GC properties for some derivatives of simplest amino acids, namely glycine (**I**) and alanine (**II**) that are among the key compounds in metabolomic studies and application of derivatization prior GC-MS analysis may lead to a better GC separation and more reliable mass spectral identification [8, 10]. We have used a sample of L-alanine and chromatographic columns with achiral stationary phase, which cannot separate the enantiomers. Due to that in the text alanine is indicated without prefix showing the chiral configuration. The structural relation between these two corresponds only to the transformation of (H $\rightarrow$ CH_3) leading to additional branching in the carbon skeleton of (**II**) (Scheme **3**).

The set of derivatives includes N-methoxycarbonyl derivatives of methyl esters (Me-MOC), other N-alkoxycarbonyl derivatives of alkyl (Alkyl = ethyl, *n*- and *iso*-propyl) esters (Alk-AlkOC), as well as N,O-*bis*- and N,N,O-*tris*-trimethylsilyl (TMS) derivatives of (**I**) and (**II**). The unusual behavior of glycine (**I**) and alanine (**II**), as well as their derivatization products, on standard non-polar phases, requires special consideration from the prospective of the contemporary theories in gas chromatography. Preliminary results of this work have been presented at the 66th ASMS conference [9].

2. EXPERIMENTAL[1]

2.1. Materials

Substrates [amino acids (AA): glycine (**I**) and L-alanine (**II**)], derivatization reagents [alkyl (methyl, ethyl, *n*-propyl and isopropyl) chloroformates, BSTFA], solvents (methanol, ethanol, 1-propanol, chloroform, and pyridine) were commercially available from Sigma-Aldrich.

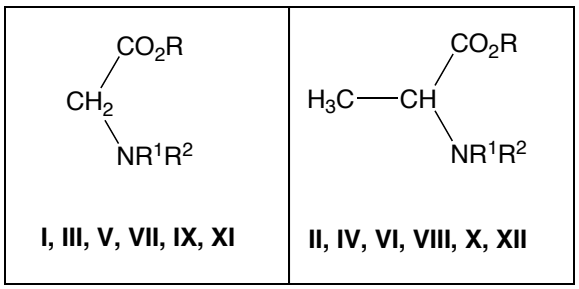
2.2. Derivatization Reactions

Derivatization of amino acids with BSTFA and alkyl chloroformates/alkanol were carried out using well-established procedures [8, 10-12]. The detailed description of the experimental procedure for preparing Me-MOC derivatives is presented below.

2.2.1. Reaction of Amino Acids with Methyl Chloroformate

1 mg AA was added to 170 μL of a solution containing 25 mmol/L aqueous hydrochloric acid, methanol, and pyri-

[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.



Compound #	R	R^1	R^2	Name
I, II	H	H	H	Glycine and alanine
III, IV	Alkyl	Alkyl	H	O-Alkyl-N-Alkoxycarbonyl-glycine and -alanine
V, VI	$Si(CH_3)_3$	$Si(CH_3)_3$	H	N,O-bis-trimethylsilyl-glycine and -alanine
VII, VIII	C_4H_9	$COCF_3$	H	O-Butyl-N-trifluoroacetyl-glycine and -alanine
IX, X	CH_3	$COCF_3$	CH_3	O-Methyl- N-methyl-N-trifluoroacetyl-glycine and -alanine
XI, XII	$Si(CH_3)_2C(CH_3)_3$	$CO_2C_2H_5$	H	O-tert.-Butyldimethylsilyl-N-ethoxycarbonyl-glycine and -alanine

Scheme 3. Structures of glycine (**I**), alanine (**II**) and their chemical modification products (**III**-**XII**).

dine in a ratio 8:4:1 (v/v). Then 5 µL of methyl chloroformate was added to this mixture during 90 s at 20 °C. The solution was vortexed for 5 s, then 100 µL of chloroform containing 1% (v/v) methyl chloroformate was added, followed by further vortexing for 10 s. After 15 min, an aliquot was analyzed by GC-MS. Similar procedures were employed for alkoxycarbonylation of AA with ethyl, *n*-propyl, and isopropyl chloroformates.

2.3. GC-MS Analysis

GC-MS analysis was carried out using a GC-MS system (Agilent 5977A, Agilent Technologies, Santa Clara, CA, USA) with EI (70 eV) and quadrupole analyzer. Two fused silica Wall Coated Open Tubular (WCOT) columns were used for GC separations: **i**) RESTEK, Rtx-5MS, 30 m length, 0.25 mm internal diameter, and 0.25 µm film thickness with semi-standard non-polar polydimethyl siloxane with 5% phenyl groups; and **ii**) RESTEK, Rxi-1MS, 15 m length, 0.25 mm internal diameter, and 0.25 µm film thickness with standard non-polar 100% dimethyl polysiloxane (100%). Both columns were used in temperature programming regimes from 60 °C to 270 °C, ramp 10 °C min^{-1}, injector and interface temperatures were 270 °C, and ion source temperatures was 230 °C.

A certified mixture of reference *n*-alkanes C_7 to C_{30} (1 mg/mL of each in hexane solution) was added to the samples for determining the linear GC retention indices (**RI**) [13]. Repeatability control was performed with the application of several (up to 4) injections.

The following derivatives of Gly and Ala were characterized by RIs on semi-standard non-polar stationary phase:

- N-methoxycarbonyl, methyl ester (R′ = R″ = CH_3, **Me-MOC**);
- N-ethoxycarbonyl, methyl ester (R′ = CH_3, R″ = C_2H_5, **Me-EOC**);
- N-ethoxycarbonyl, ethyl ester (R′ = R″ = C_2H_5, **Et-EOC**);
- N-*n*-propyloxycarbonyl, methyl ester (R′ = CH_3, R″ = C_3H_7**, Me-POC**);
- N-*n*-propyloxycarbonyl, *n*-propyl ester (R′ = R″ = C_3H_7, **Pr-POC**);
- N-isopropyloxycarbonyl, methyl ester (R′ = CH_3, R″ = iso-C_3H_7, **Me-i-POC**);

Two trimethylsilyl derivatives were characterized for comparison:

- N- trimethylsilyl, trimethylsilyl ester (**N,O-*bis*-TMS**);
- N,N-*bis*-trimethylsilyl, trimethylsilyl ester (**N,N,O-*tris*-TMS**).

Their structures (**III**, **IV**) are depicted in Scheme **3**.

Unless otherwise specified, as stated above, all RI values for standard non-polar phases are taken from NIST-17 [2]. Statistical analysis of GC-RI was carried out using Excel software (Microsoft Windows 2007). Reference data on di-

Table 6. GC retention indices for selected derivatives of glycine (Gly) and alanine (Ala) on standard non-polar phases.

Derivative	GCRI, i.u.		ΔRI(Ala-Gly)	Total Mass (Da) of Substituents (CO_2X) and (NHY)
	Alk-AlkOC Derivatives			
	Gly	Ala		
Me-MOC	1130	1133	+3	133
Me-EOC	1202	1201	-1	147
Et-EOC	1274	1270	-4	161
Me-POC	1304	1297	-7	161
Pr-POC	1471	1458	-13	189
Me-iso-POC	1239	1236	-3	161
	Other Derivatives			
N,O-*bis*-TMS	1121	1105	-16	205
N,N,O-*tris*-TMS	1314	1373	+59	277
Bu ester, N-$COCF_3$	1173	1159	-14	213
N-CH_3, N-$COCF_3$ (acid)	1157	1189	+32	143
Me ester, N-CH_3-N-$COCF_3$	1016	1042	+26	157
TBDMS ester, NEOC	1579	1561	-18	247

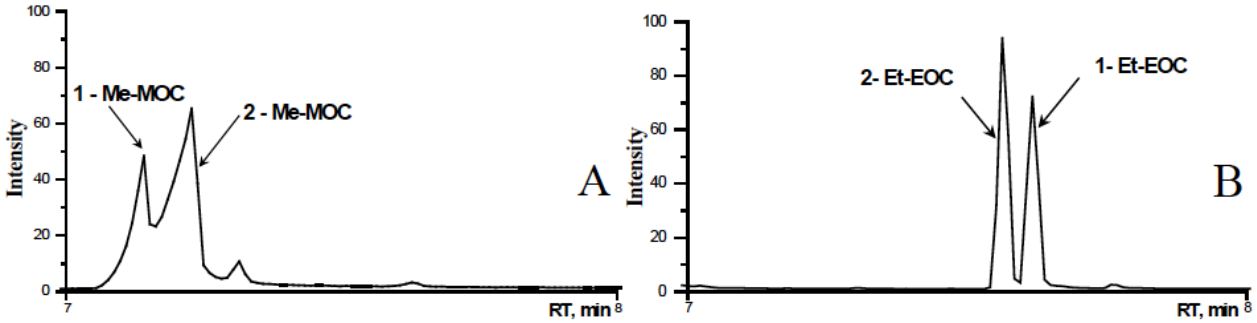


Fig. (1). Chromatograms of glycine (1) and alanine (2) derivatives on a standard non-polar phase illustrating an inversion of elution order: (A) methyl esters of N-methoxycarbonyl (Me-MOC) derivatives and (B) ethyl esters of N-ethoxycarbonyl (Et-EOC) derivatives. The inversion of elution order is observed.

electric permeability (ε) and dipole moments (μ) were taken from the reference edition [14].

3. RESULTS AND DISCUSSION

3.1. Anomalous Elution Order for Alkyl Esters of N-Alkoxycarbonyl Derivatives of Glycine and Alanine

In accordance with the homology concept, alanine (Ala, **II**) is the next level branched homologue of glycine (Gly, **I**). Hence, one could expect an average increase in GC-RI values of 61 units ($\Delta RI^3 = 61 \pm 10$, Table **3**) taking into account the GC properties of compounds with non-hindered branching of molecular carbon skeleton when moving from lower to the higher homolog. The increase of differences in GC-RI values can be even more (over 100 units) when considering sterically hindered molecular structures ($\Delta RI^3 = 123$ to 153, Table **5**). The Alk-AlkOC derivatives under consideration indicate peculiarly small ΔRI values on standard non-polar stationary phases ranging from -13 to 3 (Table **6**). This type of regularities for the series of structural analogues is being revealed for the first time in the present study, whereas several sole examples of such anomaly have been observed in gas chromatography earlier.

A formal H → CH_3 replacement reaction resulting in the appearance of branching in the skeleton (Gly → Ala) does not lead to a regular increase of GC-RI values; instead, a decrease of the GC-RI values for Alk-AlkOC (alkyl CH_3) is observed. As depicted in Fig. (**1**), while methyl esters of N-MOC derivatives for Gly and Ala illustrate a "regular" elution order (Gly < Ala, Fig. (**1A**)), the elution order for ethyl esters of N-EOC derivatives is inverted (Gly>Ala, Fig.

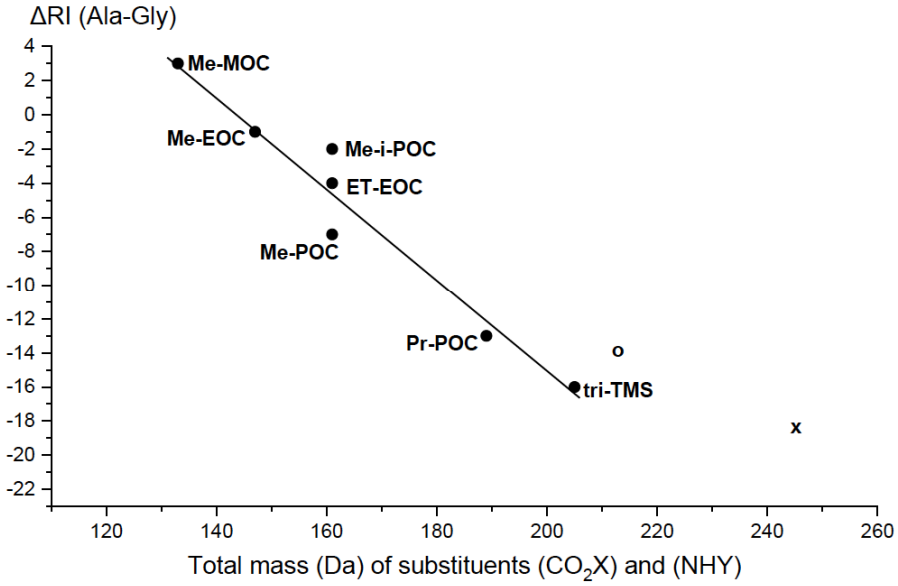


Fig. (2). Plot of the linear dependence of ΔRI = RI(Ala) – RI(Gly) *vs.* total mass (Da) of variable substituents at $C^{(2)}$ [*M*(CO_2R) + *M*(NHX)]. Parameters of the linear regression are listed in the text. The symbol "**o**" corresponds to butyl esters of N-trifluoroacetyl derivatives of alanine and glycine, the symbol "**x**" – to their *tert.*-butyldimethylsilyl esters of N-ethoxycarbonyl derivatives, r = -0.982 (correlation coefficient).

(**1B**)). These observations cannot be explained with the well-established gas chromatography theories.

The variations (from +3 up to -13) in the observed effect are just less than 20 i.u. as shown in Table **6**. The principal feature is the negative sign of these values except the Me-MOC derivatives. The small numbers do not diminish the importance of the effect. The same is true for the temperature anomalies of GC-RIs of polar compounds on non-polar stationary phases caused by dynamic modification of the stationary phase by analytes [15].

Considering the sum of the masses of functional substituents at $C^{(2)}$, such as carboxyl and amino groups, and their comparison to the GC-RI differences [ΔRI = RI(Ala) – RI(Gly)] for various analytes show sufficient correlations: the value of ΔRI is decreased with the increase of the sum of the masses of functional substituents at $C^{(2)}$ [CO_2R: R = C_nH_{2n+1} or $Si(CH_3)_3$, and NHX : X = CO_2R or $Si(CH_3)_3$].

The plot on Fig. (**2**) corresponds to the linear regression illustrating this tendency; the parameters of this regression are: a = -0.257 ± 0.03, b = 39 ± 4, r = -0.975, S_0 = 1.7. High absolute value of correlation coefficient (r = -0.975) confirms the correctness of this regression considered.

3.2. Elution Order Effects for Trialkylsilyl and Mixed Derivatives of Glycine and Alanine

3.2.1. TMS Derivatives

B*is*-TMS derivatives of glycine (**V**) and alanine (**VI**) also demonstrate similar unusual regularity that is similar to the series of homologous Alk-AlkOC derivatives. The same sign of a negative difference ΔRI = RI(Ala) – RI(Gly), namely -16 (Table **6**) is observed for the pair of N,O-*bis*-TMS derivatives. Conversely, corresponding N,N,O-*tris*-TMS derivatives do not indicate any anomaly; their ΔRI = RI(Ala) – RI(Gly) = +59 (Table **6**) is within the expected regular values for most of the chemicals.

3.2.2. n-Butyl Esters of N-Trifluoroacetates

Additionally, the described "anomaly effect" is specific for another widespread type of derivatives used for the structure elucidation of amino acids including glycine and alanine, namely butyl esters of N-trifluoroacetylated derivatives (Bu N-TFA) [9, 16, 17].

The GC-RI value for butyl ester of N-trifluoroacetyl-glycine (**VII**, RI = 1173) [16] is about (-14) i.u. less compared to the value for the corresponding alanine derivative (**VIII**, RI = 1159 [17], 1161 [16]). The total mass of substituents in this example is equal to 213 Da and this point is represented on the plot (Fig. (**2**)) with a symbol "**o**". However, the corresponding ΔRI value is not used for regression and this point is presented only as a comparison. In case of methyl esters of N-methyl-N-trifluoroacetyl-glycine (**IX**, RI = 1016) and alanine (**X**, RI = 1042) all acidic and amine hydrogen atoms are substituted [2, 18]. These derivatives do not demonstrate anomaly. The deference value +26 is within the expected regular values for most of the chemicals (ΔRI = RI(Ala) – RI(Gly) = 1042-1016=26).

3.2.3. tert.-Butyldimethylsilyl Esters of N-Ethoxycarbonyl Derivatives (TBDMS N-EOC)

tert.-Butyldimethylsilyl esters of N-ethoxycarbonyl derivatives of glycine (**XI**, RI = 1579) and alanine (**XII**, RI = 1561) exhibit the same type of anomaly [19]. In this case, the total mass of the fragments at functional groups is 247 Da, and the difference [ΔRI = RI(Ala) – RI(Gly)] is -18 i.u. The corresponding point in Fig. (**2**) is represented with a symbol "**x**". Similarly to the previously discussed symbol "**o**", it does not correspond to the linear regression for Alk-AlkOC,

Table 7. Dielectric permeability, dipole moment and GC-RI data for ethanol and its selected homologs on standard polar and non-polar phases.

Alcohol	$RI_{non-polar}$ [2]	RI_{polar} [2]	Dielectric Permeability (ε)	Dipole Moment (μ, D)
Ethanol	440 ± 13	932 ± 8	25.3	1.7
2-Propanol	489 ± 11	927 ± 15	20.2	1.7
2-Methyl-2-propanol	512 ± 5	900 ± 21	12.5	1.7

Table 8. Dielectric permeability, dipole moment and GC-RI data for propanol and its selected homologs on standard polar and non-polar phases.

Alcohol	$RI_{non-polar}$ [2]	RI_{polar} [2]	Dielectric Permeability (ε)	Dipole Moment (μ, D)
1-Propanol	546 ± 9	1036 ± 9	20.8	1.6
2-Butanol	586 ± 5	1025 ± 11	17.3	1.7
2-Methyl-2-butanol	628 ± 4	1008 ± 12	5.8	1.8

Table 9. GC-RI data for ethyl- and isopropyl nitro- and cyano-derivatives on standard polar and non-polar phases.

Alkyl-X	$RI_{non-polar}$ [2]	RI_{polar} [2]	ΔRI_{polar}
CH_3CH_2-NO_2	618 ± 2	1186 ± 7	-67
$(CH_3)_2CH$-NO_2	676 ± 8	1119 ± 10	
CH_3CH_2-CN	544 ± 2	1025 ± 6	-16
$(CH_3)_2CH$-CN	597 ± 3	1009 ± 1	

but the general tendency seems to be the same: the larger is the total mass of functional substituents connected to the central carbon atom $C^{(2)}$, the higher is the RI difference between alanine and glycine derivatives.

It is worth noting that: (a) Supelco Corp reported GC-RI values 1675 i.u. for Ala-EZ:faast and 1697 i.u. for Gly-EZ:faast derivatives obtained with the use of analytical procedure for Fast-GC analysis of amino acids after derivatization with EZ:faast kit® using ZB-AAA 10 m x 0.25 mm column [20]; the difference in GC-RI values is -22 i.u, and that is in accordance with the anomaly under discussion, as well, and (b) GC-RI values 1085 i.u. [2] and 1143 i.u. [21] were reported for methyl ester of N-dimethylaminomethylene-alanine and both publications indicated that glycine did not form such a derivative at standard conditions; it can be explained by specific chemical properties of glycine as the first member of the homologous series.

3.3. Elution Order Interpretation for Glycine and Alanine Derivatives

The elution order for Alk-AlkOC derivatives of glycine and alanine on standard non-polar stationary phases is of interest. Moreover, the observed changes of elution order just for their "methyl homologs" (Me-MOC derivatives) is out of the ordinary.

Considering the possible rationale for this effect, it should be noted that similar anomalies are revealed quite often for homologs of various series on polar stationary phases. The simplest of such examples is the elution order of ethanol, 2-propanol, and *tert.*-butyl alcohol, as well 1-propanol, 2-butanol, and 2-methyl-2-butanol (Table **7** and Table **8**). Every next member in this series contains additional methyl group and one additional branching.

Similar anomalies are often revealed for homologs of various types of compounds on polar stationary phases. The simplest example is a mixture of ethanol, 2-propanol, and *tert.*-butyl alcohol - three alkanols, where a hydroxyl is bound to a primary, secondary, or tertiary carbon. An additional methyl group is added to every molecule in this series that leads to additional branching of the carbon skeleton. No anomalies were observed when analyzing this series on a standard non-polar stationary phase [2] since their GC RI values increase with the increase of the molecular weight. However, their GC-RI values demonstrate an inverted increasing order on a standard polar column with polyethylene glycol: *tert.*-butanol < 2-propanol < ethanol. Similar elution

Table 10. Comparison of GC retention indices for homologs type CH_2XY to the type CH_3-CHXY homologs on standard polar stationary phases.

X-CH_2-Y	RI_{polar}	CH_3-CHXY	RI_{polar}	ΔRI[10]
CH_2Cl_2 Dichloromethane	933 ± 6	CH_3CHCl_2 1,1-Dichloroethane	901 ± 1	-32
$ClCH_2CO_2C_2H_5$ Ethyl chloroacetate	1337 ± 27	CH_3CHCl-$CO_2C_2H_5$ Ethyl 2-chloropropanoate	1250 ± 21	-87
$CH_2(CO_2C_2H_5)_2$ Diethyl propanedioate	1574 ± 5	$CH_3CH(CO_2C_2H_5)_2$ Diethyl methylpropanedioate	1539*	-35

*) A single reference RI value is presented without MAD.

orders are observed for homologs of other type of alkanols, nitroalkanes and cyanoalkanes (Table **9**).

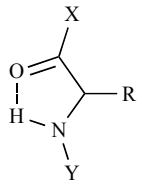
Hence, the above examples clearly demonstrate that the decrease of GC- RI values for homologs type of R-X compounds (where R is primary, secondary, and tertiary alkyl radical and X is any polar functional group) on polar stationary phases seems to be a "standard" tendency, and no anomaly is observed. The tangible reason for this effect is diminution of the polarity of analytes in the sequence *prim*. > *sec*. > *tert.*-alkanols.

Therefore, analysis of the data based on the simplest characteristics of the polarity of organic compounds [22], namely dielectric permeability (ε) and dipole moment (μ) can be beneficial. As demonstrated in the above tables, the μ-values for all alcohols mentioned above are the same, and they equal to 1.7; hence, the dipole moments do not rule the elution order for these alkanols. The constant value 1.7 D for all alcohols is determined by the constant polarity of the chemical bond C-O. On the other hand, the values of dielectric permeability (ε, the alternative characteristic of the polarity) strongly decrease in the sequence *prim*. > *sec*. > *tert.*-alkanols. At first, it is caused by so-called hyperconjugation effect of methyl groups [23], which is not typical for other alkyl fragments.

The same inversion tendency of elution order on polar stationary phases is observed for simple homologs containing two polar functional groups at the same carbon atom. GC-RI data on polar stationary phases for some dichloro-, chloro, ethoxycarbonyl- and di(ethoxycarbonyl)alkane pairs are depicted in Table **10**.

Concurrently, only a few compounds with two polar functional groups at the same carbon atom demonstrate similar tendencies with regard to the GC-RI on non-polar phases; the pairs represented in Table **10** are analogs of two simplest amino acids: glycine and alanine. The structural difference between them is the methyl group that provides the largest variations in their polarity (according to the ε-criterion) due to the hyperconjugation effect of this group. No anomalies, similar to the discussed above, were detected for any derivatives of the following members in an amino acid series, such as valine (R = *iso*-C_3H_7), norvaline (R = C_3H_7), leucine (R = *iso*-C_4H_9), isoleucine (R = 2-C_4H_9), norleucine (R = C_4H_9), *etc*.

Another reason for the anomalous chromatographic behavior of the simplest amino acid derivatives may be related to the intramolecular hydrogen bond interaction between amino and carbonyl fragments since nitrogen is less electronegative than oxygen, and the H-N bond is less polar than H-O bond (Scheme **4**):



Scheme 4. Intramolecular hydrogen bonding in N, O-disubstituted amino acids.

The well-established H-bond interactions within parent amino acids, also studied by quantum chemical calculations [24-27], cannot be directly utilized for the determination of the GC-RI anomaly; these interactions are observed for free amino acids, but not for their derivatives. Formation of similar H-bonds in molecules of derivatives may restrict the intramolecular rotation and limit vibration processes, which may lead to the increase of corresponding GC-RI values. However, there is no evidence to conclude that the H-bond interaction in glycine is stronger than in alanine. As a result, this anomaly cannot be explained based on the H-interaction concept. Nevertheless, the role of intramolecular hydrogen bonding in this process may be confirmed by the GC-RI data recorded for glycine and alanine when a complete derivatization of amino- and hydroxyl- functional groups are achieved. Two types of derivatives are presented, and in both cases the difference has a positive value: for a pair of N,N,O-*tris*-TMS derivatives, the difference is +59 (Table **6** and Fig. (**2**); ΔRI = RI(Ala) – RI(Gly) = +59), and for methyl esters of N-methyl-N-trifluoroacetyl-glycine and alanine this value equals +26.

A reasonable rationalization of the anomaly can be made with the application of a linear dependence of the differences ΔRI = RI(Ala) – RI(Gly) *vs*. the total mass of functional substituents at $C^{(2)}$, such as for carboxyl and amino groups. This type of interpretation can be made with the use of a comparative analysis of GC-RI data we obtained for Alk-AlkOC derivatives of Gly and Ala, and the literature data for *n*-alkanes

Table 11. GC retention indices of selected (*n*/2)-methyl alkanes, namely *gem*-dialkylethanes $CH_3CH(C_kH_{2k+1}$, with a chemical formula C_nH_{2n+2} ($6 \le n \le 16$).

Isoalkane	RI [2]	$\Delta RI = RI(C_nH_{2n+2}) - 100(n-1)$
3-Methylpentane	584 ± 2	84
4-Methylheptane	767 ± 1	67
5-Methylnonane	961 ± 1	61
6-Methylundecane	1154 ± 2	54
7-Methyltridecane	1351*	51
8-Methylpentadecane	1539*	39

*) Single reference RI value is presented without a MAD.

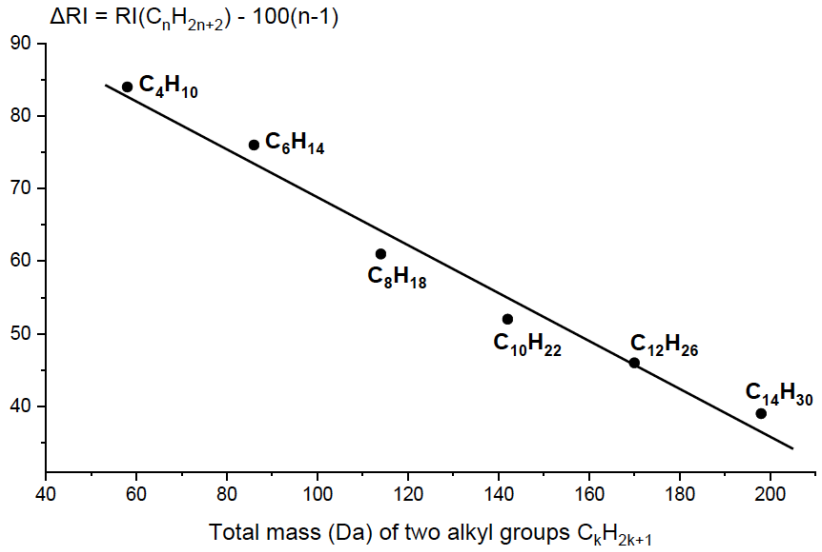


Fig. (3). Plot of the differences of retention indices for (*n*/2)-methyl alkanes and retention indices for normal alkanes C_nH_{2n+2} [RI = 100(*n*-1)] *vs.* total mass of two alkyl fragments C_kH_{2k+1} in a molecule, $r = -0.974$ (correlation coefficient).

containing methyl substituent in the middle of a chain, namely (*n*/2)-methylalkanes C_nH_{2n+2} ($k = n/2 - 1$).

Table **11** includes GC-RI data for methyl-substituted *n*-alkanes covering pentane - pentadecane hydrocarbons, where each alkane contains a methyl substituent in the middle of a chain. The ΔRI values calculated and depicted in Table **11** are then used for the presentation of a plot in Fig. (**3**) depicting $\Delta RI = RI[(n/2)\text{-methyl alkane}] - RI(n\text{-}C_{n-1}H_{2n}]$ as a function of total mass of two alkyl fragments C_kH_{2k+1}.

The plot in Fig. (**3**) clearly displays the same general tendency of ΔRI variations as that observed for derivatives of amino acids and depicted in Fig. (**2**). Parameters of linear regression are: $a = -0.30 \pm 0.03$, $b = 97 \pm 4$, $R = -0.982$, $S_0 = 3.4$. This dependence of RI values *vs.* position of branching in a carbon skeleton can be confirmed by other examples, as well.

CONCLUSION

The peculiar behavior of Alk-AlkOC derivatives of glycine and alanine agrees with the concepts of gas chromatography and known GC-RI regularities of organic compounds. The observed decrease of GC-RI is a result of an introduction of an additional methyl group to a carbon atom connected to two polar fragments in a molecule CH_2XY. The dependence of ΔRI values for homologs of the types of CH_3-CHXY and CH_2XY *vs.* the total mass of fragments (X + Y) is similar to those for other sub-groups of analytes.

LIST OF ABBREVIATIONS

Ala	=	Alanine
Alk	=	Alkyl
AlkOC	=	Alkoxycarbonyl
Bu N-TFA	=	N-trifluoroacetyl Butyl ester
DMAM	=	Dimethylaminomethylene
EI-MS	=	Electron Ionization Mass Spectra
EOC	=	Ethoxycarbonyl
Et	=	Ethyl
GC	=	Gas Chromatography

GC-MS	=	Chromatography-Mass Spectrometry
Gly	=	Glycine
i-POC	=	Iso-Propyloxycarbonyl
i.u.	=	Index Units
MAD	=	Median Absolute Deviation
Me	=	Methyl
MOC	=	Methoxycarbonyl
MS	=	Mass Spectrometry
POC	=	Propyloxycarbonyl
Pr	=	Propyl
RI	=	Retention Index
TBDMS	=	tert.-Butyldimethylsilyl
TMS	=	Trimethylsilyl

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable

HUMAN AND ANIMAL RIGHTS

No animals/humans were used in this research.

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

REFERENCES

[1] Kovats' retention index system.Cazes, J., Ed.; *Encyclopedia of Chromatography,* 3rd Edn; Taylor & Francis. N.Y., **2010**, 2, pp. 1304-1310.

[2] The NIST 17 Mass Spectral Library (NIST17/2017/EPA/NIH). Software/DataVersion (NIST17); NIST Standard Reference Database, Number 69, June 2017. National Institute of Standards and Technology, Gaithersburg, MD 20899: https://www.nist.gov/srd/nist-standard-reference-database-1a-v17http://webbook.nist.gov

[3] Kovats, E. Gas chromatographische Characterisierung organischer Verbindungen. *Helv. Chim. Acta,* **1958**, *41*, 1915-1931 [http://dx.doi.org/10.1002/hlca.19580410703]

[4] Fieser, L.F.; Fieser, M. *Advanced Organic Chemistry*; Reinhold Publ. Corp.: N.Y., **1961**.

[5] Zenkevich, I.G. Dependence of gas chromatographic retention indices on dynamics characteristics of molecules. *J. Phys. Chem. A (Rus.),* **1999**, *73*(5), 905-910.

[6] Zenkevich, I.G.; Marinichev, A.N. Comparison of the topological and dynamic characteristics of molecules for calculating retention indices of organic compounds. *Rus. J. Struct. Chem.,* **2001**, *42*(5), 747-754 [http://dx.doi.org/10.1023/A:1017961115124]

[7] Zenkevich, I.G.; Kostikov, R.R. Prediction of gas-chromatographic elution sequence of diastereomers and enantiomers using the molecular dynamics methods. *Russ. J. Org. Chem.,* **2003**, *39*(8), 1057-1063 [http://dx.doi.org/10.1023/B:RUJO.0000010170.81727.ae]

[8] Todua, N.G.; Camara, J.E.; Murray, J.A.; Mikaia, A.I. Stepwise extraction, chemical modification, GC-MS separation, and determination of amino acids in human plasma. *Sep. Sci. plus,* **2018**, *1*, 177-189.

[9] Todua, N.G.; Zenkevich, I.G.; Mikaia, A.I. Peculiar behavior of N-alkoxycarbonyl derivatives of simplest amino acid methyl esters under GC-MS analysis. San-Diego, USAAbstr. 66th ASMS Conference on Mass Spectrom. & Allied Topics.July 3-7**2018**.

[10] Zaikin, V.; Halket, J. *A Handbook of Derivatives for Mass Spectrometry*; IM Publ.: Chichester, **2009**.

[11] Zampolli, M.G.; Basaglia, G.; Dondi, F.; Sternberg, R.; Szopa, C.; Pietrogrande, M.C. Gas chromatography – mass spectrometry analysis of amino acid enantiomers as methyl chloroformate derivatives: application to space analysis. *J. Chromatogr. A,* **2007**, *1150*, 162-172.

[12] Villas-Boas, S.G.; Delicado, D.G.; Akesson, M.; Nielsen, J. Simultaneous analysis of amino and nonamino organic acids as methyl chloroformate derivatives using gas chromatography – mass spectrometry. *Anal. Biochem.,* **2003**, *322*, 134-138.

[13] Van den Dool, W.A.; Kratz, P.D. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J. Chromatogr.,* **1963**, *11*, 463-471.

[14] Handbook of Chemistry and Physics.CRC Press, Taylor & Francis Group: Boca Raton, **2018**.

[15] Pavlovskii, A.A.; Heberger, K.; Zenkevich, I.G. Anomalous temperature dependence of gas chromatographic retention indices of polar compounds on non-polar phases. *J. Chromatogr. A,* **2016**, *1445*, 126-134.

[16] Gamerith, G. Gas chromatography of various N(O,S)-acyl esters of amino acids. *J. Chromatogr. A,* **1983**, *268*, 403-415 [http://dx.doi.org/10.1016/S0021-9673(01)95439-1]

[17] Schneider, K.; Neupert, M.; Spiteller, G.; Henning, H.V.; Matthaei, D.; Scheler, F. Gas chromatography of amino acids in urine and haemofiltrate. *J. Chromatogr. A,* **1985**, *345*(1), 19-31 [http://dx.doi.org/10.1016/0378-4347(85)80131-6] [PMID: 4086581]

[18] Todua, N.G.; Tretyakov, K.V.; Mikaia, A.I. Mass spectrometry of analytical derivatives. 1. Cyanide cations in the spectra of N- alkyl-N-perfluoroacyl- α-amino acids and their methyl esters. *Eur J Mass Spectrom (Chichester),* **2015**, *21*(3), 183-190 [http://dx.doi.org/10.1255/ejms.1374] [PMID: 26307698]

[19] Paik, M.-J.; Lee, H.-L.; Kim, K.-R. Simultaneous retention index analysis of urinary amino acids and carboxylic acids for graphic recognition of abnormal state. *J. Chromatogr. B,* **2005**, *82*, 94-104.

[20] Analysis of amino acids using fast-GC/MS and metabolite database. Application News. Shimadzu Corp. **2011**, No M246. Accessed as c3162802.workcast.net/10211_GCMS246_2011105145013.pdf

[21] Zenkevich, I.G.; Pushkareva, T.I. Chromatographic and chromato-mass-spectral characterization of amino acids derivatives formed via the interaction with dimethyl acetal of dimethylformamide. *Russ. J. Gen. Chem.,* **2015**, *85*(8), 1918-1926 [http://dx.doi.org/10.1134/S1070363215080204]

[22] Héberger, K.; Zenkevich, I.G. Comparison of physicochemical and gas chromatographic polarity measures for simple organic compounds. *J. Chromatogr. A,* **2010**, *1217*(17), 2895-2902 [http://dx.doi.org/10.1016/j.chroma.2010.02.037] [PMID: 20236649]

[23] Alabugin, I.V.; Bresch, S.; Gomes, J.P. Orbital hybridization: a key electronic factor in control of structure and reactivity. *J. Phys. Org. Chem.,* **2015**, *28*, 147-162 [http://dx.doi.org/10.1002/poc.3382]

[24] Ramek, M. Intramolecular hydrogen bonding in neutral glycine, β-alanine, γ -aminobutyric acid, and δ -aminopentane acid. Int. *J. Quant. Chem.,* **1990**, *38*(17), 45-53 [http://dx.doi.org/10.1002/qua.560381708]

[25] Pakari, A.H.; Jamshidi, Z. Intramolecular dihydrogen bond in the amino acid. *J. Mol. Struct. THEOCHEM,* **2004**, *685*(1), 155-161 [http://dx.doi.org/10.1016/j.theochem.2004.06.053]

[26] Ramaniah, L.M.; Kamal, C.; Kshirsagar, R.J. How universal are hydrogen bond correlations? A density functional study of intramolecular hydrogen bonding in low-energy conformers of α -amino acids. *J. Mol. Phys.,* **2013**, *111*(20), 3067-3076 [http://dx.doi.org/10.1080/00268976.2013.770174]

[27] Dake, Yu.; Armstrong, D.A.; Rauk, A. Hydrogen bonding and internal rotation barriers of glycine and its zwitterions (hypothetical) in the gas phase. *Can. J. Chem.,* **1992**, *72*(6), 1762-1772 [http://dx.doi.org/10.1139/v92-221]