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IMPACT OF SUBSTITUENTS OF VARIOUS NATURE AT C-3 AND C-5 OF 4*H*-1,2,4-TRIAZOLE ON COMPOUNDS' BEHAVIOR UNDER THE CONDITIONS OF GC-MS ANALYSIS

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Abstract

Pharmaceutical products require appropriate quality control, which comprises a set of tests aimed at the determination of assays of the substances. Chromatographic methods are one of the most abundant analytical methods applied in routine analyses of pharmaceuticals. Information on the behavior of the compounds under certain analytical conditions is valuable in the process of analytical method development. This work was aimed at revealing the impact of substituents of various natures at two positions of triazole ring on the behavior of new 3-thio-1,2,4-triazoles under GC-MS conditions and evaluating the applicability of GC-MS method for the analysis of these compounds. Gas chromatography was used in combination with mass spectrometry. New 1,2,4-triazole derivatives were obtained at the Department of Natural Sciences for International Students and Toxicological Chemistry of Zaporizhzhia State Medical University. For the experiment, two groups of compounds were formed, each having different substituents at C-3 and C-5 of triazole molecule, respectively. The analysis was held using Agilent 7890B GC system coupled with Agilent 5977B mass selective detector. Compounds were separated on a non-polar column. The following parameters were assessed and compared for identification and study of chromatographic behavior of the compounds: mass spectra, retention times, symmetry factors, and relative responses (against reference compound). It was shown that there is no direct relation between melting point and retention time; instead, the nature of substituents significantly affects the chromatographic behavior. The strongest response belongs to compounds that have phenyl moiety at C-5 and alkyl substituent attached through thiol group at C-3 of the triazole ring. Thiophen-2-yl and methyl at C-5, as well as acetate and ester groups attached through thiol group at C-3 of the cycle, decrease chromatographic response. GC-MS can be applied for the analysis of biologically active 1,2,4-triazoles, though with several limitations. The higher the polarity of the compound, the worse the response and peak shape; these circumstances complicate GC-MS analysis of 1,2,4-triazole derivatives. Apart from providing information on physicochemical properties of the compounds, the obtained experimental data provide some valuable insights into permeability of the new triazole derivatives through biological membranes, which could be useful in drug design.

Keywords: gas chromatography; mass spectrometry; heterocyclic compounds; 1,2,4-triazole derivatives; retention time; response; peak area; symmetry factor; melting point; polarity.

ВПЛИВ ЗАМІСНИКІВ РІЗНОЇ ПРИРОДИ У 3- ТА 5-МУ ПОЛОЖЕННЯХ 4*H*-1,2,4-ТРИАЗОЛУ НА ПОВЕДІНКУ ПОХІДНИХ В УМОВАХ ГХ-МС-АНАЛІЗУ

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Анотація

Інформація про поведінку сполук у певних умовах аналізу може бути корисною при розробці аналітичних методик. Дослідження було направлене на визначення впливу замісників різної природи у двох положеннях триазольного кільця на поведінку нових 3-тіо-1,2,4-триазолів в умовах ГХ-МС. Застосували метод газової хроматографії у поєднанні з мас-спектрометрією. Для експерименту було сформовано дві групи речовин, кожна з яких мала різні замісники у 3- та 5-му положенні триазольного циклу відповідно. Аналіз проведений використовуючи газовий хроматограф Agilent 7890B із одноквадрупольним мас-спектрометричним детектором Agilent 5977B. Речовини були розділені на неполярній колонці. Для ідентифікації та дослідження хроматографічної поведінки отримали та порівняли такі параметри: мас-спектр, час утримування, фактор симетрії піка та відносний відгук (до речовини-стандарту). Показали, що між температурою плавлення та часом утримування похідних 1,2,4-триазолу прямої залежності немає;

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хроматографічна поведінка сполук значним чином залежить від природи замісника. Встановили, що найвищий відгук мають сполуки, які містять фенільний радикал у 5-му положенні та алкільний замісник, приєднаний через тіогрупу у 3-му положенні триазольного циклу. Тіофен-2-іл та метил у 5-му положенні, а також карбоксильна та естерна групи, приєднані через сульфур у 3-му положенні циклу, знижують хроматографічний відгук. Таким чином, полярність ускладнює аналіз похідних 1,2,4-триазолу за допомогою ГХ-МС.

Ключові слова: газова хроматографія; мас-спектрометрія; гетероциклічні сполуки; похідні 1,2,4-триазолу; час утримування; відгук; площа піка; фактор симетрії; температура плавлення; полярність.

ВЛИЯНИЕ ЗАМЕСТИТЕЛЕЙ РАЗЛИЧНОЙ ПРИРОДЫ В 3- И 5-М ПОЛОЖЕНИЯХ 4*H*-1,2,4-ТРИАЗОЛА НА ПОВЕДЕНИЕ ПРОИЗВОДНЫХ В УСЛОВИЯХ ГХ-МС-АНАЛИЗА

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Аннотация

Информация о поведении веществ в определённых условиях анализа может оказаться полезной при разработке аналитических методик. Исследование было направлено на определение влияния заместителей различной природы в двух положениях триазольного кольца на поведение новых 3-тио-1,2,4-триазолов в условиях ГХ-МС. Использовали метод газовой хроматографии в сочетании с масс-спектрометрией. Для эксперимента было сформировано две группы веществ, каждая из которых имела разные заместители в 3- и 5-м положении триазольного цикла соответственно. Анализ проведен с использованием газового хроматографа Agilent 7890B с одноквадрупольным масс-спектрометрическим детектором Agilent 5977B. Соединения разделены на неполярной колонке. Для идентификации и исследования хроматографического поведения получили и сравнили следующие параметры: масс-спектр, время удерживания, фактор симметрии пика и относительный отклик (к веществу-стандарту). Показали, что между температурой плавления и временем удерживания производных 1,2,4-триазола прямой зависимости нет; хроматографическое поведение значительным образом зависит от природы заместителя. Установили, что наиболее высокий отклик принадлежит соединениям, которые имеют фенильный радикал в 5-м положении и алкильный заместитель, присоединённый через тиогруппу в 3-м положении триазольного цикла. Тиофен-2-ил и метил в 5-м положении, а также карбоксильная и сложноэфирная группы, присоединённые по сере в 3-м положении, снижают хроматографический отклик. Таким образом, полярность осложняет анализ производных 1,2,4-триазола с помощью ГХ-МС.

Ключевые слова: газовая хроматография; масс-спектрометрия; гетероциклические соединения; производные 1,2,4-триазола; время удерживания, отклик; площадь пика; фактор симметрии; температура плавления; полярность.

Introduction

Heterocyclic compounds garner more and more attention within the scientific community due to their broad spectrum of biological activities. Triazoles, heterocyclic systems that contain three atoms of nitrogen, are one of such substances. Some of the structures that include 1,2,4-triazole have already found applications in medicine and agricultural chemistry. In this way, anastrozole is used as cancer treatment, and in particular, as breast cancer medication [1]; fluconazole is an antifungal drug [2]; rizatriptan is used to mitigate migraine and headache [3]; myclobutanil is a fungicide that has low carcinogenicity [4]. However, these compounds have one common feature: they do not contain a 1,2,4-triazole ring as a central system but feature it as a side substituent of the main structure.

Drugs, in which 1,2,4-triazole cycle is the central structural component, are currently of high scientific and commercial interest due to wide range of properties they possess [5]. Scientists from various research laboratories across the globe are actively searching for new 1,2,4-triazoles that exhibit antimicrobial, antifungal, antiviral, diuretic, anti-inflammatory, and other types of biological activities [6]. Therefore, many of these heterocyclic compounds are potential medicinal products.

An important step in the production of pharmaceuticals is a pertinent quality control. Gas chromatography (GC) coupled to mass spectrometric (MS) detection is a powerful research tool for many classes of compounds. However, GC has one significant limitation: analytes must be volatile enough to be detected by the instrument. Majority of biologically active compounds have high molecular weight due to presence of phenyl and alkyl groups as well as heterocyclic substituents [6]. Various groups alter physicochemical properties of 1,2,4-triazoles in different ways, which affects GC-MS results. That is why studying the possibility of application of this hyphenated analytical method for qualitative and quantitative determination of 3-thio-1,2,4-triazole derivatives that contain various substituents is of high significance.

There is no universal method for the determination of 1,2,4-triazole derivatives. The

most popular approaches to their analysis include high-performance liquid chromatography (HPLC), GC-MS, and spectrophotometry. Only a few chromatographic methods have been officially proposed for triazole analysis. In this way, antifungal drug fluconazole that may be present in serum and amniotic fluid in animals is analyzed using GC-MS [7]; this method is also proposed for determination of impurities in rizatriptan [8]. However, due to high molar weight and polarity of 1,2,4-triazoles, HPLC continues to be the most used method for their analysis [9; 10; 11; 12]. Apart from that, researchers also suggest studying the effect of various substituents on antioxidant activity of 1,2,4-triazoles using spectrophotometry method [13]. In our work, the attempt to determine the effect of substituents at C-3 and C-5 of 3-thio-1,2,4-triazole on chromatographic behavior is made for the first time.

Results and discussion

The results of this investigation are based on chromatograms obtained during the analysis of new 3-thio-1,2,4-triazoles. The results of GC-MS, specifically the retention times t_R, peak areas A and peak symmetries A_S along with mass spectra of the compounds in RRX group, are given in Table 1, XRR group – in Table 2.

Table 1

GC-MS results for the RRX group

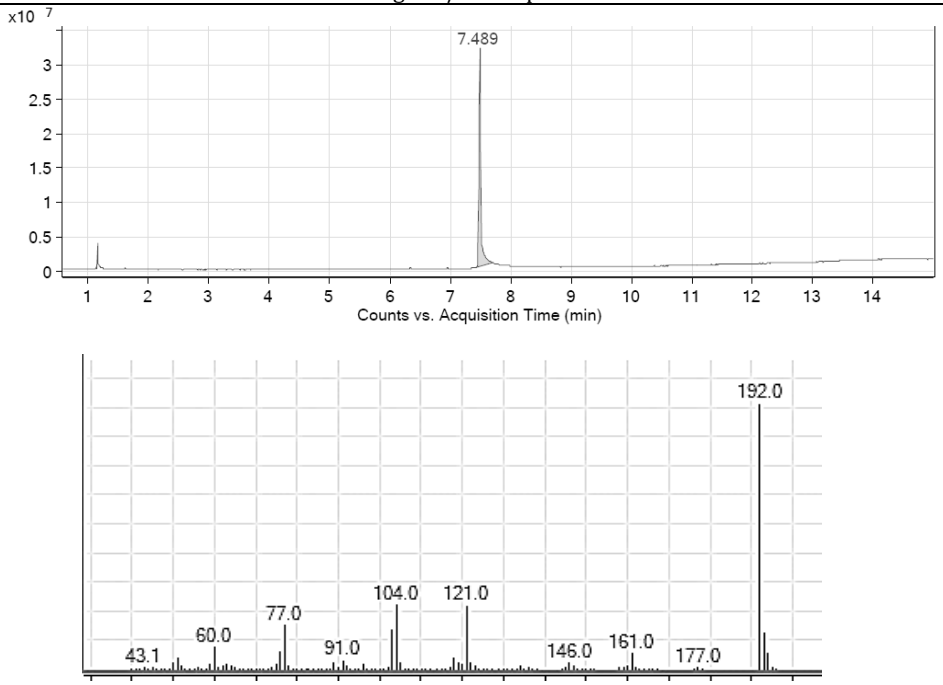
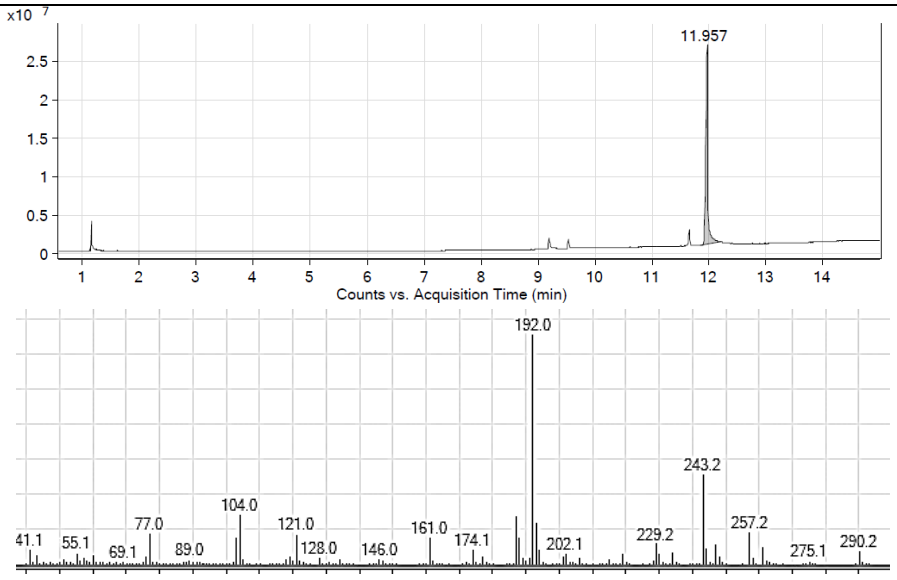
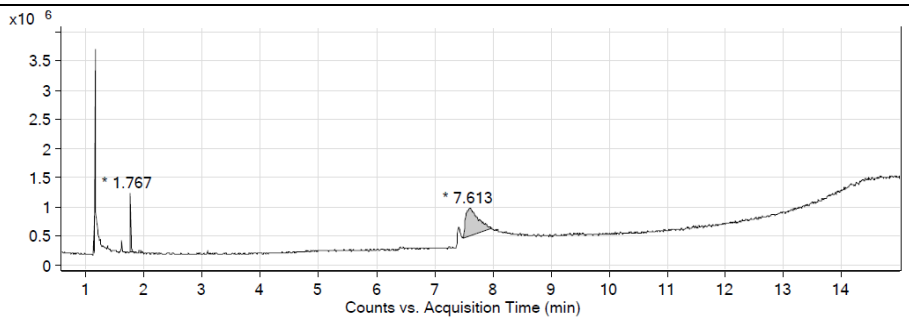
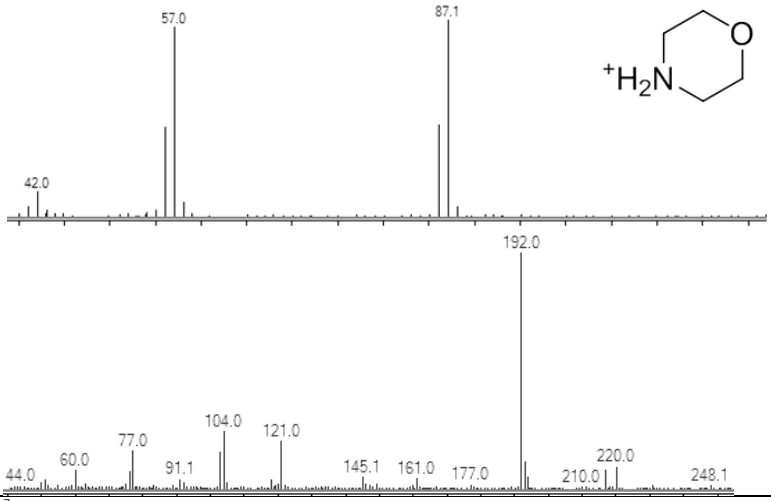
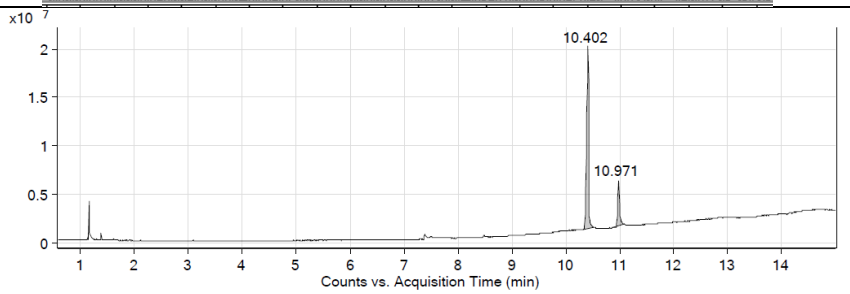
Chromatogram/Mass spectrum	Information
	Compound **1a** [ST] "-thiol" t_R = 7.489 A = 69158736 $A_{\%}$ = 100% A_S = 1.00 $A_{S\%}$ = 100%
	Compound **1b** "-(thio)heptyl" t_R = 11.957 A = 75391344 $A_{\%}$ = 109% A_S = 1.67 $A_{S\%}$ = 167%

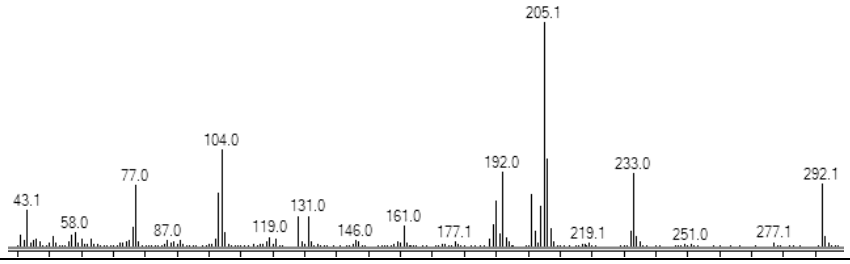
Table 1





Compound **1c**
"morpholinium"
t_{R1} = 1.767
A_1 = 876101
"-(thio)acetate"
t_{R2} = 7.613
A_2 = 8404085
$A_{\%2}$ = 12%
A_S = ---
$A_{S\%}$ = ---





Compound **1d**
"-(thio)ester"
t_R = 10.402
A = 18745763
$A_{\%}$ = 27%
A_S = 1.67
$A_{S\%}$ = 167%

Table 2

GC-MS results for the XRR group

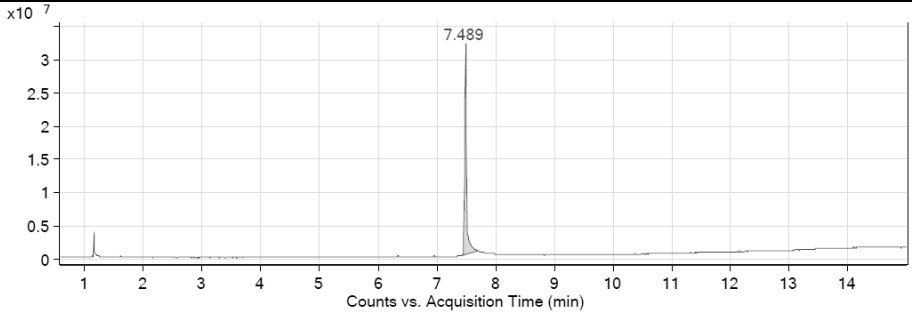
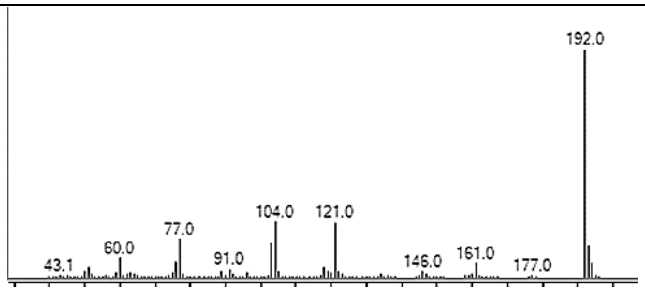
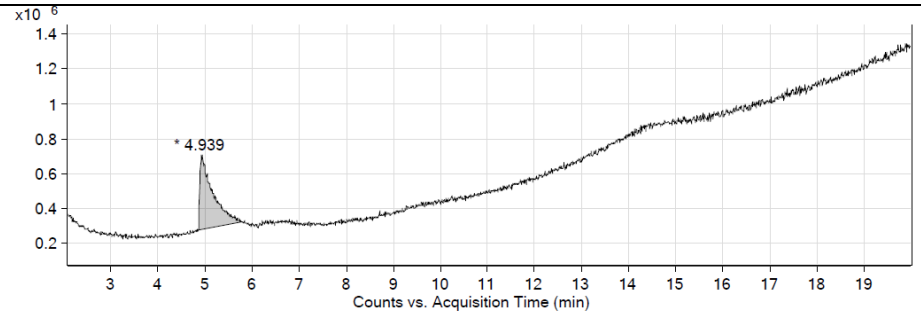
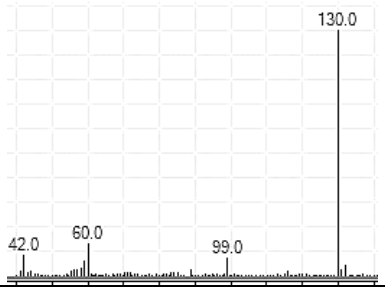
Chromatogram/Mass spectrum	Information
	Compound **1a** "-phenyl" t_R = 7.489 A = 69158736 $A_{\%}$ = 100% A_S = 1.00 $A_{S\%}$ = 100%

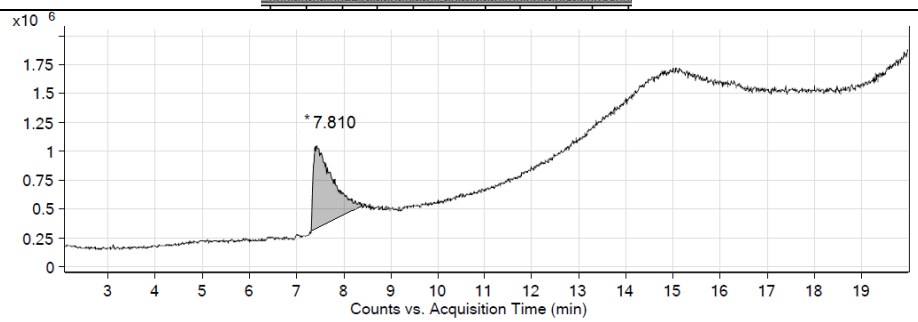
Table 2



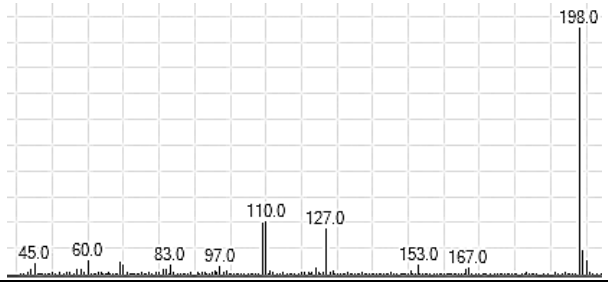


Compound **2a**
"-methyl"
t_R = 4.939
A = 8043863
$A_{\%}$ = 12%
A_S = ---
$A_{S\%}$ = ---





Compound **3a**
"-thiophen-2-yl"
t_R = 7.810
A = 12228575
$A_{\%}$ = 18%
A_S = ---
$A_{S\%}$ = ---



All three compounds of RRX group appeared on the corresponding chromatograms. The best response belonged to compounds **1a** and **1b** (thiol and (thio)alkyl, respectively), the worst belonged to compound **1c.** For all compounds, the mass-to-charge ratios matched molecular weights of the corresponding compounds; compound **1c** was identified based on two peaks on the chromatogram that corresponded to morpholin-4-ium cation and acidic residue.

All compounds of XRR group appeared on all corresponding chromatograms as well. Only compound **1a** (5-phenyl derivative) produced sufficient peak area and appropriate symmetry. Peak areas of compounds **2a** and **3a** (5-methyl and 5-thiophen-2-yl derivative, respectively) turned out to be 5-10 times lower than peak area

of standard compound. That is why symmetry factors for these peaks were not evaluated. Molecular peaks on the corresponding mass spectra matched molecular weights of all compounds in this group.

The relationship "retention time *vs.* substituent" was plotted based on data provided in Tables 1 and 2; the relationship "response *vs.* substituent" was plotted using values for relative peak areas $A_{\%}$. All corresponding relations are depicted in Figures 1, 2, 3, and 4. Molecular weights of the derivatives are given in parentheses along with designations of the corresponding substituents. Melting points (T_{mp}) of the compounds are also reflected in all graphs.

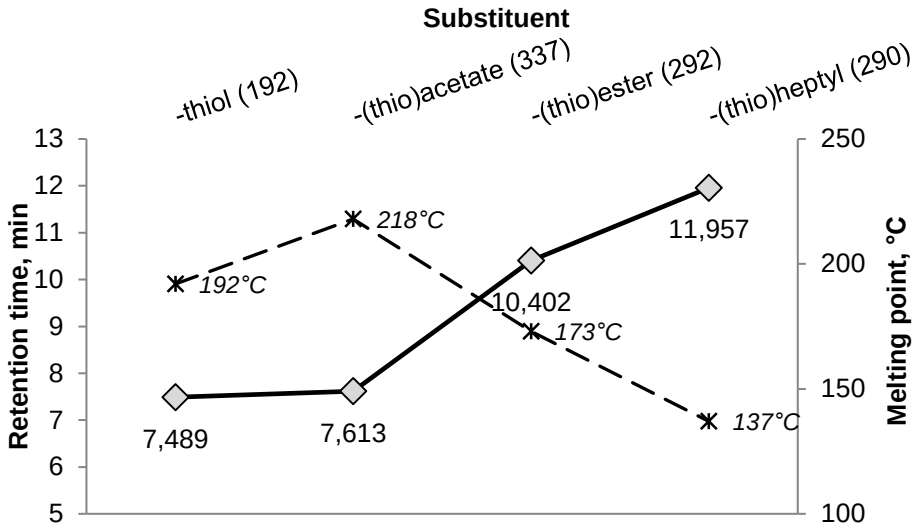


Fig. 1. The relationship between the nature of substituent at C-3 of the triazole ring and retention time (RRX group)

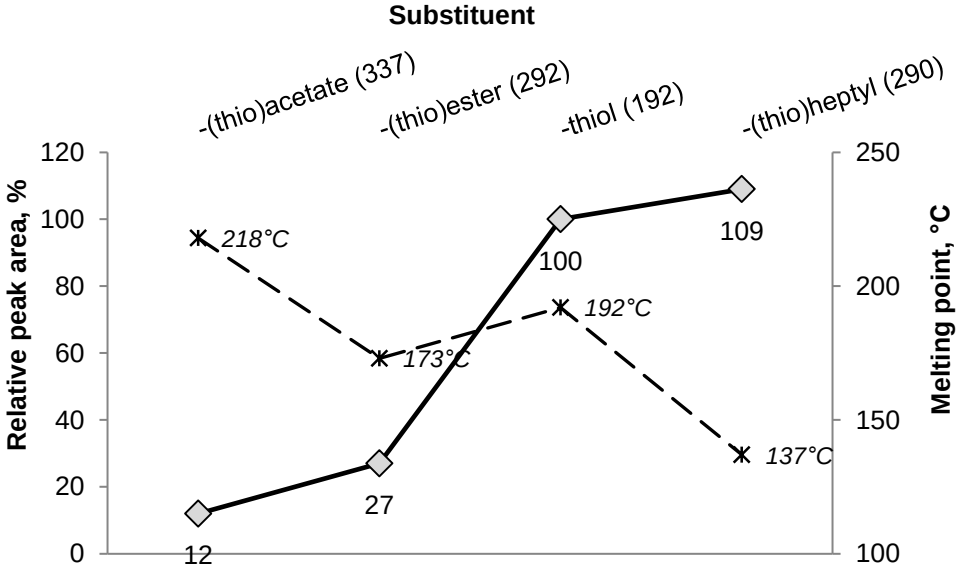


Fig. 2. The relationship between the nature of substituent at C-3 of the triazole ring and relative response (RRX group)

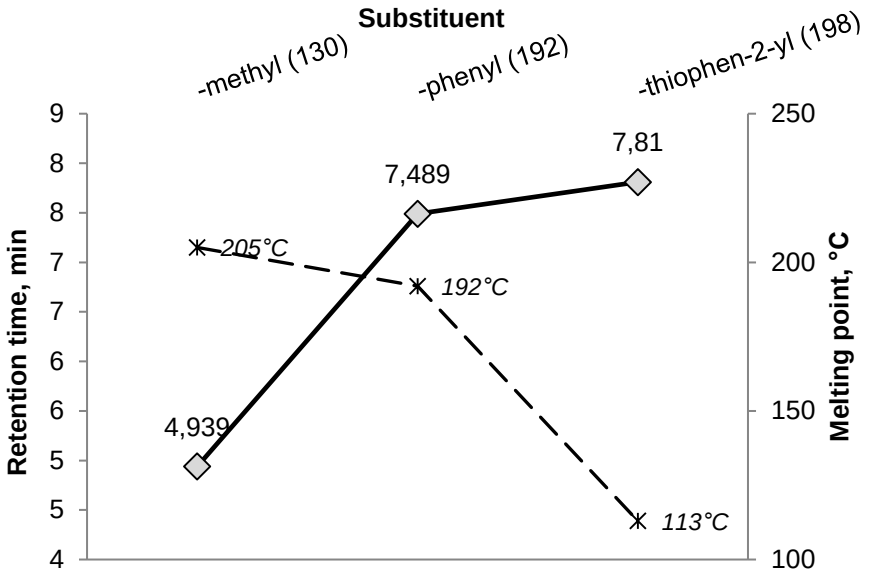


Fig. 3. The relationship between the nature of substituent at C-5 of the of triazole ring and retention time (XRR group)

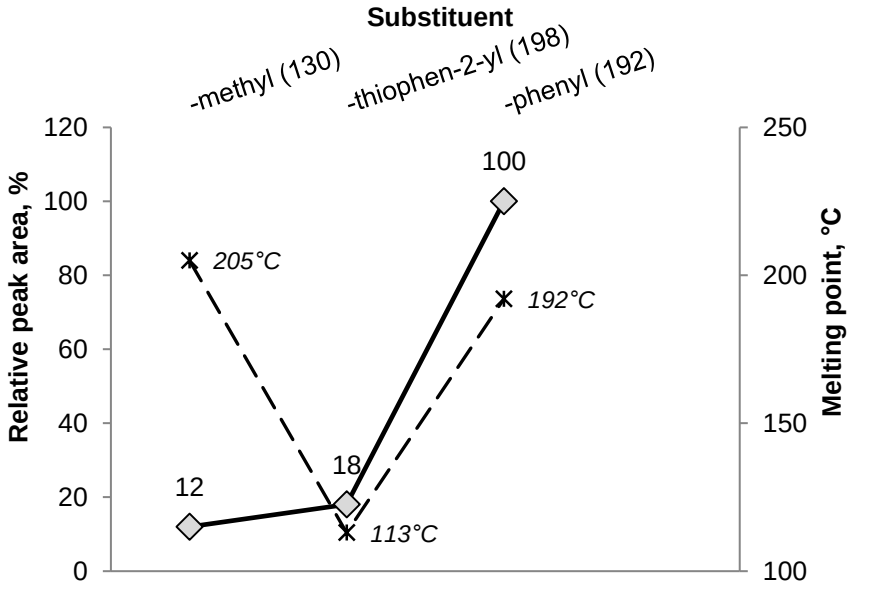


Fig. 4. The relationship between the nature of substituent at C-5 of the triazole ring and relative response (XRR group)

Mass spectrometry was used for the identification of the compounds; the latter were ionized using electron ionization (EI) mode at 70 eV. This is considered a hard ionization technique causing high fragmentation, which is suitable for the identification of specific functional groups and fragments in the molecule. The detachment of thiol group from the molecular ion as it had been shown in previous studies [14; 15] was not observed.

All compounds of RRX group were positively identified by their molecular ion peaks on the corresponding mass spectra. The same was true for compounds within XRR group, which, at the same time, exhibited the highest stability under ionization: molecular ion peaks were also basic in the respective spectra. Compound **1c** of RRX group exhibited the least stability under the ionization. High fragmentation of **1c** may be explained, firstly, by the detachment of morpholinium cation from the acidic residue as early as at the stage of injection (evaporation). Secondly, due to charge distribution, the acidic residue is unstable, and carboxyl group is easily eliminated from the anion.

As for chromatographic behavior of the compounds, the first thing to consider is retention times of the analytes. It is worth noting that stationary phase of the GC column was non-polar, and that is why components that had

aliphatic substituents were retained in the column for longer time, which is confirmed by the investigation of RRX group. In this regard, compound **1b** was the most retained component within this group, although its melting point was considerably lower than that of **1a**, the least retained analyte. In addition, despite the anion of **1c** had the highest molecular weight and T_{mp}, it eluted from the column twice earlier than **1b**; it indicates that **1c** is highly polar. As for XRR group, low retention time of the methyl derivative **2a** might have been affected by higher polarity and lower molecular weight, as compared to phenyl- and thiophen-2-yl derivatives **1a** and **3a**, respectively.

The second most important parameter in chromatographic analysis is peak area (response). For convenience, relative peak areas (to standard compound) were calculated. The highest responses within RRX group belong to **1b** and **1a** ((thio)alkyl and thiol, respectively); they were the least polar. The lowest response within the XRR group belongs to the compound that has methyl substituent attached to C-5 of triazole ring (**2a**); thiophen-2-yl derivative (**3a**) showed a slightly better result. In the case of methyl derivative, its high polarity and T_{mp} might have affected the response. Phenyl ring attached to triazole at C-5 stabilizes the molecule, possibly due to high aromaticity of the substituent [16]. Given the poor response of the highly polar analytes such as **1c** and **2a**, derivatization step may be required in order to enable their determination in complex sample matrices, including biological fluids, tissue samples, plant material, etc. Since the analytes represent various classes of compounds, there would be no uniform derivatization step that would suit every analyte. However, derivatization step would be based on modification of substituents connected through thiogroup. In this regard, both **1c**, through reduction with appropriate reagents, and **2a**, through alkylation directly, may be derived into (thio)alkyl, which would potentially have a largely better response.

Peak symmetry was calculated only for those analytes that had responses of at least 1/5 of peak area of standard compound **1a**. According to European Pharmacopoeia, acceptance range for peak symmetry lies between 0.8 and 1.5 [17]. Sensitivity significantly impacts the value of peak symmetry, and that is why peak symmetry was not calculated for peaks with low responses (less than 1/5 of the peak of standard compound **1a**). Standard compound **1a** had the best peak symmetry. Peak symmetries of all other analytes did not fit the acceptance range. In this way, compounds **1b** and **1d** showed slight broadening of the left part of the peak: their symmetries were above 1.5.

The obtained data will be useful in the development of chromatographic techniques within the field of analytical chemistry and provide new information on certain physicochemical properties of triazoles. However, apart from that, the results provide insights into some of the pharmacological properties of the potential drugs. The evaluation of bioavailability is an important step in drug design. This parameter is comprised of such properties as molecular shape and size, solubility, lipophilicity, ionization, presence of hydrogen bonds, polarity, and more [18]. Lipophilicity and solubility of the bioactive compound are considered by far the most important among the mentioned properties. Lipophilicity (log*P*) is a measure of affinity of the substance to non-polar solvents and lipids, which is defined as the logarithm of the relation of the concentration of the substance in octane to the concentration of this substance in water. Scientists have described several ways of lipophilicity determination using chromatographic methods. For instance, HPLC technique for the determination of lipophilicity of cytotoxic complexes has been developed and validated [19]. At the same time, HPLC has been shown to be effective in the evaluation of the lipophilicity of 1,2,4-triazole-3-thiones [20]. Thin layer chromatography has proven its efficiency in lipophilicity assessment [21]. In particular, it has been used in calculation of log*P* for anti-inflammatory drugs, specifically ibuprofen and its analogues [22]. Many lipophilic compounds may be successfully identified using gas chromatography, including both plants phytochemicals [23] and highly lipophilic medicinal products [24]. However, due to the absence of liquid-liquid partition mechanisms in gas chromatography process, this method has not found applications for lipophilicity determination.

On the other hand, compound solubility, which is connected to lipophilicity in a certain way, often depends on the polarity of the molecule and presence of specific groups. Poor solubility of medicinal products in biological liquids, primarily in saliva and gastric environment, is one of the major problems in drug design [25]. Particularly, polarity is an important parameter that affects the formation of hydrogen bonds, which is closely connected to permeability of chemicals through biological membranes [26]. Ability to form

hydrogen bonds is established by the presence of donor and acceptor groups that possess or may receive a proton [18]. In the experiment, assuming the chromatographic behavior, compounds **1c** (salt) within RRX and **2a** (5-methyl) within XRR group turned out to be the most polar in their groups. The first compound contains ionized carboxyl group stabilized by morpholinium cation. Although it has high polarity, such a combination most probably decreases the ability to form hydrogen bonds to the optimal level. The second compound has high polarity due to electroneutrality of methyl group at C-5 and comparatively high electronegativity of the groups at two other positions of triazole (C-3 and N-5). Amino group at N-5 of **2a** may behave as a donor and open atoms of nitrogen as acceptors of protons in the formation of hydrogen bonds.

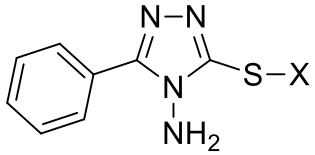
Experimental

New derivatives of 1,2,4-triazole were obtained at the Department of Natural Sciences for International Students and Toxicological Chemistry of Zaporizhzhia State Medical University. Two groups of compounds were formed to establish the experimental relationship between the structure and behavior. Groups were labeled as "RRX" and "XRR" (by analogy with the spatial arrangement of functional groups in the molecules), where R designates permanent groups, X – a certain substituent. RRX group incorporates mercaptoderivatives (3-thio-derivatives of 1,2,4-triazole), XRR comprises C-5 derivatives of 1,2,4-triazole. The list of all compounds, as well as properties that were used for the interpretation of the chromatographic behavior under GC-MS conditions, is given in Tables 3, 4.

Table 3

The compounds of the RRX group

Basic structure:



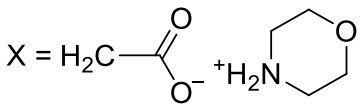
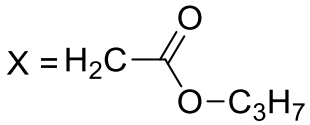
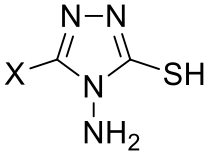
Label	Substituent	Data & Properties
1a	X = H	Name: 4-amino-5-phenyl-4*H*-1,2,4-triazol-3-thiol Gross formula: $C_8H_8N_4S$ Molecular weight: 192.24 T_{mp}: 191-193°C
1b	X = C_7H_{15}	Name: 3-(heptylthio)-5-phenyl-4*H*-1,2,4-triazol-4-amine Gross formula: $C_{15}H_{22}N_4S$ Molecular weight: 290.43 T_{mp}: 136-138°C
1c		Name: morpholin-4-ium 2-((4-amino-5-phenyl-4*H*-1,2,4-triazol-3-yl)thio)acetate Gross formula: $C_{14}H_{19}N_5O_3S$ Molecular weight: 337.4 T_{mp}: 217-219°C
1d		Name: propyl 2-((4-amino-5-phenyl-4*H*-1,2,4-triazol-3-yl)thio)acetate Gross formula: $C_{13}H_{16}N_4O_2S$ Molecular weight: 292.36 T_{mp}: 172-174°C

Table 4

The compounds of the XRR group

Basic structure:



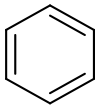
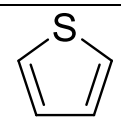
Label	Substituent	Data & Properties
1a	X = 	Name: 4-amino-5-phenyl-4*H*-1,2,4-triazol-3-thiol Gross formula: $C_8H_8N_4S$ Molecular weight: 192.24 T_{mp}: 191-193°C

Table 4

2a	X = CH_3	Name: 4-amino-5-methyl-4*H*-1,2,4-triazol-3-thiol Gross formula: $C_3H_6N_4S$ Molecular weight: 130.17 T_{mp}: 203-206°C
3a	X = 	Name: 4-amino-5-(thiophen-2-yl)-4*H*-1,2,4-triazol-3-thiol Gross formula: $C_6H_6N_4S_2$ Molecular weight: 198.27 T_{mp}: 112-114°C

Compound **1a** was chosen as standard (marked as [ST] in Tables 1, 2); retention times, peak areas and symmetries were compared to these parameters of the standard compound. Relative peak areas and symmetries were calculated in relation to standard compound **1a**, in %. Compounds were identified by their mass spectra. Chromatographic behavior of the compounds was described based on their retention times, relative peak areas and symmetries. In addition, molecular weight, melting point, and presence of certain substituents were assumed during the evaluation of the behavior. Mass spectrometric characterization of the molecules was conducted using the corresponding mass spectra and their comparison with molecular weights and structures of the analytes.

Once the groups were formed, a solution of each analyte was prepared. To prepare the solutions, 1 mg of a given analyte was weighed and dissolved in 1.5 mL of methanol (GC-MS grade). The following system was used for GC-MS analysis: Agilent 7890B (USA) GC system fitted with Gerstel CIS 4 (Germany) cooled injection system and coupled with Agilent 5977B (USA) mass selective detector. Compounds were separated on J&W Agilent DB-5ms (USA) column. Parameters of GC separation were selected and optimized based on Agilent's method development recommendations, expected behavior of the compounds, and system parameters limitations for the MSD [27] and were as follows. Injector temperature: programmed, 230°C → 12°C/s → 270°C; oven temperature: programmed, 80°C (hold for 1 min) → 40°C/min → 240°C (hold for 0.5 min) → 10°C/min → 280°C (hold for 0.5 min) → 5°C/min → 300°C (hold for 1 min) → 2°C/min → 310°C; total run time: 20 minutes; injection volume: 0.5 μL at split ratio of 1:30. Helium was used as the carrier gas with a flow rate of 1.6 mL/min. Column parameters: stationary phase: poly[95%-methyl][5%-phenyl]siloxane (non-polar); dimensions: 30 m × 250 μm × 0.25 μm. MSD parameters were as follows. GC-MS interface temperature: 290°C; ion source: 230°C; quadrupole: 150°C; ionization mode: electron ionization at 70 eV; scanned range of mass-to-charge ratio: 40-500 *m/z*. The obtained chromatograms and mass spectra were processed using Agilent MassHunter for Qualitative Analysis software.

Conclusions

The obtained data suggests that the most GC suitable behavior belonged to the standard compound 1a, which showed low retention time (under 10 min), high response, acceptable peak symmetry, as well as molecular stability under hard ionization. This compound contains phenyl ring attached to C-5 of triazole, amino group at N-4, and thiol group at C-3. The introduction of a non-polar substituent (alkyl) through the thiol group leads to the increase of retention time, while response and peak shape remain largely unaffected. Conversely, the introduction of polar substituents through thiol group causes significant decrease in response and, thus, in sensitivity. On the other hand, the substitution of the aromatic group with a less aromatic one leads to the destabilization and polarization of the molecule, which is observed through a considerably decreased response. The response of the analytes that had comparatively low peak areas may be improved by adjusting chromatographic parameters as well as implementing a derivatization step prior to analysis. In this way, increasing injection volume, injector temperature, decreasing split ratio, and using pulsed injection technique could improve the response of the analytes. Apart from that, the obtained experimental data provides some valuable insights into permeability of the new triazole derivatives through biological membranes, which could be useful in drug design.

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