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Posted Date: 23 April 2025

doi: 10.20944/preprints202504.1975.v1

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 doi:10.20944/preprints202504.1975.v1

Article

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Margarita A. Tokareva [1,2,*], Evgeny S. Melnikov [1,2], Elizaveta N. Fisher [1,3], Tatiana A. Rodina [2], Igor E. Shohin [4,5] and Maria V. Belova [1,6]

[1] A. P. Nelyubin Institute of Pharmacy. I. M. Sechenov First MSMU of the Ministry of Health of the Russian Federation (Sechenov University), 8/2, Trubetskaya str., Moscow, 119991, Russia

[2] Clinical Hospital. I. V. Davidovsky Department of Health of the city of Moscow, 11, Yauzskaya str. Moscow, 119027, Russia

[3] LLC " Laboratory of Pharmaceutical Research" (LLC "LPhR"), 20, building. 3, Nauchny Drive str, Moscow, 117246, Russia

[4] LLC "Center of Pharmaceutical Analytics" (LLC "CPHA"). 8, Simferopol Boulevard, Moscow, 117149, Russia

[5] National Research Nuclear University MEPhI (Moscow Engineering Physics Institute). 31, Kashirskoe highway, Moscow, 115409, Russia

[6] N.V. Sklifosovsky Research Institute for Emergency Medicine, 3, Bolshaya Sukharevskaya Sq., Moscow, 129090, Russia

* Correspondence: tokareva_m_a@student.sechenov.ru

Abstract: The application of HPLC-MS/MS in the analysis of peptide therapeutics demonstrates its capacity to achieve high sensitivity and selectivity, which are essential qualities for the expanding peptide therapeutic industry. Given the challenges posed by hydrophilic peptides in reversed-phase chromatography, we investigated the necessity of a derivatization procedure to improve chromatographic separation and quasimolecular ion fragmentation during MS/MS detection. We investigated how eight different derivatizing agents react with a hydrophilic lysine- and arginine-containing tetradecapeptide to identify the most suitable one. The findings revealed propionic anhydride as the most effective derivatizing agent, enabling high and reproducible product yield that demonstrate sufficient retention on RP column and easily detectable in MRM mode.

Keywords: peptide; HPLC-MS/MS; derivatization; optimization of chromatographic systems

1. Introduction

The study of peptides is currently at the cutting edge of research interest. The scientific community is primarily involved in two distinct areas of investigation: proteomics and peptide drug discovery.

Proteomic research aims to study the structure, functions and interactions of proteins in biological systems and their role in cellular processes. Thus, the research is relevant to both fundamental and applied research, such as clinical trials [1]. Peptide drug discovery focuses on improving the efficacy of the peptides used, for example, by searching for alternative routes of administration [2], increasing membrane permeability, oral bioavailability and *in vivo* stability [3].

In both proteomics and peptide drug discovery, there is a need to identify and quantify peptides, for which there are various techniques, including immunological methods, such as radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), and mass-spectrometry (MS) methods [4]. The latter, especially high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS), have found the greatest application [5,6].

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Proteomics has two main approaches: top-down and bottom-up [7]. In top-down proteomics, whole proteins are analyzed by matrix-assisted laser desorption ionization source (MALDI)-MS/MS to identify and characterize, whereas in bottom-up proteomics, enzymatic hydrolysis is performed. The resulting peptides are then separated and analyzed by HPLC-MS/MS [8].

For peptide therapeutics, HPLC-MS/MS is a powerful technique with applications in many areas, starting with peptide characterization [6] and quality control [9], followed by investigation in clinical trials [10,11]. Quantification for pharmacokinetic studies is of special concern because therapeutic peptides are known for their high potency at low doses[12], which makes analysis difficult due to low concentrations in biological samples, such as blood [13]. Although HPLC-MS/MS can be considered as highly sensitive and selective method [14], additional difficulties may occur.

A peptide is a chain of up to 40 amino acids that vary in structure and properties [12,15]. Among the variety of amino acids, lysine and arginine are of particular interest. Lysine- and arginine-containing peptides have great potential as cell-penetrating peptides (CPPs) for delivery vectors [16]. In addition, peptides enriched with these amino acids tend to exhibit antimicrobial properties [17,18]. Such peptides show high hydrophilicity, which can be a challenge during chromatographic separation in reversed-phase (RP) HPLC. In addition, the fragmentation of such peptides can be difficult due to the presence of numerous basic groups that require increased collision energy [19]. For some of these peptides, hydrophilic interaction liquid chromatography (HILIC) is an option [20,21]. However, it does not improve the fragmentation properties. In such a case, derivatization is often used to solve both problems [10,22,23].

Derivatization-based HPLC-MS is highly effective tool to solve many analytical problems and greatly expand the applications of HPLC-MS in various research areas [23,24]. Derivatization methods are widely used for quantitative proteomics analysis, such as isotope-coded affinity tag (ICAT) or isotope-coded protein label (ICPL), isobaric tag for relative and absolute quantitation (iTRAQ), and tandem mass tag (TMT) [22]. There are multiple derivatization methods for the analysis of active compounds in peptide drug researches [25–28]. Therefore, derivatization-based HPLC-MS has become an important tool for peptide analysis [23].

Despite the need for additional reaction steps, the presence of interference caused by excess reagents and byproducts, and the need to account for additional matrix effects, derivatization have been considered by many scientists as a last resort to overcome detection and occasional separation problems [29].

The determination of lysine and arginine rich peptides in biological matrices by RP-HPLC-MS is quite challenging. It is difficult to achieve optimal conditions for separation. In addition, the choice of fragmentation conditions can be rather energy and time consuming. Therefore, derivatization process can be a desirable way for analytical scientists, although there is still appropriate choice of derivatization reagent is required, which can be time-consuming.

The aim of the present study is to provide a step-by-step derivatizing agent selection for hydrophilic lysine- and arginine-containing peptide - threonyl-glutamyl-lysyl-lysyl-arginyl-arginyl-glutamayl-threonyl-valyl-glutamyl-arginyl-glutamyl-lysyl-glutamate - for further analysis by HPLC-MS/MS in human plasma.

2. Results and Disscussion

2.1. Peptide Therapeutic to Study

The subject is a tetradecapeptide (TDP) consisting of 14 consecutive amino acids: threonyl-glutamyl-lysyl-lysyl-arginyl-arginyl-glutamayl-threonyl-valyl-glutamyl-arginyl-glutamyl-lysyl-glutamate, that is supposed to have immunomodulator and antiviral effect [30]. The molecule shows pronounced basic properties due to the presence of seven basic centers.

In order to study the pharmacokinetics of the drug for different routes of administration, it became necessary to develop a method that would be highly sensitive and selective for the determination of the peptide.

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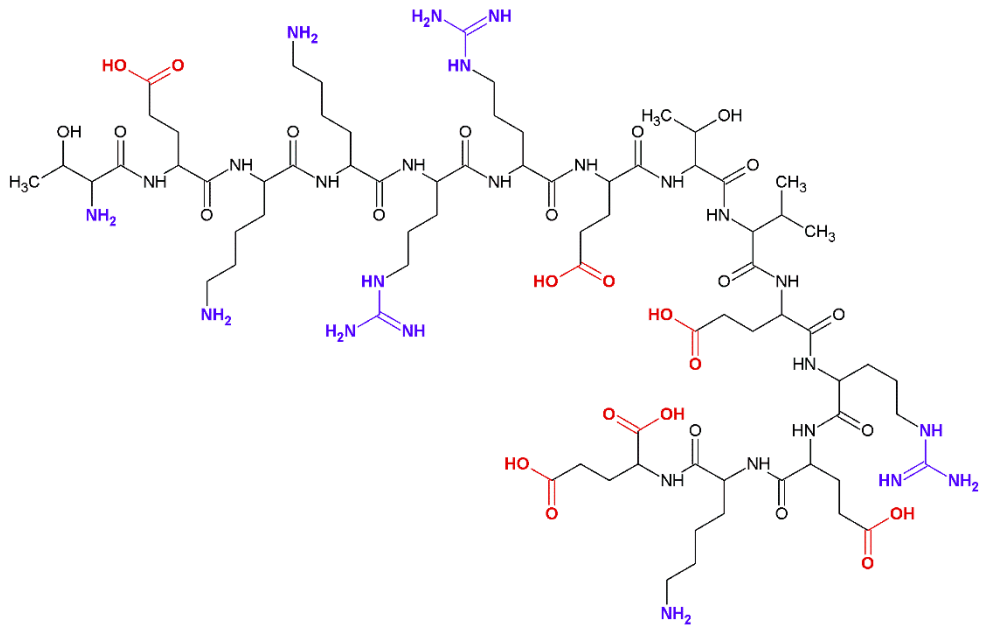

Figure 1. Tetradecapeptide molecule, acidic sites are highlighted in red and basic sites are highlighted in blue.

2.2. Mass-Spectrum

Based on the calculated monomolecular mass of TDP, which is 1817.0 Da [31], the m/z values for single- and multicharged quasimolecular ions that can be detected in the mass spectrum of the total ion current during electrospray ionization in the positive mode were predicted (Table 1). The computed values matched the experimental values, but there were some deviations. These can be explained by the limited resolution of the detector (Figure A1). No single charged ions were detected, probably due to the seven basic amino acid residues of TDP, which favor multicharged cations. This can be beneficial because, according to Dongre et al [19], multi-protonated peptides fragment more easily than the single-protonated forms of the same peptides.

Table 1. The calculated and obtained mass-spectrum values of the tetradecapeptide (TDP), which can be detected in scan mode.

n (H+)	m/z calculated	m/z obtained
1	1818,0	-
2	909,5	909,6
3	606,7	607,1
4	455,3	455,5
5	364,4	364,6

To establish the conditions for TDP detection in the multiple reaction monitoring (MRM) mode, we analyzed the fragmentation mass spectra of three precursor ions (m/z: 607.1; 455.5, 909.6). However, the reliable fragmentation data was not observed regardless of the collision energy applied and the argon pressure inside the cell. It is known, that lysine and arginine rich peptides at a given collision energy undergo fragmentation less easily than similar protonated peptides without a basic residue [19].

As a result, it proved challenging to identify the most suitable conditions for mass spectrometry (MS) detection of TDP in its native form, due to the considerable energy required for the hydrophilic lysine- and arginine-containing peptide fragmentation. Furthermore, it was observed that TDP studied had a relatively low affinity for reversed-phase sorbents due to its high hydrophilicity. The potential of peptide derivatization was considered to overcome these challenges.

 doi:10.20944/preprints202504.1975.v1

2.3. Derivatizing Agent Selection

There are many basic amine groups in TDP, three lysine and three arginine residues and no N-terminal modification for threonyl so we focused on finding amine derivatization agents. Furthermore, it was considered that derivatization can reduce the gas-phase basicity of the peptide, which was expected to reduce the energy required for fragmentation by collision-induced dissociation (CID) [19].

After a comprehensive analysis of the available literature on peptide derivatization reagents, the following candidates were selected for further investigation:

1. Dimethylaminonapthalene-5-sulfonyl chloride (dansyl chloride) [32];
2. 4-Toluenesulfonyl chloride (tosyl chloride) [33];
3. Phenylisothiocyanate (PITC) [34,35];
4. N-(9H-fluorene-2-ylmethoxy)succinimide (Fmoc-OSu) [36–38];
5. Di-tert-butyl dicarbonate (Boc anhydride) [39];
6. N-(Benzyloxycarbonyloxy)succinimide (Cbz-OSu) [40];
7. Benzoic anhydride [41];
8. Propionic anhydride [36,42,43]

All of the substances considered are capable of interacting with the amino groups of TDP under certain conditions [35]. It was postulated that at pH less than 10, modification of the N-terminal amino group and the ε-amino groups of lysine residues is the most possible outcome [43]. Given the high basicity of guanidine fragments, it was deemed unlikely that arginine residues would interact with derivatizing reagents [43,44]. These assumptions were subsequently confirmed by experiments in which no cases of more than quadruple substituted derivatives were observed. Three arginine residues did not appear to be derivatized under the postulated conditions.

The interaction of TDP with dansyl chloride, tosyl chloride, and PITC reagent was conducted in a carbonate buffer at pH 9 in a mixed solvent (water-acetonitrile) with heating in an ultrasonic bath [33,35,45,46].

The use of dansyl chloride in the reaction mixture resulted in the detection of 3- and 4-substituted peptide derivatives, whose observed mass spectra matched with the theoretically calculated values (Table 2A, Figure A2). However, as with the native peptide, the derivatized molecular ions had insufficient fragmentation to obtain informative mass spectra; therefore, it was decided not to perform further derivatization with dansyl chloride.

Similar trends were observed when working with tosyl chloride (Table 2A, Figure A3), although minor fragmentation of quasi-molecular ions was observed for the tosylated derivatives. Furthermore, MRM transitions were developed (570.95->84.00; 570.95->91.00; 570.95->101.90; 570.95->154.90; 570.95->237.90; 760.95->83.90; 760.95->102.00; 760.95->154.90; 760.95->238.00). However, we did not find convenient conditions to control the yield of 3- and 4-tosylated derivatives. As a result, the process of tosylating TDP was found to be inefficient and too challenging to control, and the search for a superior derivatizing reagent was continued.

The interaction of TDP with PITC showed characteristics and complications analogous to those observed with dansyl chloride (Table 2A, Figure A4). Consequently, further investigation with PITC derivatives was considered inadvisable.

The interaction of TDP with Fmoc-OSu, Boc anhydride, and Cbz-OSu was conducted in triethylamine (pH = 9) in the dark for 30 minutes. The derivatives of Fmoc-OSu, Boc anhydride, and Cbz-OSu were identified as 1-, 2-, 3-, and 4-substituted compounds (Table 2A, Figure A5, Figure A6, Figure A7). The 4-charged ions of the 4-substituted derivatives were fragmented most efficiently, and MRM were developed for two of them (Boc anhydride: 740.30->57.05; 740.30->84.05; 740.30->129.00; 740.30->606.90; Cbz-Osu: 785.40->91.05; 1178.00->1504.40). However, the detector signal intensity was reasonable only at relatively high TDP concentrations. As with the previously discussed reagents, the optimal reaction conditions for the highest yield of the target 4-substituted derivatives were not identified.

 doi:10.20944/preprints202504.1975.v1

Derivatization with benzoic anhydride was conducted at room temperature overnight. Among the reaction products, 2-, 3-, and 4-substituted peptide derivatives were identified, but their quasimolecular ions did not appear to undergo fragmentation in the collision cell. (Table 2A, Figure A8).

In the experiment with propionic anhydride, TDP predominantly yielded 4-substituted derivatives. The ratio of reaction products was easily controlled by adjusting the amount of propionic anhydride added to the reaction mixture, the reaction temperature, and the reaction time. There were no detectable MRM of derivatization products after conducting the reaction at room temperature, but it yielded 4-substituted derivatized peptide after 30 min 50 °C ultrasonic bath in acidic condition. The mass spectrum for the substance was found to correspond with the calculated one (Table 2A, Figure A9); moreover, the fragmentation of multi-charge ions of the 4-propionylated derivative was observed(681.30->73.95; 681.30->84.00; 681.30->101.90; 681.30->140.10), which made it possible to select and optimize detection conditions in the MRM mode. Consequently, derivatization of TDP with propionic anhydride was determined to be the most efficient and was used in subsequent investigations.

Table 2. Derivatizing agents, reaction conditions and method development progress.

Derivatizing agent	Reaction condition	Derivative product mass spectra	Informative product ion scan	MRM developed	Controlled yield reaction	Applied in plasma	Achieved high sensetivity
Dansyl chloride	50°C for 30 minutes in a carbonate buffer (pH 9) with a mixed solvent (water-acetonitrile, 1:1)	+	-	-	-	-	-
Tosyl chloride		+	+	+	-	-	-
PITC		+	-	-	-	-	-
Fmoc-OSu	room temperature in the dark for 30 minutes in triethylamine at pH 9	+	+	+	-	-	-
Boc anhydride		+	+	+	-	-	-
Cbz-OSu		+	+	+	-	-	-
Benzoic anhydride	room temperature overnight	+	+	-	+	-	-
Propionic anhydride	50°C for 30 minutes in a propionic acid solution	+	+	+	+	+	+

In the next phase of the research, different methods for the preparation of blood plasma samples were evaluated, with simultaneous selection of optimal conditions for TDP derivatization with propionic anhydride.

2.4. Sample Preparation Selection

Historically, three different sample purification methods have been used to prepare blood plasma samples. These include solid-phase extraction, liquid-liquid extraction, and plasma protein precipitation, as well as combinations and modifications of these methods [47,48]. In terms of

 doi:10.20944/preprints202504.1975.v1

complexity, protein precipitation is considered the simplest method, followed by liquid-liquid extraction and then solid-phase extraction. In order to obtain a more economical technique, particular attention has been paid to the protein precipitation method.

The three basic methods of blood plasma protein precipitation were studied using acetonitrile, methanol, and a 50% solution of trifluoroacetic acid (TFA) in water as precipitants. This was done in conjunction with propionylation of TDP performed directly in blood plasma or in the supernatant after protein precipitation. The schematics of the sample preparation procedures are presented below.

Table 3. A sample preparation scheme for three different precipitators, with derivatization conducted in either plasma or supernatant.

Stage	Precipitant		
	Methanol	**Acetonitrile**	**50% solution of TFA in water**
Derivatization in plasma			
Preparation of the test sample	10 µl of 1 TDP stock solution + 190 µl of intact blood plasma		20 µl of 1 TDP stock solution + 380 µl of intact blood plasma
Stirring	Vortex shaker, 10 seconds		
Derivatization	50 µl propionic anhydride→ stirring on a Vortex shaker, 10 seconds → ultrasonic bath, 50°C, 30 minutes		
Addition of precipitant	600 µl	600 µl	200 µl
Stirring	Vortex shaker, 10 seconds		
Centrifugation	15 min with relative centrifugal acceleration 15294 g		
Derivatization in the supernatant after protein precipitation			
Preparation of the test sample	10 µl of 1 TDP stock solution + 190 µl of intact blood plasma		20 µl of 1 TDP stock solution + 380 µl of intact blood plasma
Stirring	Vortex shaker, 10 seconds		
Addition of precipitant	600 µl	600 µl	200 µl
Stirring	Vortex shaker, 10 seconds		
Centrifugation	15 min with relative centrifugal acceleration 15294 g		
Derivatization	50 µl propionic anhydride→ stirring on a Vortex shaker, 10 seconds → ultrasonic bath, 50°C, 30 minutes		

In addition, parallel assays with blank blood plasma were performed to confirm the specificity of the technique. For this purpose, the same procedures were performed sequentially with 200 µl of intact blood plasma, without the addition of TDP and IS.

The experiment resulted in the detection of a 4-propionylated TDP peak in all samples where derivatization was performed in blood plasma. No peak corresponding to the test substance was observed in the chromatograms of samples derivatized in the supernatant. This finding suggests that the native TDP has a low extraction rate with acetonitrile and methanol. Consequently, it can be hypothesised that TDP is co-precipitating with plasma proteins. However, further research is required to verify this hypothesis. In the case of precipitation with 50% TFA solution, there was no propionylation of TDP, likely due to the presence of a strong acid. Therefore, derivatization in the supernatant after protein precipitation with TFA as a precipitant is deemed an inappropriate procedure.

Derivatization of TDP in blood plasma using methanol as a precipitant yielded the strongest signal intensity. However, when samples with different concentrations were analyzed, no linearity was observed in the results obtained for the target 4-propionylated derivative. Therefore, we assumed a side reaction to occur and analyzed the sample in total ion current. The analysis revealed the presence of a substance with a molecular mass of approximately 2056 Da (m/z 514.9, 686.4, 1029.0), which had a later retention time than the target compound (2.55 min for the by-product and 2.42 min for the target product). It was hypothesized that elevated temperatures within a water-methanol

 doi:10.20944/preprints202504.1975.v1

solution would induce a side reaction with acidic residues of TDP. This hypothesis was supported by the observation that the mass spectrum showed correspondence with calculated mass spectrum of methylated derivative of 4-propionylated TDP.

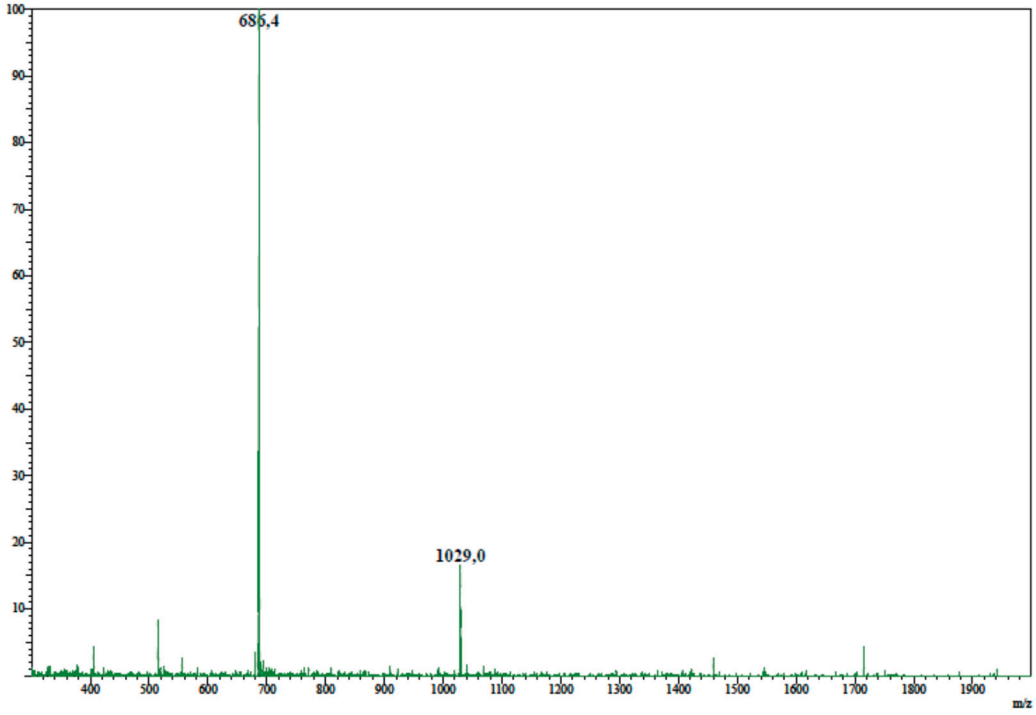

Figure 2. Mass spectrum for MeOH byproduct of propionylated TDP.

On the chromatogram of the byproduct three poorly separated peaks were detected. It can be explained, that esterification of carboxylic groups by methanol may occur on different side chains of the TDP [49]. Therefore, future research was directed to prevent the formation of methylated byproducts during derivatization and to increase the sensitivity of the technique by concentrating the sample. We decided to use methanol for precipitation, but evaporate the extracted supernatant with non-derivatized TDP for further derivatization with acetonitrile, which excludes methylated byproducts.

It was founded that TDP recovery was higher in methanol acidified with 4.76% propionic acid. After complex investigation, we achieved a linearity range of 5.00-1000.00 ng/ml and validated the method for quantitative determination in human plasma by HPLC-MS/MS by the following parameters: selectivity, matrix effect, calibration curve, accuracy and precision, recovery, lower limit of quantification, sample carry-over, stability [50].

3. Materials and Methods

3.1. Chemicals and Reagents

Next reagents were used during investigation: acetonitrile (ACN) (LC-MS grade, Biosolve, France), formic acid (98%, PanReac, Spain), chloroform (99,9%, Honeywell, Germany), propionic acid (99,84%, BASF, Germany), propionic anhydride (>99,8%, Sigma-Aldrich, USA), dimethylaminonapthalene-5-sulfonyl chloride (dansyl chloride, >98%, Sigma-Aldrich, USA), phenylisothiocyanate (PITC, 98%, Sigma-Aldrich, USA), 4-toluenesulfonyl chloride (tosyl chloride, >98%, Merck KGaA), benzoic anhydride (98%, Acros Organics, Belgium); methanol (MeOH, Chemically Pure), sodium bicarbonate (Reagent grade), sodium carbonate (Reagent grade) were purchased from Chimmed Group, Russia; N-(9H-fluorene-2-ylmethoxy)succinimide (Fmoc-Osu,

 doi:10.20944/preprints202504.1975.v1

>98), di-tert-butyl dicarbonate (Boc anhydride, >95%), N-(Benzyloxycarbonyloxy)succinimide (Cbz-Osu, >98%, TCI, Japan), triethylamine (TEA, >99%) were obtained from TCI, Japan. Ultrapure water was prepared with a Milli-Q water purification system (Millipore, France).

3.2. Apparatus and HPLC Condition

Chromatographic separation and detection were performed using a Nexera high-performance liquid chromatograph with tandem mass spectrometric detector LCMS-8040 (triple quadrupole, Shimazu, Japan), equipped with a gradient pump LC-30AD (Shimazu, Japan), column and sample thermostat CTO-20AC (Shimazu, Japan), degasserDGU-20A$_{5R}$ (Shimazu, Japan), automatic sampler SIL-30AC (Shimazu, Japan). Lab Solutions software (Ver. 5.97), Shimadzu Corporation, Japan, was used for primary data processing.

The chromatography method was performed using Waters XBridge C18 Column, 5 µm, 4.6 mm X 50 mm. Gradient elution was performed using 0,1% formic acid in water (eluent A) and 0,1% formic acid in acetonitrile (eluent B), the flow rate was 1.0 mL/min for 7 min and, gradient is presented in table. Electospray ionization (ESI) interface was operated in positive mode, with the following set of operation parameters: capillary voltage, 5000 V; nebulizing gas flow, 5 L/min; desolvatation line temperature, 250 °C, heating block temperature 400 °C, drying gas flow, 20 L/min

Table 4. Mobile phase gradient.

Time, min	Eluent B, %
0,00 – 0,50	5
0,50 – 2,50	5 → 30
2,50 – 2,60	30 → 100
2,60 – 3,60	100
3,60 – 4,10	100 → 5
4,10 – 5,00	5

3.3. Preparation of Stock Solution and Working Solutions

The stock standard solution of TDP was prepared in ACN/Water 1:1 in concentration of 1 mg/mL by dissolving 2 mg of the drug substance in 2 mL ACN/Water 1:1, and stored at −20 °C, protected from light.

Work solutions of derivatizing agents (dansyl, tosyl chloride, PITC, Fmoc-Osu, boc anhydride, Cbz-Osu, benzoic anhydride) were prepared at the concentration of 1 mg/mL by dissolving the calculated amount of the reagent in acetonitrile.

Carbonate-bicarbonate buffer was prepared by mixing 850 mg of sodium carbonate with 840 mg of sodium bicarbonate followed by ultrapure water added up to 100 mL (0.08 M sodium carbonate and 0.1 M of sodium bicarbonate).

Work solution of TEA was prepared at the concentration of 1 mg/mL by dissolving the calculated amount of the reagent in acetonitrile.

3.4. Preparation of Analytical Samples

3.4.1. Preparation of Dansyl, Tosyl, and PITC Derivative Samples of TDP

To 20 µl of TDP stock solution 20 µl of carbonate-bicarbonate buffer was added, followed by the addition of 20 µl of 1 mg/mL work solution of the derivatizing agent (dansyl, tosyl or PITC) and 40 µl of water. Then the solution was stirred on a vortex shaker for 10 seconds, followed by ultrasonic bath with the 50°C temperature for 30 minutes. The mix was diluted by 300 µl of acetonitrile, stirred and transferred to chromatographic vial.

 doi:10.20944/preprints202504.1975.v1

3.4.2. Preparation of Fmoc-Osu, Boc Anhydride, Cbz-Osu Derivative Samples of TDP

In the dark environment to 20 µl of TDP stock solution 20 µl of 1% TEA was added, followed by the addition of 20 µl of 1 mg/mL work solution of the derivatizing agent (Fmoc-Osu, boc anhydride or Cbz-Osu) and 40 µl of water. Then the solution was stirred on a vortex shaker for 10 seconds, followed by standing in a dark place with the room temperature for 30 minutes. The mix was diluted by 300 µl of acetonitrile, stirred and transferred to chromatographic vials.

3.4.3. Preparation of Benzoic Anhydride Derivative Samples of TDP

To 20 µl of TDP stock solution 20 µl of of 1 mg/mL work solution of the benzoic anhydride was added, followed by the addition of 60 µl of acetonitrile. Then the solution was stirred on a vortex shaker for 10 seconds, followed by night-standing with the room temperature. The mix was diluted by 300 µl of acetonitrile, stirred and transferred to chromatographic vials.

3.4.4. Preparation of Propionic Anhydride Derivative Samples of TDP in Blood Plasma

To 190 µl of intacted blood plasma added 10 µl of ACN/Water 1:1 TDP solution, following stirring and adding 420 µl of 4.76% propionic acid solution in methanol, stirred on a vortex shaker for 10 seconds, followed by a 20-minute standing period, then centrifuged for 15 min at a relative centrifugal acceleration of 15294 g. The supernatant was then transferred to 2 ml microcentrifuge tubes. In order to remove the methanol, 800 µl of chloroform was added to the supernatant. Following stirring and centrifugation, approximately 250 µl of the aqueous-methanol phase (upper layer) was obtained, with the majority of the methanol retained in the lower layer. Methanol-water layer was dried in a nitrogen current at room temperature and then 60 µl of 16.67% propionic acid solution in water and 55 µl of 9.09% propionic anhydride solution in acetonitrile was added. The derivatization reaction was conducted at 50°C for 30 minutes. The excess propionic anhydride and acetonitrile were removed by evaporating the sample, after which the dry residue was redissolved in 100 µl of 1:1 acetonitrile-water mixture, stirred and transferred to chromatographic vials.

Author Contributions: Conceptualisation E.S.M., M.V.B; Devolopement and validation, E.S.M., M.A.T., E.N.F. and. T.A.R.; Resourseces and study organization, I.E.S, E.N.F. and M.V.B.; writing—review and editing, E.S.M., M.A.T. M.V.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ELISA	enzyme-linked immunosorbent assay
MS	mass spectrometry
HPLC-MS/MS	high performance liquid chromatography with tandem mass spectrometry
MALDI	matrix-assisted laser desorption ionization source
CPP	cell-penetrating peptide
RP	reversed-phase
HILIC	hydrophilic interaction liquid chromatography
ICAT	isotope-coded affinity tag
ICPL	isotope-coded protein label
iTRAQ	isobaric tag for relative and absolute quantitation
TMT	tandem mass tag
TDP	tetradecapeptide
MRM	multiple reaction monitoring

 doi:10.20944/preprints202504.1975.v1

MS	mass spectrometry
CID	collision-induced dissociation
dansyl chloride	dimethylaminonapthalene-5-sulfonyl chloride
tosyl chloride	4-Toluenesulfonyl chloride
PITC	phenylisothiocyanate
Fmoc-OSu	N-(9H-fluorene-2-ylmethoxy)succinimide
Boc anhydride	Di-tert-butyl dicarbonate
Cbz-OSu	N-(Benzyloxycarbonyloxy)succinimide
ESI	electrospray ionization
TFA	trifluoroacetic acid
ACN	acetonitrile

Appendix A

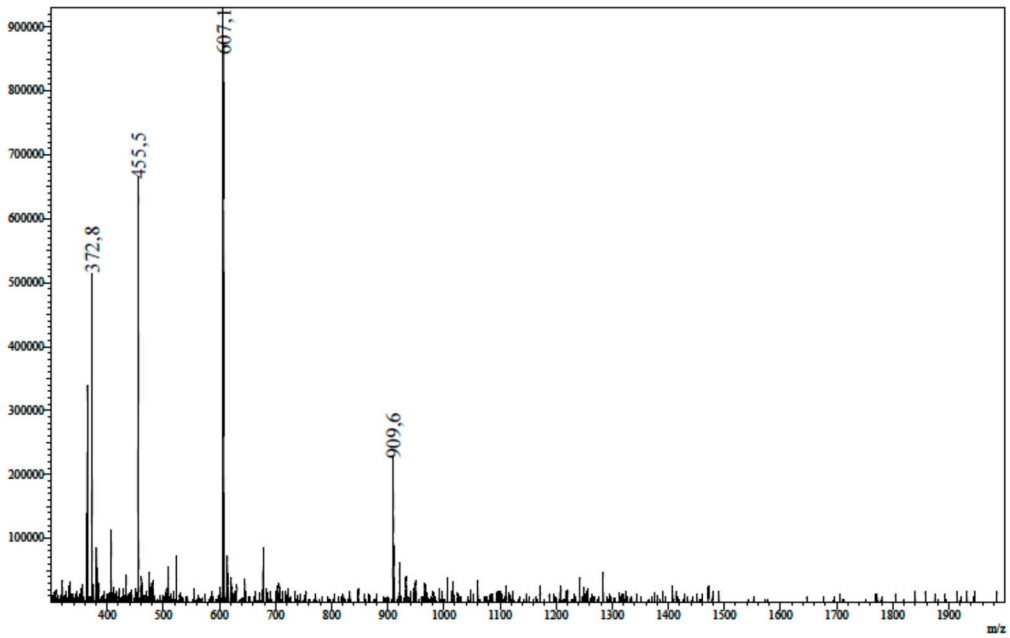

Figure A1. The mass spectrum of the native peptide under positive electrospray ionization.

Table A1. The calculated and observed m/z values for quasimolecular ions of the tetradecapeptide derivates, (green color marks founded in mass-spectrum m/z).

Dansyl group monoisotopic mass, Da						**234,1**	
Derivatized groups	**Charge**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1	2051,0	1026,0	684,3	513,5	411,0	342,7	293,9
2	2284,1	1142,5	762,0	571,8	457,6	381,5	327,2
3	2517,1	1259,1	839,7	630,0	504,2	420,4	360,5
4	2750,2	1375,6	917,4	688,3	550,8	459,2	393,7
5	2983,2	1492,1	995,1	746,6	597,5	498,0	427,0
6	3216,3	1608,6	1072,8	804,8	644,1	536,9	460,3
7	3449,3	1725,2	1150,4	863,1	690,7	575,7	493,6
Tosyl group monoisotopic mass, Da						**155,0**	
Derivatized groups	**Charge**						

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	1	2	3	4	5	6	7
1	1972,0	986,5	658,0	493,8	395,2	329,5	282,6
2	2126,0	1063,5	709,3	532,3	426,0	355,2	304,6
3	2280,0	1140,5	760,7	570,8	456,8	380,8	326,6
4	2434,0	1217,5	812,0	609,3	487,6	406,5	348,6
5	2588,0	1294,5	863,3	647,8	518,4	432,2	370,6
6	2742,0	1371,5	914,7	686,3	549,2	457,8	392,6
7	2896,0	1448,5	966,0	724,8	580,0	483,5	414,6
PITC group monoisotopic mass, Da						**135,0**	
Derivatized groups	**Charge**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1	1952,0	976,5	651,3	488,8	391,2	326,2	279,7
2	2086,0	1043,5	696,0	522,3	418,0	348,5	298,9
3	2220,0	1110,5	740,7	555,8	444,8	370,8	318,0
4	2354,0	1177,5	785,3	589,3	471,6	393,2	337,1
5	2488,0	1244,5	830,0	622,8	498,4	415,5	356,3
6	2622,0	1311,5	874,7	656,3	525,2	437,8	375,4
7	2756,0	1378,5	919,3	689,8	552,0	460,2	394,6
(fluoren-9-ylmethoxy)carbonyl (Fmoc) group monoisotopic mass, Da						**223,1**	
Derivatized groups	**Charge**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1	2040,0	1020,5	680,7	510,8	408,8	340,8	292,3
2	2262,1	1131,6	754,7	566,3	453,2	377,9	324,0
3	2484,2	1242,6	828,7	621,8	497,6	414,9	355,7
4	2706,2	1353,6	902,8	677,3	542,1	451,9	387,5
5	2928,3	1464,7	976,8	732,8	586,5	488,9	419,2
6	3150,4	1575,7	1050,8	788,4	630,9	525,9	450,9
7	3372,4	1686,7	1124,8	843,9	675,3	562,9	482,6
Trert-butoxycarbonyl (Boc) group monoisotopic mass, Da						**101,1**	
Derivatized groups	**Charge**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1	1918,0	959,5	640,0	480,3	384,4	320,5	274,9
2	2018,1	1009,5	673,4	505,3	404,4	337,2	289,2
3	2118,1	1059,6	706,7	530,3	424,4	353,9	303,5
4	2218,2	1109,6	740,1	555,3	444,4	370,5	317,7
5	2318,2	1159,6	773,4	580,3	464,5	387,2	332,0
6	2418,3	1209,6	806,8	605,3	484,5	403,9	346,3
7	2518,3	1259,7	840,1	630,3	504,5	420,6	360,6
Benzyloxycarbonyl (Cbz) group monoisotopic mass, Da						**135,0**	
Derivatized groups	**Charge**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1	1952,0	976,5	651,3	488,8	391,2	326,2	279,7
2	2086,0	1043,5	696,0	522,3	418,0	348,5	298,9
3	2220,1	1110,5	740,7	555,8	444,8	370,9	318,0
4	2354,1	1177,6	785,4	589,3	471,6	393,2	337,2
5	2488,2	1244,6	830,1	622,8	498,4	415,5	356,3

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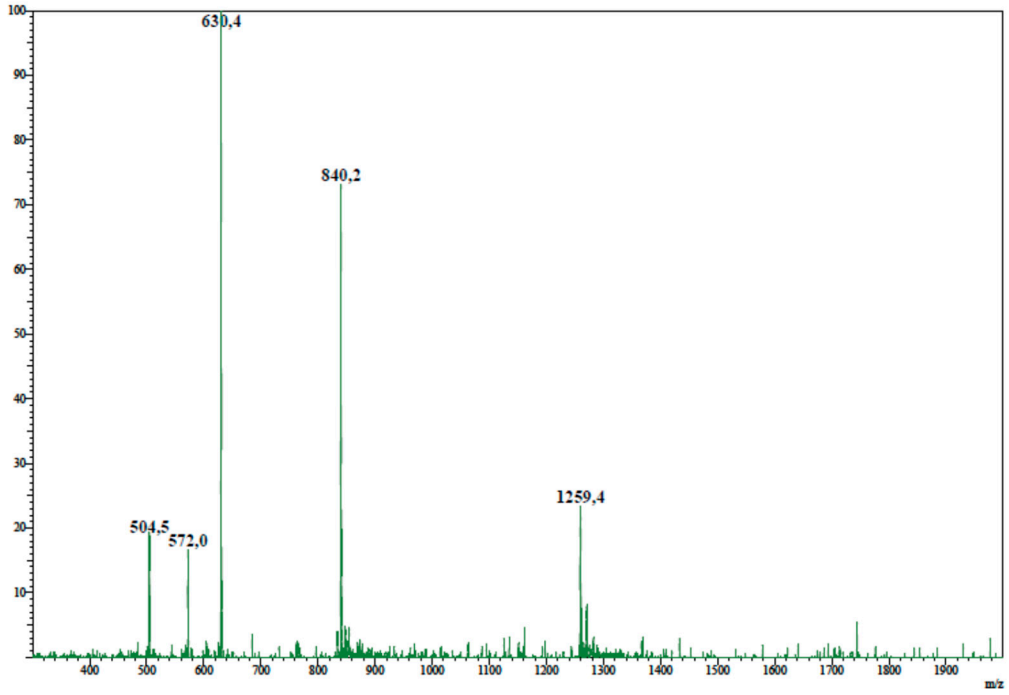
6	2622,2	1311,6	874,7	656,3	525,2	437,9	375,5
7	2756,2	1378,6	919,4	689,8	552,1	460,2	394,6
Benzoyl group (from benzoic anhydride) monoisotopic mass, Da						**105,0**	
Derivatized groups	**Charge**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1	1922,0	961,5	641,3	481,3	385,2	321,2	275,4
2	2026,0	1013,5	676,0	507,3	406,0	338,5	290,3
3	2130,1	1065,5	710,7	533,3	426,8	355,8	305,2
4	2234,1	1117,5	745,4	559,3	447,6	373,2	320,0
5	2338,1	1169,6	780,0	585,3	468,4	390,5	334,9
6	2442,1	1221,6	814,7	611,3	489,2	407,9	349,7
7	2546,2	1273,6	849,4	637,3	510,0	425,2	364,6
Propionyl group (from propionic anhydride) monoisotopic mass, Da						**57,0**	
Derivatized groups	**Charge**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1	1874,0	937,5	625,3	469,3	375,6	313,2	268,6
2	1930,0	965,5	644,0	483,3	386,8	322,5	276,6
3	1986,0	993,5	662,7	497,3	398,0	331,9	284,6
4	2042,1	1021,5	681,4	511,3	409,2	341,2	292,6
5	2098,1	1049,6	700,0	525,3	420,4	350,5	300,6
6	2154,1	1077,6	718,7	539,3	431,6	359,9	308,6
7	2210,2	1105,6	737,4	553,3	442,8	369,2	316,6



(a)

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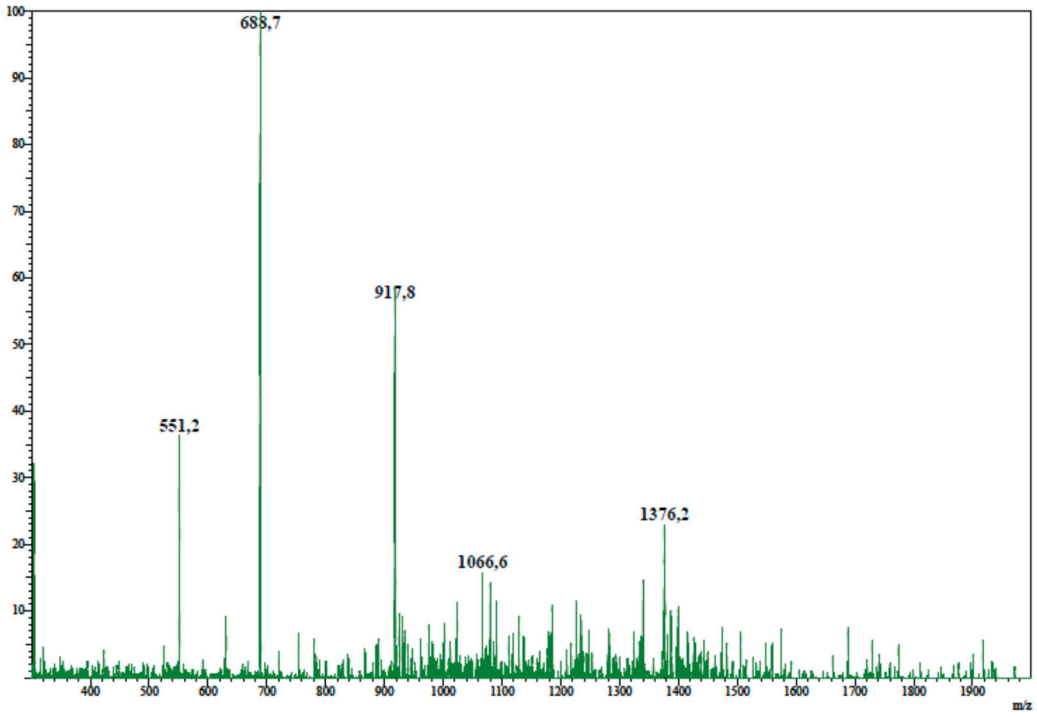
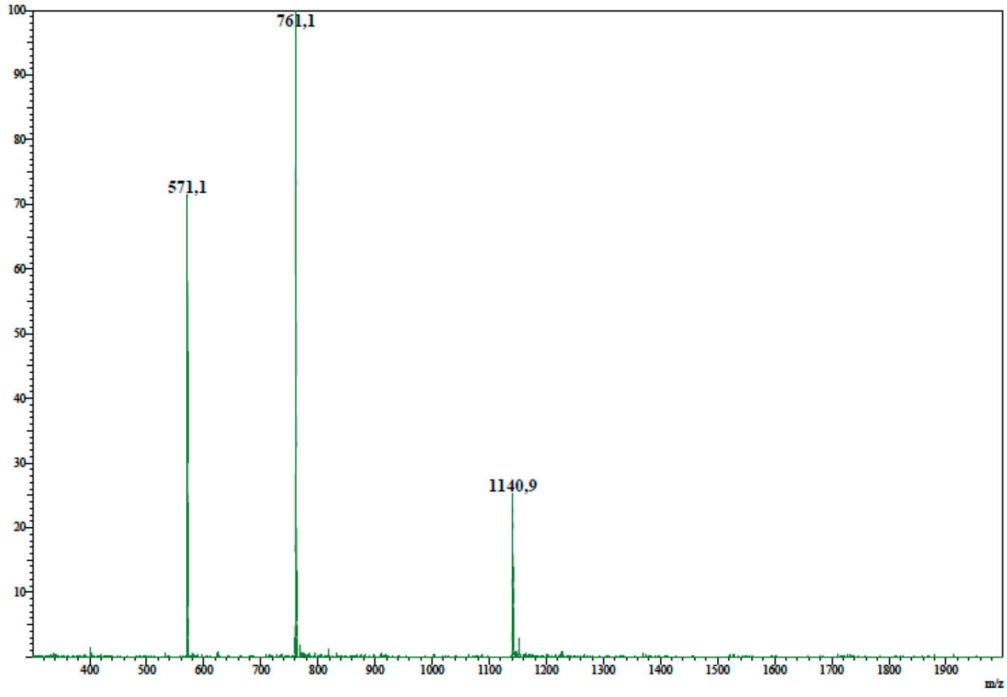


(b)

Figure A2. Mass spectrum for dansyl derivatized TDP. (a) Mass spectrum of triply derivitized TDP by dansyl; (b) Mass spectrum of quadruply derivatized TDP by dansyl.



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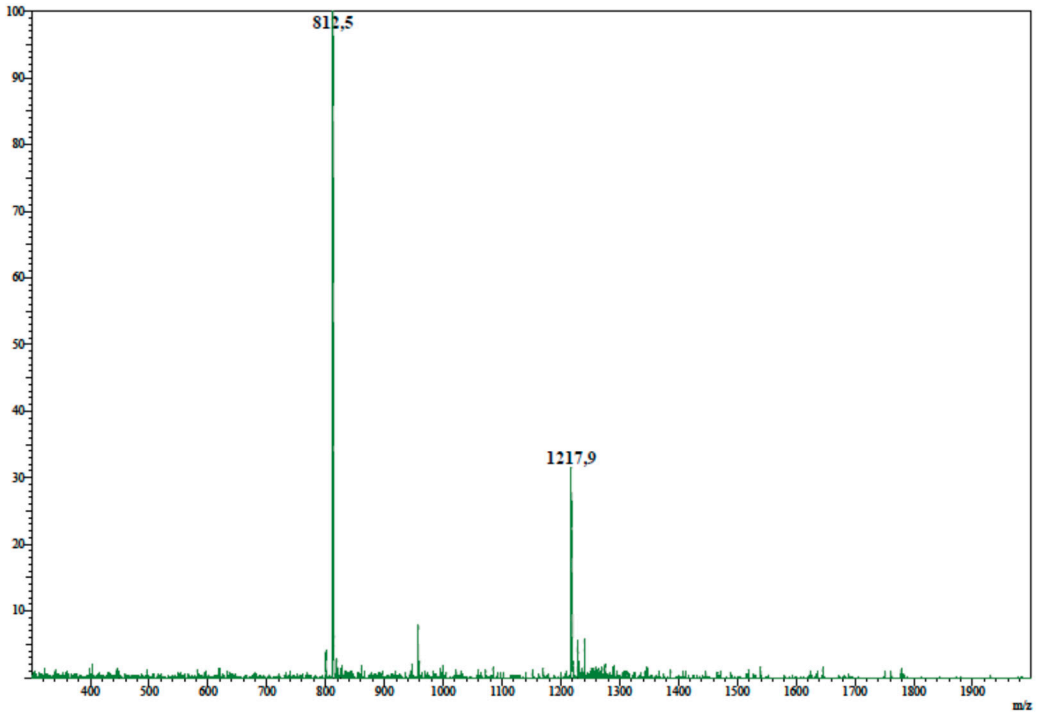
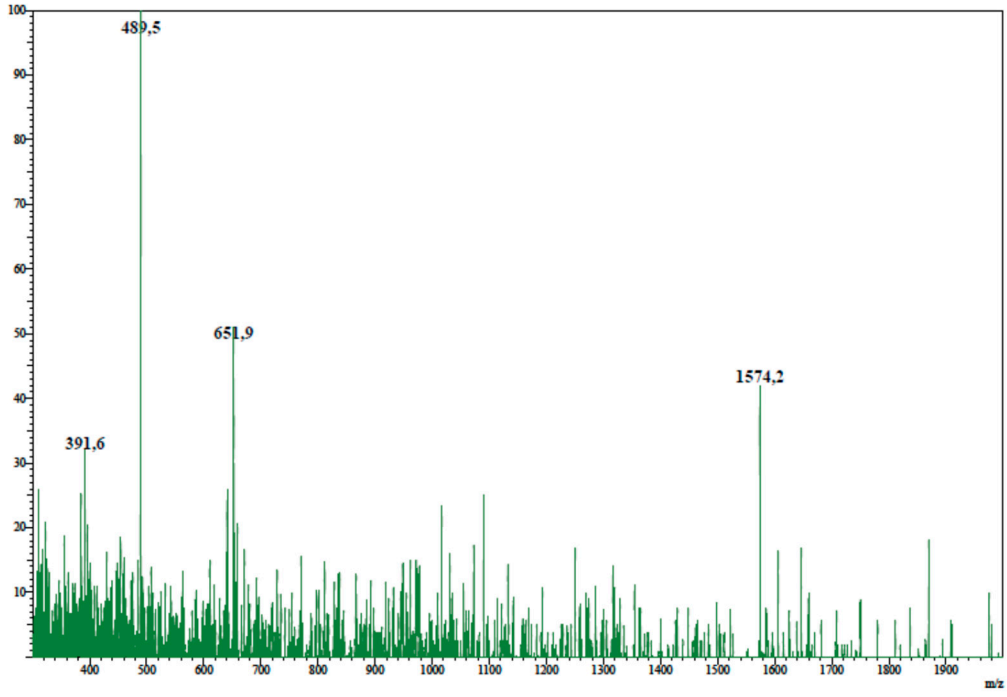


(b)

Figure A3. Mass spectrum for tosyl derivatized TDP. (a) Mass spectrum of triply derivitized TDP by tosyl; (b) Mass spectrum of quadruply derivatized TDP by tosyl.



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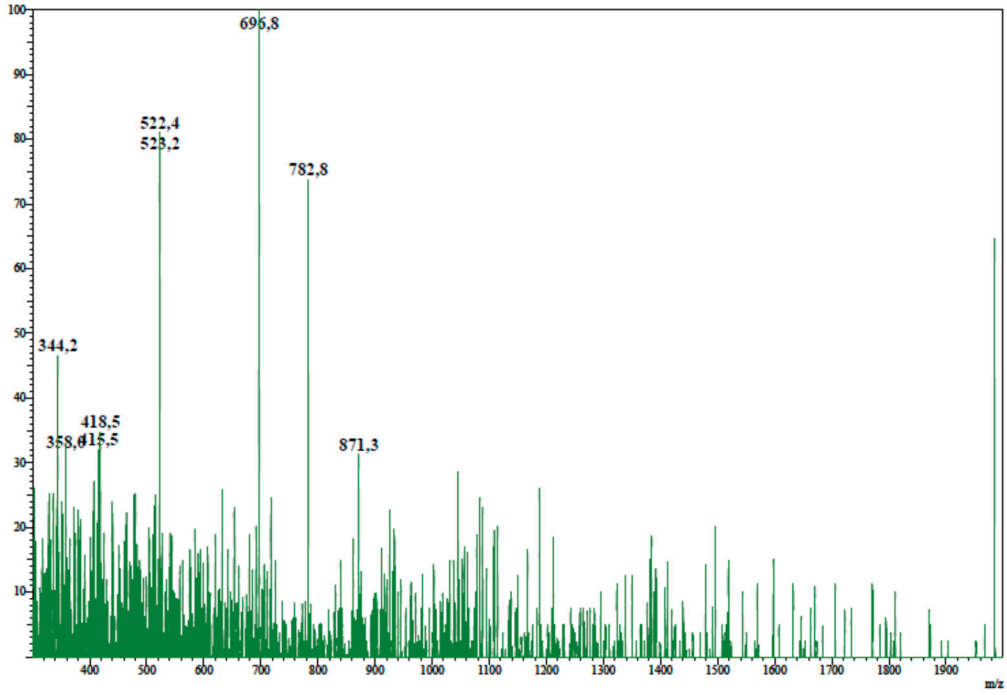
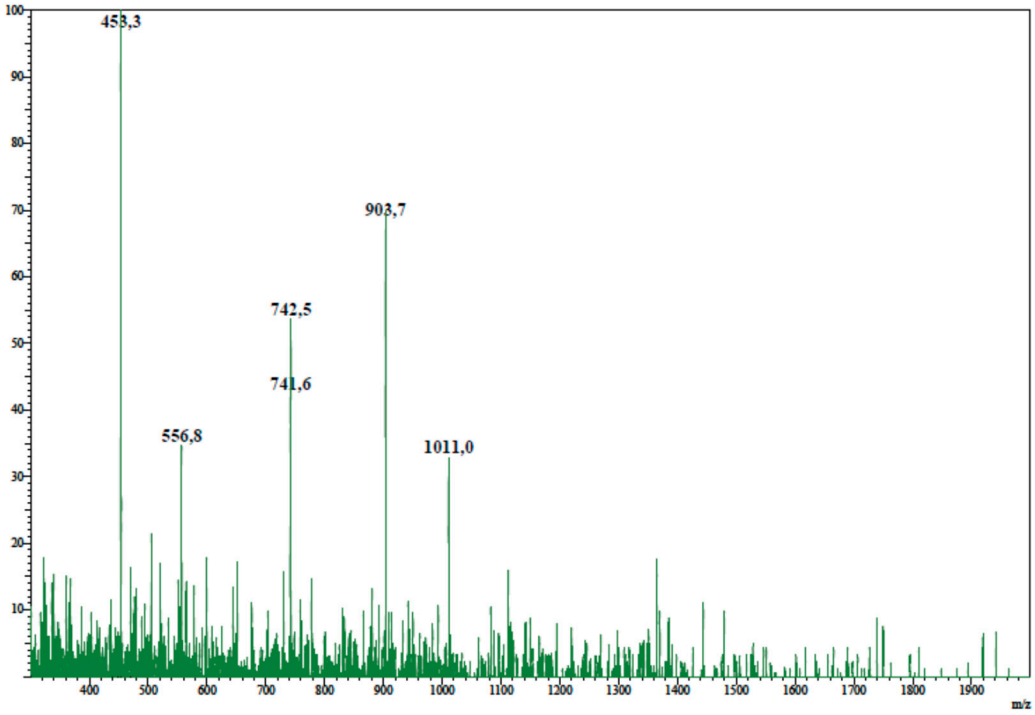


(b)



(c)

Figure A4. Mass spectrum for PITC derivatized TDP. (a) Mass spectrum of singly derivitized TDP by PITC; (b) Mass spectrum of doubly derivatized TDP by PITC; (c) Mass spectrum of triply derivatized TDP by PITC.

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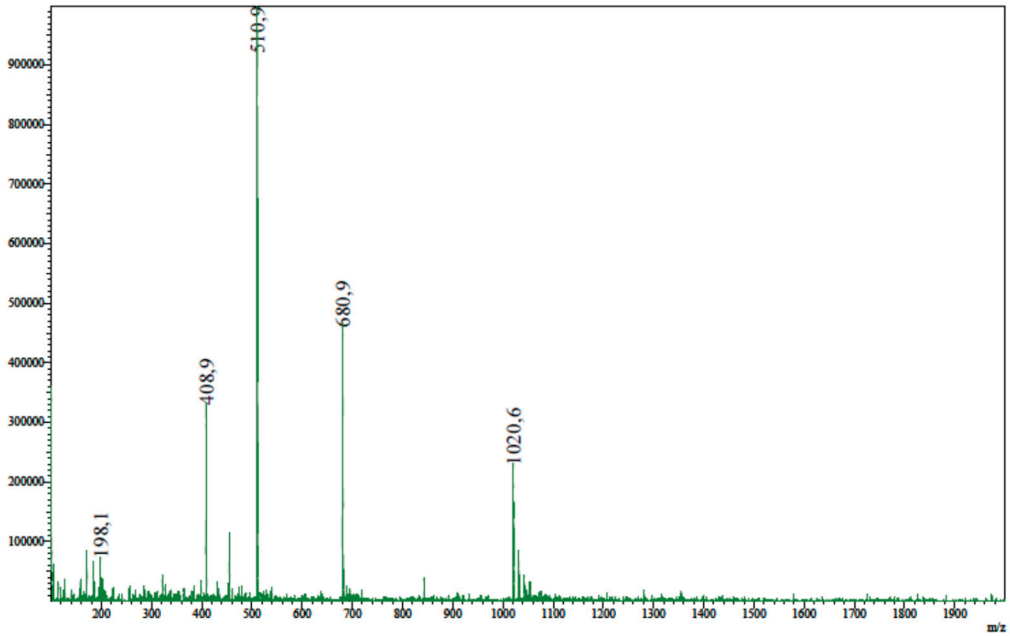
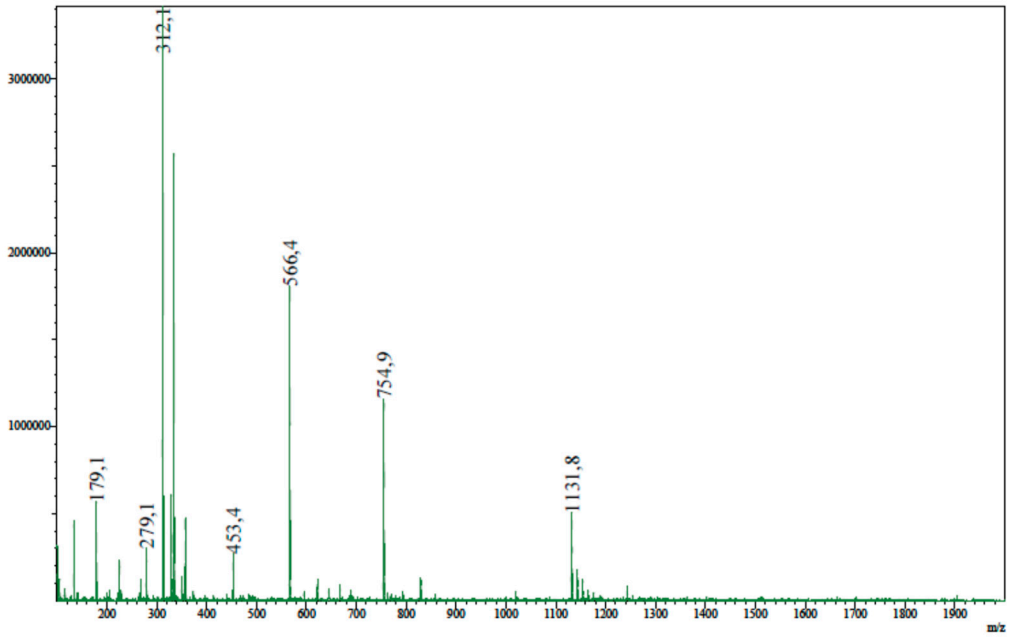


A

(a)



(b)

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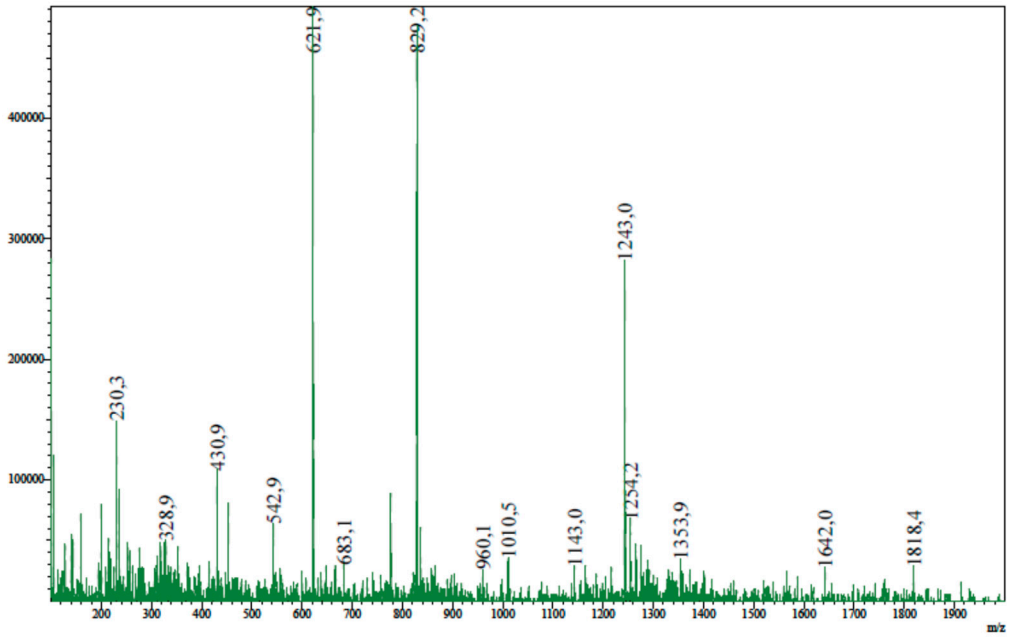
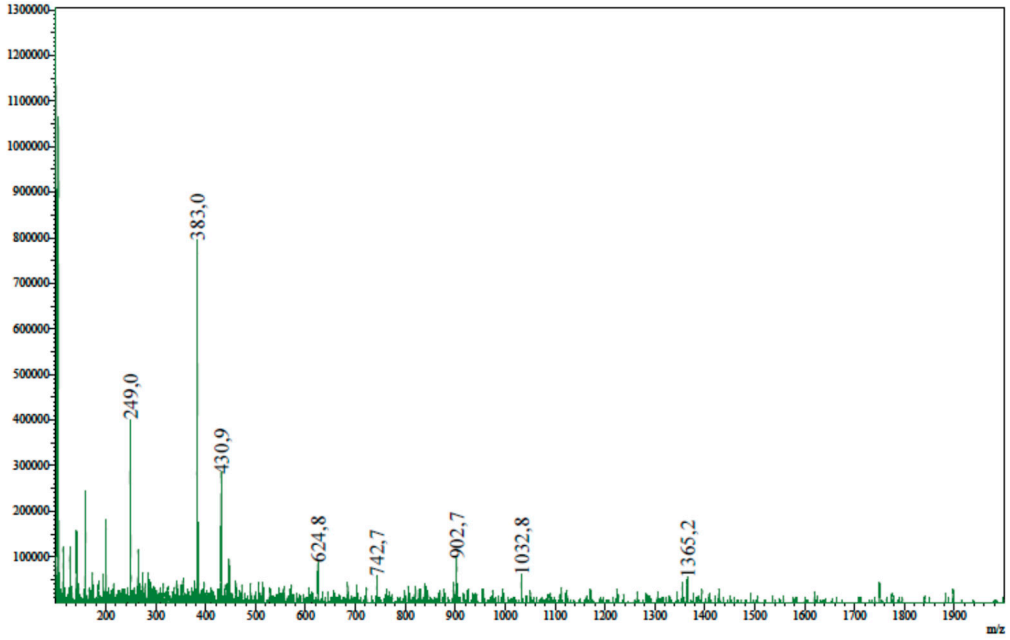


(c)



(d)

Figure A5. Mass spectrum for Fmoc-OSu derivatized TDP. (a) Mass spectrum of singly derivitized TDP by Fmoc-OSu; (b) Mass spectrum of doubly derivatized TDP by Fmoc-OSu; (c) Mass spectrum of triply derivatized TDP by Fmoc-OSu; (d) Mass spectrum of quadruply derivatized TDP by Fmoc-OSu.

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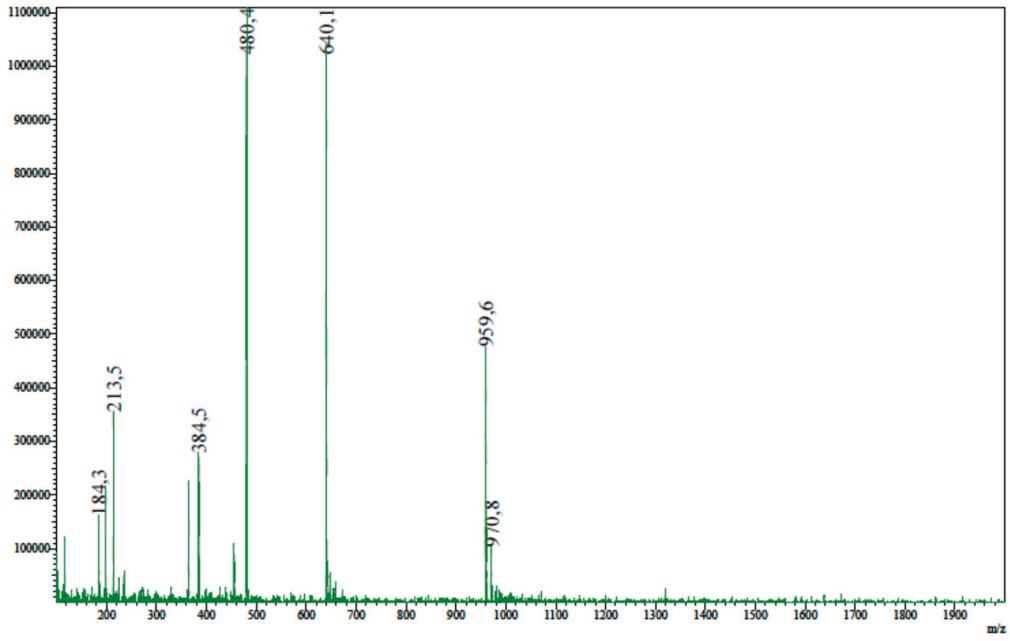
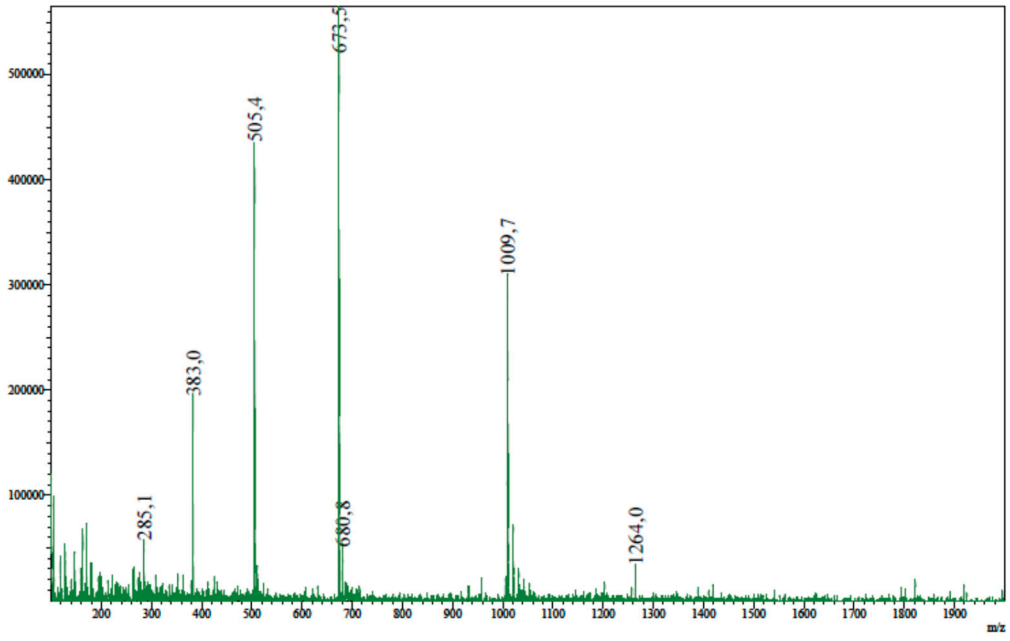


(a)



(b)

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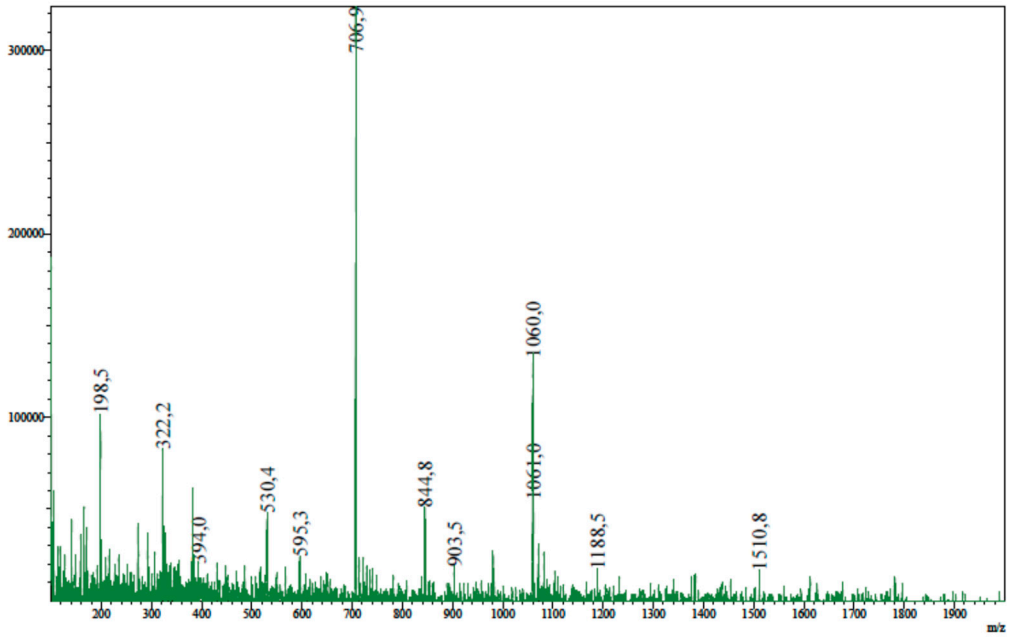
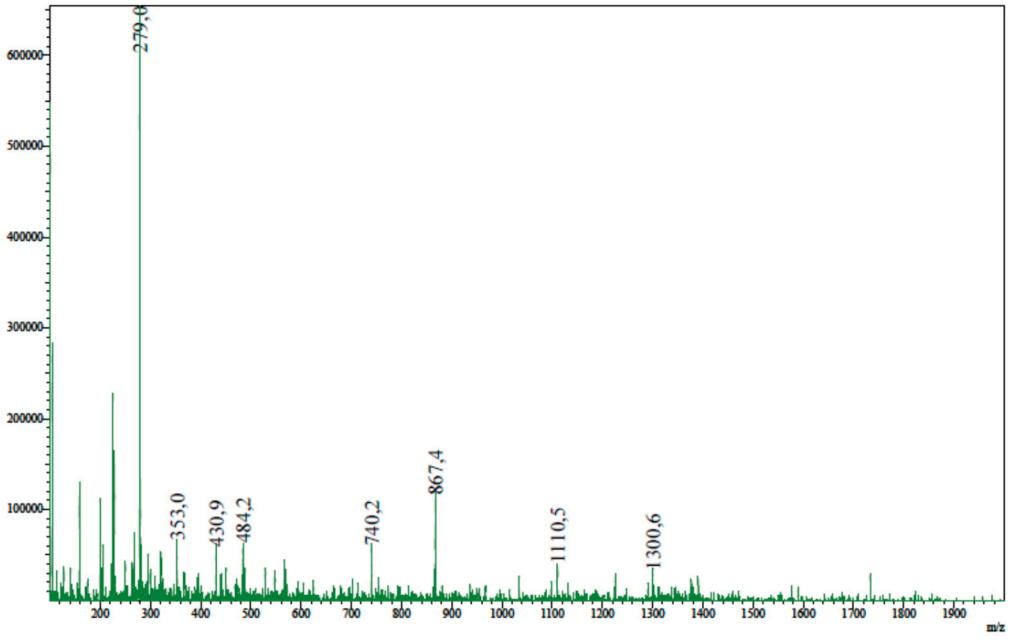


(c)



(d)

Figure A6. Mass spectrum for Boc anhydride derivatized TDP. (a) Mass spectrum of singly derivatized TDP by Boc anhydride; (b) Mass spectrum of doubly derivatized TDP by Boc anhydride; (c) Mass spectrum of triply derivatized TDP by Boc anhydride; (d) Mass spectrum of quadruply derivatized TDP by Boc anhydride.

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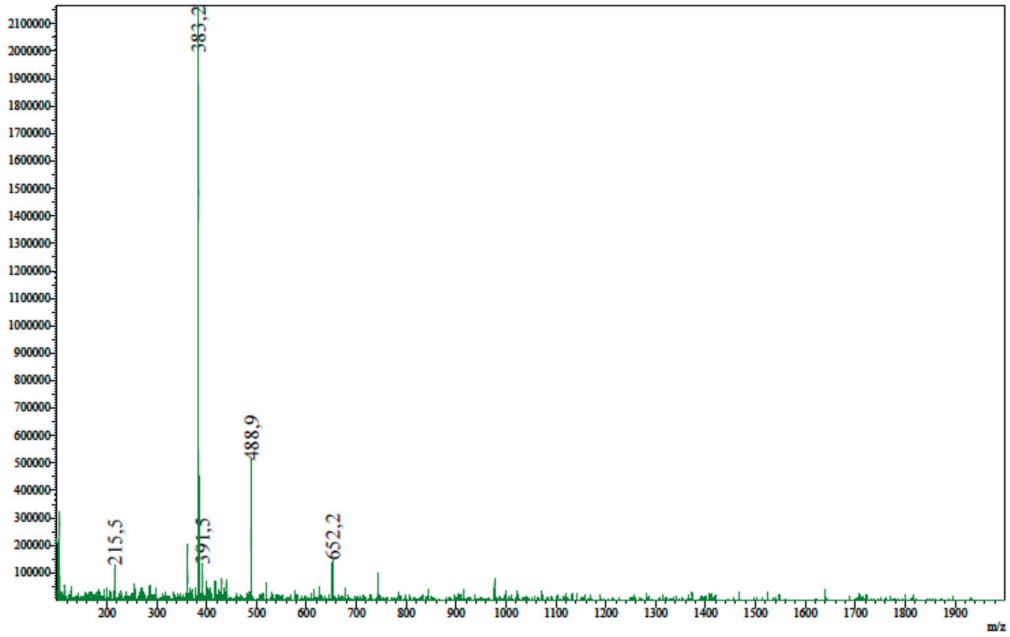
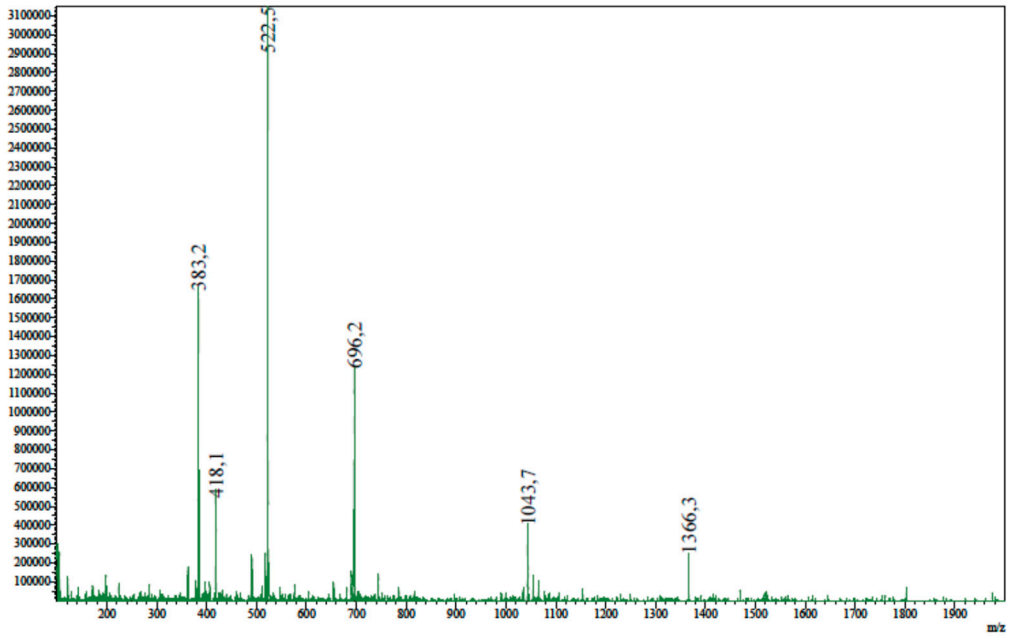


(a)



(b)

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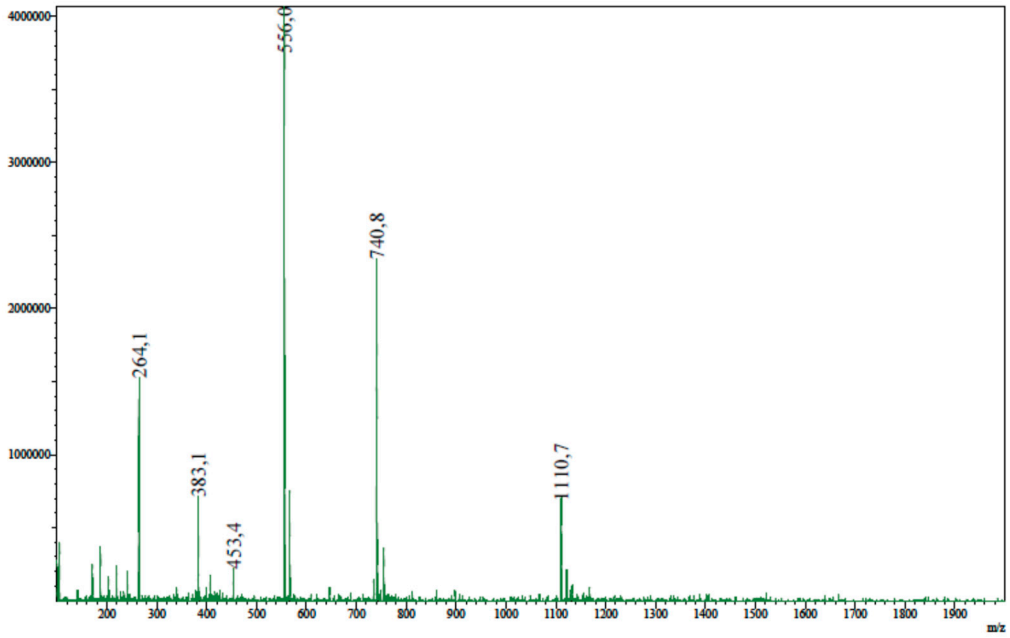

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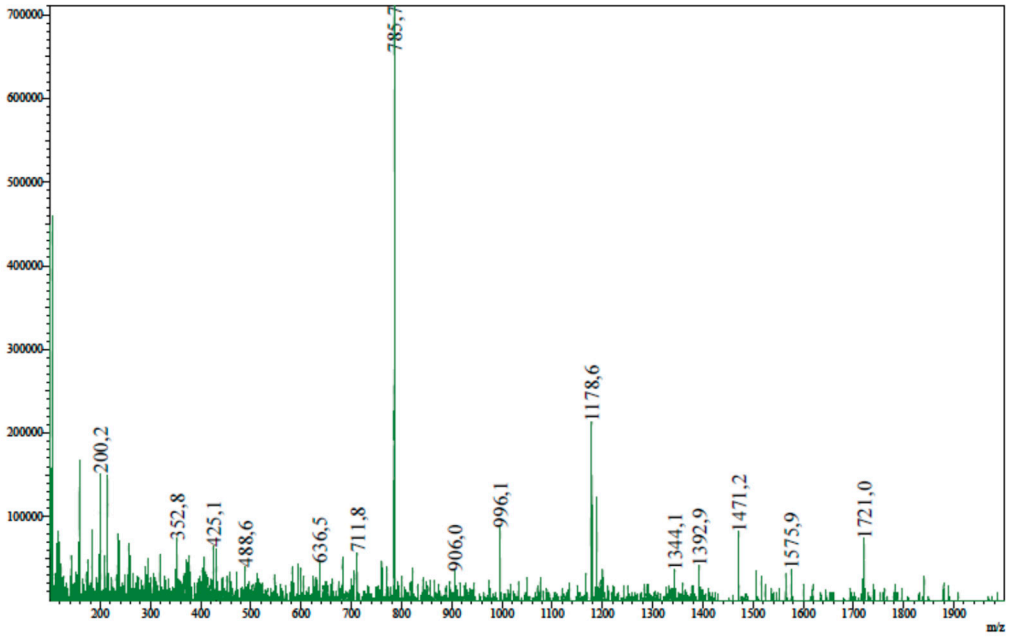

(d)

Figure A7. Mass spectrum for Cbz-OSu derivatized TDP. (a) Mass spectrum of singly derivatized TDP by Cbz-OSu; (b) Mass spectrum of doubly derivatized TDP by Cbz-OSu; (c) Mass spectrum of triply derivatized TDP by Cbz-OSu; (d) Mass spectrum of quadruply derivatized TDP by Cbz-Osu.

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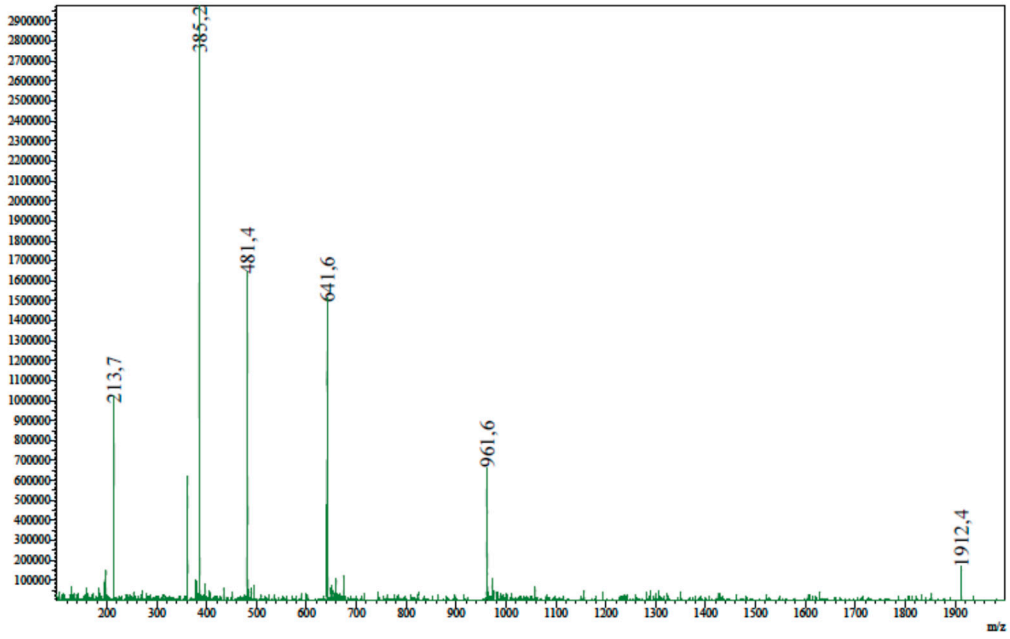

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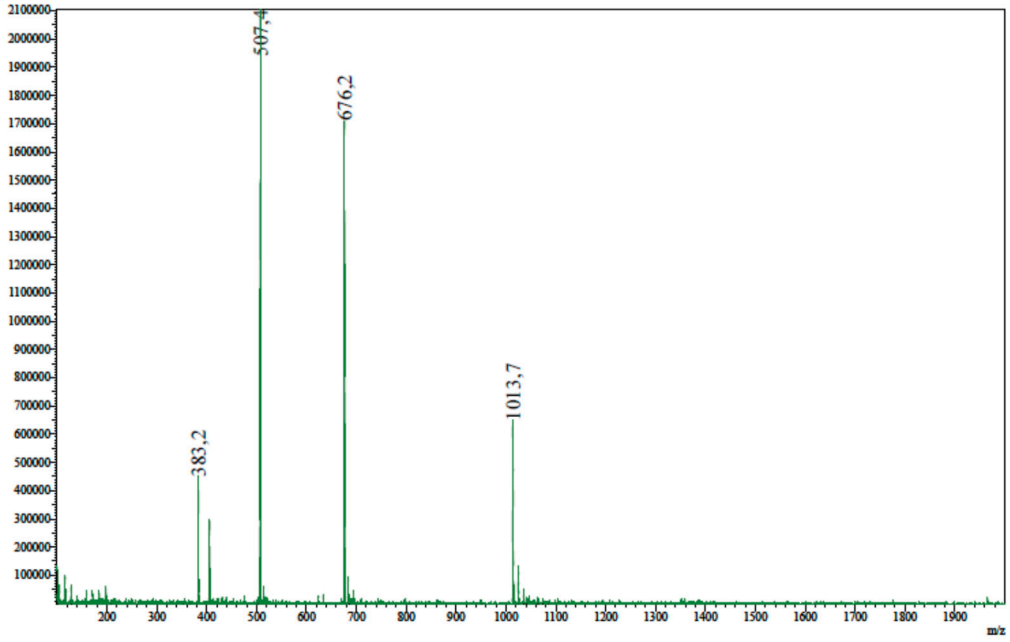

(b)

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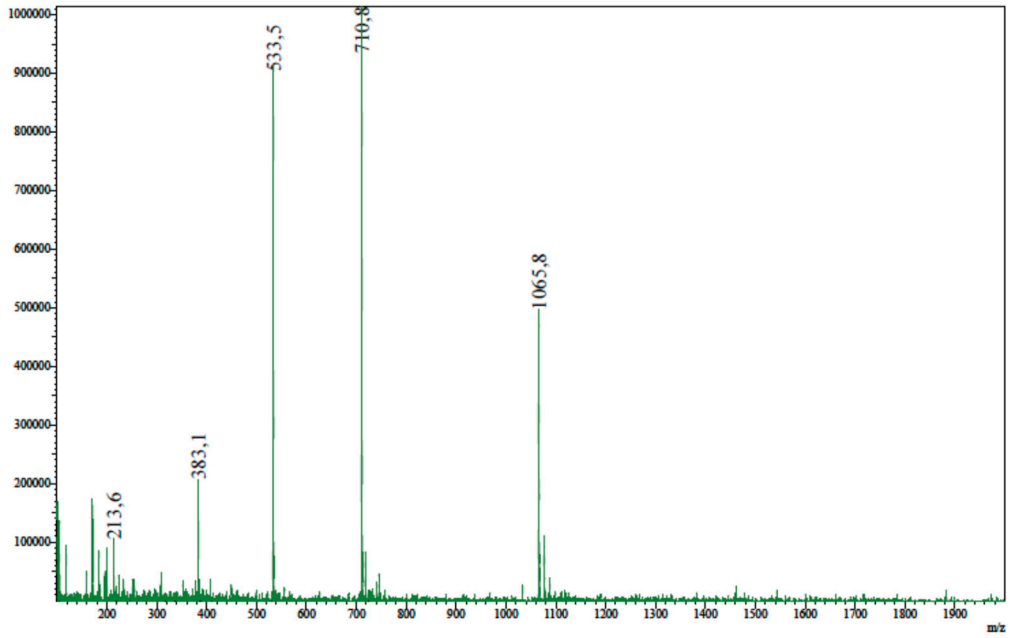
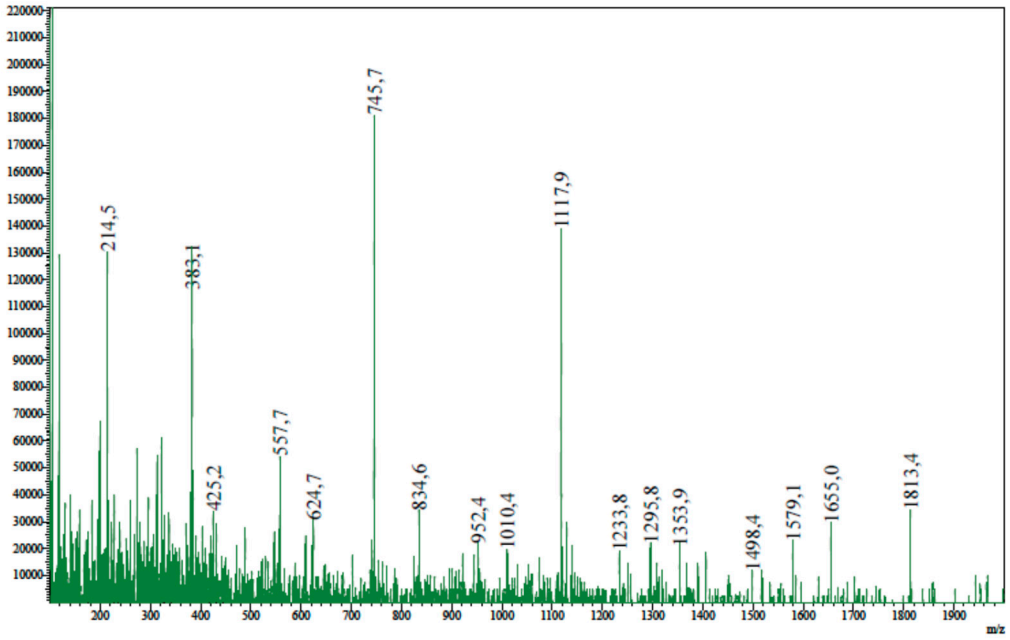


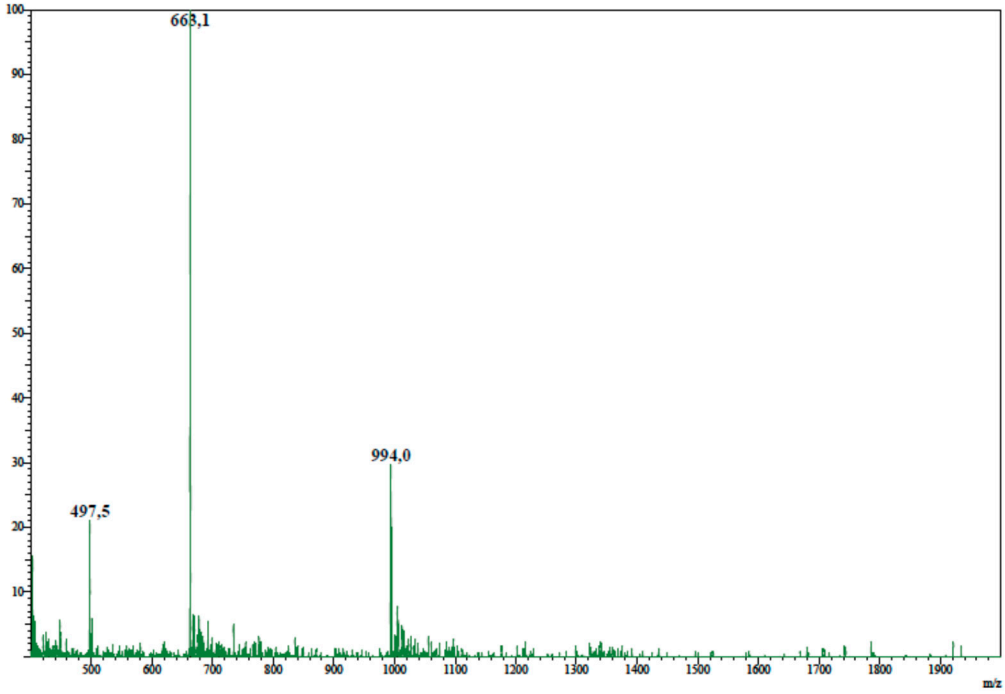
(c)



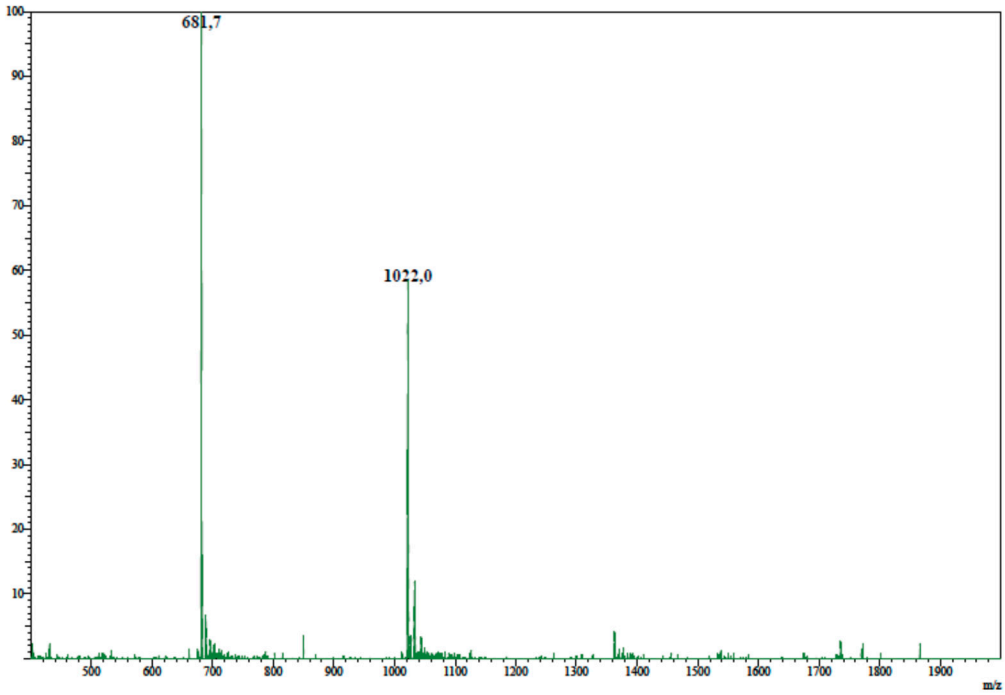
(d)

Figure A8. Mass spectrum for benzoic anhydride derivatized TDP. (a) Mass spectrum of singly derivatized TDP by benzoic anhydride; (b) Mass spectrum of doubly derivatized TDP by benzoic anhydride; (c) Mass spectrum of triply derivatized TDP by benzoic anhydride; (d) Mass spectrum of quadruply derivatized TDP by benzoic anhydride.

 doi:10.20944/preprints202504.1975.v1



(a)



(b)

Figure A9. Mass spectrum for propionic anhydride derivatized TDP. (a) Mass spectrum of triply derivatized TDP by propionic anhydride; (b) Mass spectrum of quadruply derivatized TDP by propionic anhydride.

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