Ricin Detection from Algerian Cultivar *Ricinus communis* species: Optimization and Validation Method

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***Abstract*— Ricin, a highly toxic protein derived from the seeds of *Ricinus communis*, poses significant threats due to its potential misuse as a biotoxin. Detecting ricin in complex matrices remains a critical challenge, particularly in regions where validated methods tailored to local cultivars are lacking. This study presents the development and validation of a robust method for the qualitative detection and screening of ricin peptides extracted from an Algerian Ricinus communis cultivar using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The extraction relied on an acetone-based method, followed directly by trypsin digestion without any enrichment step, enabling a streamlined workflow. Five tryptic peptides were identified as reliable markers (T7A, T12A, T13B, T10B-SS, and T14B-SS-T16B) for unambiguous identification. The method was validated for its limit of detection (LOD), selectivity, repeatability, and reproducibility, achieving LODs ranging from 0.27 µg/mL to 2.65 µg/mL. This work addresses the urgent need for validated detection tools adapted to local contexts and provides a valuable framework for forensic ricin detection.**

***Keywords*— Toxins, Ricin, LC-MS/MS, Method validation, Tryptic peptides.**

I. INTRODUCTION

Ricin, a potent and long-lasting toxin, has been recognized for centuries and remains a significant concern today due to its persistent relevance in toxicology and bioterrorism [1] [2]. This highly toxic protein is found in the seeds of *Ricinus communis* plant, a widely known as the castor plant, which is thought to have originated in North Africa or Asia and has been cultivated for over 4000 years [3]. The concentration of ricin in castor beans is estimated to range between 9.3 mg/g and 9.9 mg/g of the seed's solid material [4]. As a type II ribosome-inactivating protein (RIP II), ricin consists of two chains linked by a disulfide bond: the catalytic A-chain (32 kDa) and the lectinic B-chain (34 kDa) [5]. The B-chain enables the toxin to enter cells by binding to galactose receptors on the cell membrane, while A-chain inactivates ribosomes by depurinate specific adenosine in the 28S ribosomal RNA, effectively halting protein synthesis and leading to cell death [6].

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Ricin is unique in that it is the only naturally occurring protein listed under both the Chemical Weapons Convention (CWC) and the Biological Weapons Convention (BWC), emphasizing its status as a prohibited chemical agent [7][8]. The widespread availability of *Ricinus communis* and the relative ease of extracting ricin from its seeds have made this substance central to numerous bioterrorism incidents and cases of poisoning worldwide [9][10]. This is despite its potential use in medicine, particularly in targeted cancer therapies, which remains an area of active research [11]. Consequently, many countries have developed strategies and techniques for detecting and profiling ricin, particularly in forensic investigations involving specific *Ricinus communis* cultivars. This is crucial because different cultivars of *Ricinus communis* can contain varying levels and profiles of ricin [12]. Furthermore, these events have led to the development of methods aimed at detecting ricin across various substances and matrices, with detection limits ranging from many ppms to sub-ppt levels, depending on the matrix and technique used [13] [14].

Understanding the complexity of ricin detection and its variability across different matrices has been the focus of numerous studies. Recent efforts have also expanded to explore the broader biological and genetic context of *Ricinus communis,* the plant from which ricin is derived. Fredriksson et al. [15] developed a forensic profiling approach using biomarkers for precise ricin detection, while Ma et al. [16] proposed a highly specific method combining magnetic immunocapture and LC-MS/MS for analyzing ricin in biomedical samples. Complementing these forensic and toxicological approaches, Wang et al. [17] introduced a simplified LC-MS method for detecting ricinine, a key ricin metabolite, demonstrating the need for accessible solutions for routine screening. Fakiha [18] emphasized the significance of LC/MS techniques in understanding ricin's forensic toxicity. Beyond analytical methods, Huarg et al. [19] and Memon et al. [20] explored the genetic and biochemical traits of *Ricinus communis*, including seed size, weight, and population diversity. These multidisciplinary studies collectively highlight the need for integrating advanced analytical techniques with genetic and biochemical research to comprehensively address the diverse applications and risks associated with ricin and its source plant.

Building on these scientific advancements, forensic investigations have become central to addressing ricin-related incidents. These investigations not only provide crucial evidence for legal action but also play a key role in tracing the geographical origin of the material used. Comprehensive ricin analysis requires the ability to detect minute quantities and assess its enzymatic activity. Various techniques, including immunological, spectrometric, and functional assays—or combinations thereof—are commonly employed to detect and identify ricin. However, while immunological and functional assays offer rapid detection, they are often limited in specificity and may fail to distinguish ricin from RCA120, a closely related lectin also derived from *Ricinus communis* [21].

In contrast, mass spectrometry-based methods offer more precise identification of ricin, even in complex mixtures containing similar proteins [22]. Despite advances in analytical instrumentation, particularly in Liquid Chromatography–Mass Spectrometry (LC-MS)[23][24], challenges persist. These include selecting appropriate reference standards, dealing with cultivar variability, and optimizing of sample preparation processes such as extraction, cleanup, and concentration.

The analysis is further complicated by the fact that proteotypic peptides generated from ricin can vary depending on the digestion reagents used and ricin cultivar. Additionally, databases reflect significant differences in peptide profiles between cultivars. Most studies, focus on a single reference cultivar, and there is a lack of validated methods encompassing multiple cultivars[25][26]. This has left a gap in the development of fully validated methods for ricin analysis in various contexts.

Recognizing the absence of validated methods for ricin detection to local environment "Algerian *Ricinus communis* cultivars", our laboratory—designated by the Organization for the Prohibition of Chemical Weapons (OPCW) for the analysis of chemical warfare agents (CWAs) in environmental matrices—aims to extend its expertise to biotoxins. By developing and validating a method for detecting and profiling ricin from Algerian cultivars, we will not only fill this critical gap but also lay the groundwork for participation in the OPCW Biotoxins sample preparation and Proficiency Tests. This study positions our laboratory to potentially gain OPCW designation in the domain of biotoxin analysis as well, in the near future [27]. To achieve this, we will employ a combination of mass spectrometry and peptide profiling techniques to develop a robust and validated method, specifically tailored to Algerian cultivars of *Ricinus communis*.

II. EXPERIMENTAL SECTION

A. Safety Considerations

Due to the high toxicity of ricin, all experiments were conducted by trained personnel. To ensure safety, all manipulations were performed in a fume hood equipped with a high-efficiency particulate air (HEPA) filter. It is recommended that ricin be inactivated through heat treatment, and all waste materials were decontaminated using NaOH solution

B. Chemicals and Reagents

Ricin reference solution (100 µg/mL) was provided by the Organization for the Prohibition of Chemical Weapons (OPCW). Acetonitrile (LC-MS grade), acetone (LC-MS grade), acetic acid (>98%), and formic acid (>98%) were purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). Ultra-purified water was produced using a Milli-Q system (Millipore, USA). Trypsin and dithiothreitol were obtained from VWR.

C. Sourcing of Seed and Ricin Cultivar Material

For this study, *Ricinus communis* seeds Fig .1S were sourced during the growing season on 31 July 2023, from a cultivar grown in a province called Mila, a region situated in the northeastern of Algeria (Latitude: 36° 27' 1.01" N, Longitude: 6° 15' 51.98" E). Mila province features a distinctive climate, characterized by short, hot, dry summers and long, cold, wet winters, as well as a soil composition that includes a mixture of sandy loam, clay, limestone, and marl [28], a voucher (RALG-001) was deposited at Fone and Flora department at the NICC. Leaves and seeds of the plant were dried in the shade.

D. Preparation of ricin samples from the Algerian cultivar

Ricin-containing samples were obtained from a crude castor bean extract of an Algerian *Ricinus communis* cultivar using an acetone-based extraction method, which is the most commonly used in studies, as described in the literature [29]. Briefly, the seeds were dehulled and ground with a mortar and pestle to remove the outer integument, then transferred to an Ultra-Turrax Tube Disperser (IKA) with 5 mL of acetone for dispersion and grinding. The resulting fine powder was centrifuged at room temperature (3000 rpm for 30 minutes) to separate and discard the acetone containing castor oil. This centrifugation and re-extraction process was repeated with fresh acetone. The resulting solid was transferred to a low-profile drying platform to accelerate the evaporation of residual acetone for 10 minutes and then transferred into a Falcon tube containing 15 mL of 5% acetic acid in water. The mixture was thoroughly blended, filtered through 0.22 µm filters, and concentrated using a 30 kDa molecular weight cutoff (MWCO) filter device, yielding approximately 8 mL concentrated extract.

E. Ricin Biological Activity Test

The ricin activity of the extract was assessed using a lateral flow immunoassay (LFA) method, employing the commercially available "BADD Ricin Toxin Biowarfare Detection Test Kit". The extract was first dissolved in PBS buffer ensuring a consistent medium for testing. The BADD detection kit was then used according to the manufacturer's instructions. To ensure accuracy and reliability, the activity of the extract was compared with the OPCW reference standard, which was obtained during the 6th OPCW Biotoxin Exercise. This direct comparison allowed for pre-evaluation of ricin activity level in the extract comparative to the recognized OPCW standard.

F. Tryptic Digestion of Ricin

The ACN-assisted trypsin digestion protocol, slightly modified from previously established method [24], was applied to a 100 µL ricin-containing solution. The process involved the sequential addition of 5 µL of 1M NH4HCO3, 10 µL of acetonitrile (ACN), and 10 µL of trypsin (1 mg/mL) dissolved in 50 mM acetic acid. Digestion was performed at a controlled temperature of 45 °C for 4 hours and quenched by adding 5% formic acid. The digested sample was then transferred to a vial for the analysis by Auto MS/MS and target MS/MS experience.

G. Accurate Automatic MS/MS Qualitative Analysis

Tryptic digests were analyzed to identify both specific and non-specific peptides for ricin identification using an LC-QTOF system. LC-(MS/MS) experiments were conducted in automatic MS/MS acquisition mode to enable a smooth transition between MS and MS/MS analyses. The instrument alternated between full MS mode, which scans for all ions present in the sample, and targeted MS/MS mode, which focuses on the most intense ions. This feature allows for the acquisition of crucial MS/MS fragmentation spectra necessary for structural elucidation of the predominant compounds [30].

The analyses were performed using an HPLC 1290 series coupled to a Q-TOF 6545 series (Agilent Technologies), managed by Mass Hunter Workstation software (Agilent Technologies). Separation was carried out on an Agilent Zorbax Eclipse Plus RRHD C18 column (100 mm x 2.1 mm x 1.8 µm) at 30 °C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B), with the following linear gradient at a flow rate of 0.3 mL/min: (0 to 2.5 min: 5% solvent B, 2.5 to 3 min: 5% to 14% solvent B, 3 to 6.5 min: 14% solvent B, 6.5 to 7 min: 14% to 23% solvent B, 7 to 9.5 min: 23% solvent B, 9.5 to 10 min:

23% to 32% solvent B, 10 to 12.8 min: 32% solvent B, 12.8 to 13 min: 32% to 100% solvent B, 13 to 17 min: 100% solvent B, 17 to 17.1 min: 100% to 5% solvent B, 17.1 to 21 min: 5% solvent B.). The injection volume was 5 µL and the system was equilibrated before each sample.

The Q-TOF 6545 detector, equipped with an Agilent Jet Stream electrospray ionization (ESI) source for accurate mass measurement, was operated in positive mode with the following parameters: gas temperature of 320 °C, gas flow of 8 L/min, nebulizer pressure of 35 psig, sheath gas temperature of 350 °C, and sheath gas flow of 11 L/min. Additional settings included a VCap voltage of 3500 V, nozzle voltage of 1000 V, fragmentor voltage of 110 V, skimmer voltage of 65 V, octopole at 750 RF, and collision energies of 5, 10, and 20 V.

H. Targeted MS/MS Qualitative Analysis

The experimental analysis was performed to ensure a non-contradistinction to the guidelines proposed for proteomic identification of unidentified proteins in complex mixtures [31], and adhering to the minimum criteria outlined by OPCW for identifying the presence and activity of ricin. The targeted assay relies on the careful selection of sensitive peptide markers, defined in the automatic MS/MS experiments, which provide clear evidence of the presence of the target compound. The selected tryptic peptides include T7A and T12A from the A chain, T13B and T10B-SS from the B chain, and the disulfide bridge peptide T14B-SS-T16B, which has been reported as a novel and reliable marker for the unambiguous identification of ricin [24],[32].

The targeted MS/MS experiments were performed using a Q-TOF hybrid instrument (Agilent Technologies, 6545) coupled with a 1290 liquid chromatograph and interfaced with Mass Hunter software. The analysis was conducted in targeted MS/MS acquisition mode, using the predefined sensitive marker masses as the precursor ions. A linear gradient of eluents was applied at a flow rate of 0.3 mL/min with the following steps: (0 to 2.5 min: 5% solvent B, 2.5 to 3 min: 5% to 14% solvent B, 3 to 6.5 min: 14% solvent B, 6.5 to 7 min: 14% to 23% solvent B, 7 to 9.5 min: 23% solvent B, 9.5 to 10 min: 23% to 32% solvent B, 10 to 12.8 min: 32% solvent B, 12.8 to 13 min: 32% to 100% solvent B, 13 to 17 min: 100% solvent B, 17 to 17.1 min: 100% to 5% solvent B, 17.1 to 21 min: 5% solvent B.

The Q-TOF was operated with a nozzle voltage of 1000 V, using nitrogen as the nebulizer (35 psi) and desolvation gas (8 L/min). MS/MS product ion spectra were recorded using collision energies of 5, 10, and 20 V, with nitrogen as the collision gas and a medium isolation width of 4 m/z.

I. Method Validation

To eensure compliance with our quality system (ISO 17025), the method was qualitatively validated according to the criteria established in the IUPAC single-laboratory validation guidelines [33]. Due to the limited availability of standard solutions, key parameters such as repeatability (intra-day) and intermediate precision (inter-day reproducibility) were evaluated across different days and operators (OP1, OP2, OP3) using a spiked known concentration over nine analyses (n=9) Fig .2S and 3S. The limit of detection (LOD) was calculated based on the signal-to-noise ratio (S/N) and the standard deviation (s). Selectivity was assessed by analyzing ten samples (n=10) to evaluate the method's robustness in the presence of potential interfering compounds.

This validated approach was applied to the qualitative analysis of crude extracts from *Ricinus communis* seeds of an Algerian cultivar. Using the assisted trypsin digestion method, the resulting tryptic peptides identified in previous experiments were used to confirm the presence of ricin, adhering to the minimum required criteria.

III. RESULTS

A. Ricin Activity Test of the Extracted Sample from the Algerian Cultivar

To confirm the successful extraction of ricin from the Algerian *Ricinus communis* cultivar, its activity was evaluated using a lateral flow immunoassay (LFA). The results indicated a notably positive outcome, with the detection kit successfully identifying and confirming the presence of ricin in the sample (Fig.1). The activity of the ricin extracted from the cultivar was found to be higher compared to observations made with the OPCW reference standard. The detection kit featured both a control and test line, and a positive result was determined by the appearance of both lines. In the case of the extract, both lines were clearly visible, confirming a positive result. In contrast, the OPCW references standard showed a weaker or faint appearance of the test line, where further analysis was suggested due to the lower concentration of ricin in the reference material. This finding confirms the obtention of ricin and underscores the kit's sensitivity in detecting ricin concentration at a LOD of 10 ng/mL [26], [34].



Fig 1. Ricin Lateral Flow Activity Assessment of: a) Ricin extract from the Algerian cultivar of Mila, b) Ricin OPCW reference standard.

B. Marker Peptide Screening and Specific Peptide Target

The objective of the LC-ESI-MS/MS analysis was to identify all tryptic peptides present in the Algerian castor bean extract and confirm the presence of ricin in the extract prepared from the Algerian *Ricinus communis* cultivar. Fig. 2 presents the base peak chromatograms of the tryptic digests of the extract, with some tryptic peptides identified and used later for the method validation.

To identify ricin-related peptides, the tryptic fragments automatically recorded via MassHunter software were converted to MGF file format and submitted to the online MS/MS ion search software Mascot 2.5.1 (MATRIX SCIENCE). The following search parameters were used: instrument type ESI-QUAD-TOF, enzyme Trypsin, peptide charges of 1+, 2+, 3+, and 4+, peptide tolerance of 1.2 Da, MS/MS tolerance of 0.6 Da, and allowance for up to 1 missed cleavage. The selected databases included NCBIprot (225419 for ricin chain B), SwissProt, and UniProt (P02879 for ricin D).

As shown in Table I the experimentally matched molecular ions of the observed peptides are highlighted in bold. Peptides previously reported in the literature that can differentiate ricin D from ricin E are indicated with underlined bold italic typeface [5].

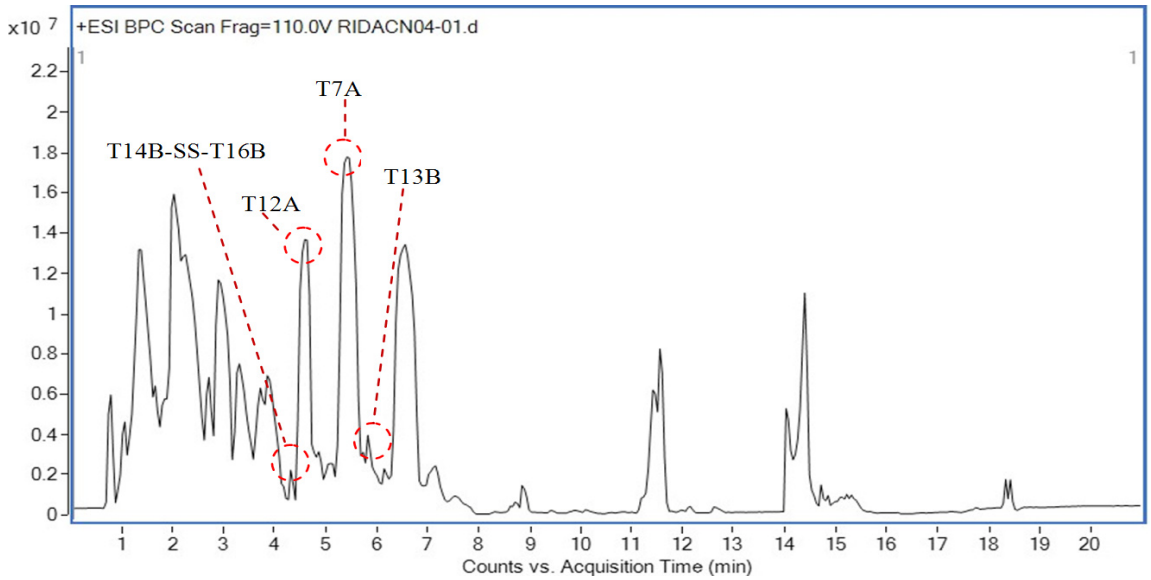


Fig. 2. Base peak chromatogram of trypsin digests of the extract obtained from the Algerian R. communis cultivar.

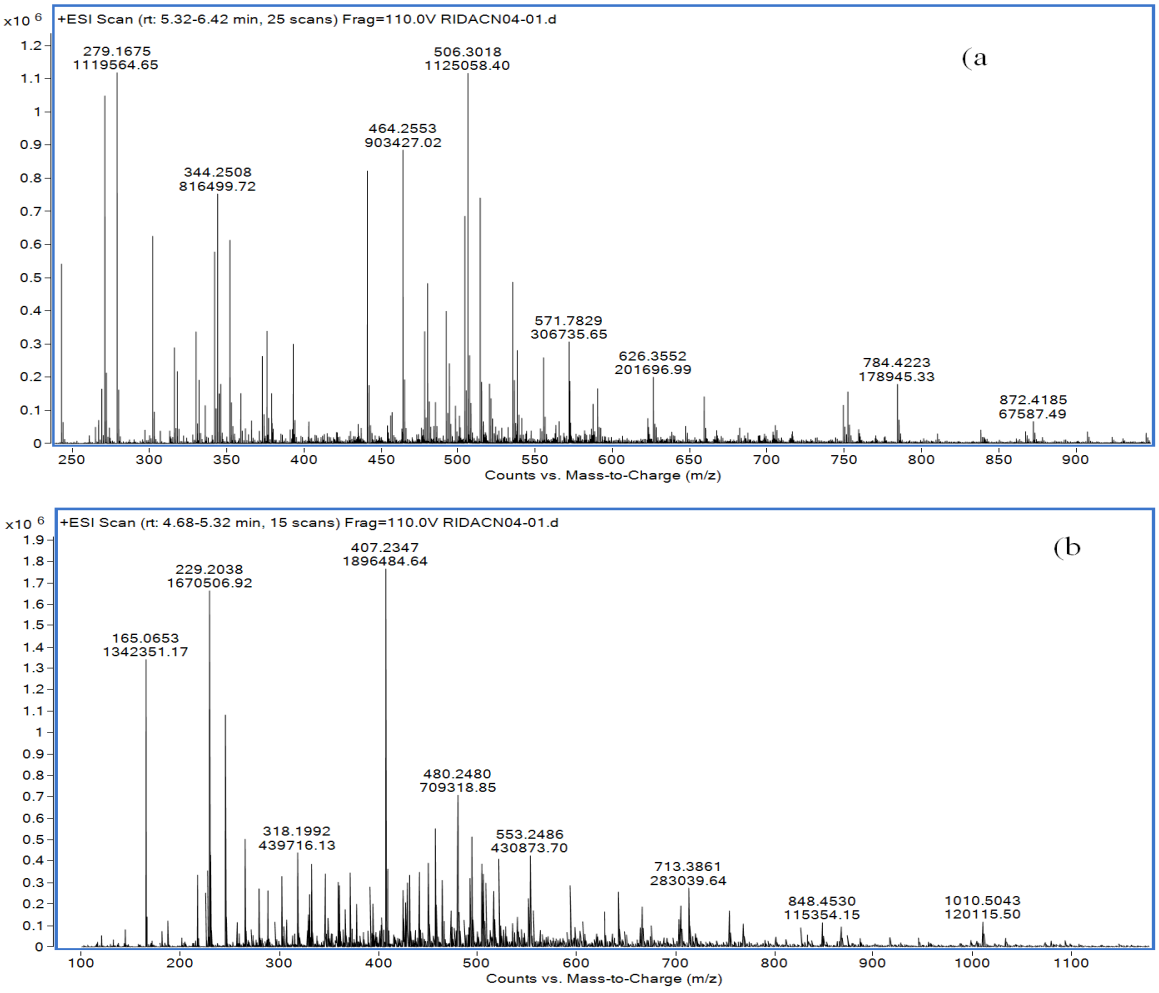


Fig. 3. ESI MS spectrum of a) T7A; b) T12A targeted tryptic peptides of ricin after digestion.

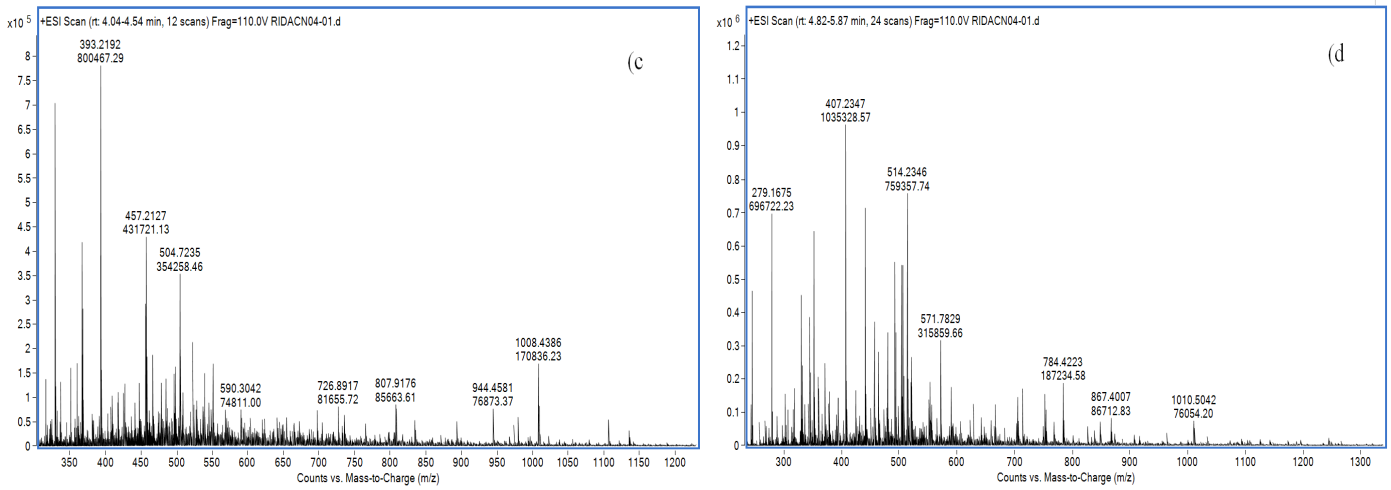


Fig. 4. ESI MS spectrum of c) T14B-SS-T16B; d) T13B targeted tryptic peptides of ricin after digestion.

Table I

Tryptic peptide markers identified from Auto MS/MS Analysis using Mascot software of a crude castor bean extracted from Algerian cultivar.

Acquisition MS and MS/MS parameters	**Ions (m/z)**					
Plot and centroid data storage: **MS** Abs.threshold = 200 Rel.threshold = 0.01 % **MS/ MS** Abs.threshold = 5 Rel.threshold = 0.01 % **Active Exclusion** Exclude after = 2 spectra Released after = 0.5 min Max Precursor per cycle = 5	**2277.2431*#**	**1116.0512*#**	872.4167	668.0261	531.2715	441.2924
	2066.9287	**1103.1728*#**	**860.9359*#**	665.9697	528.2572	431.2100
	1862.0018*#	1089.4841	855.4093	663.3769	524.2407	429.2091
	1603.8584*#	1071.4786	852.9256	660.6386	521.2765	427.2022
	1584.7741*#	1070.9625#	840.4247	659.2813	517.8855	424.7307
	1430.7877	1045.9679	833.4644	**655.7936*#**	514.2346	419.2729
	1420.3190	1041.9639	830.8927	**647.7882*#**	513.3344	413.2140
	1410.9017	1033.9688	**819.9207*#**	642.2916	512.4108	409.1779
	1390.6961*#	1026.2966	811.4830	637.4213	508.2448	406.2458
	1360.6224	**1021.7893*#**	**804.0410†*#**	628.4925	**507.2905*#**	399.1720
	1310.5800*#	1008.4386	**792.8906*#**	**600.7979*#**	504.2760#	393.2092
	1294.5692*#	1001.5437	784.4224	593.3327	498.2497	389.1993
	1278.2865	**997.7684*#**	775.4260	589.4221	495.7211	385.1570
	1276.5883	989.9368	773.8890	584.4667	487.3553	379.1939
	1272.2538	**983.1031*#**	**759.7525*#**	579.4920	484.2852	376.7639
	1262.2695	974.4925	748.3865	576.2563	480.2420	374.2245
	1245.5251	950.5117	**744.3699†*#**	**575.3399*#**	478.2710	371.1792
	1253.9127	946.5481	**737.5791†*#**	571.7828	475.2025	366.1623
	1252.7747	939.5841	719.9862	563.3225	470.4146	355.2785
	1236.6310	939.5841	714.6553	561.3920	468.3850#	351.1027
	1205.5579*#	937.4770	710.4367	559.2526	463.2983	**348.4295*#**
	1200.5886*#	**932.4618*#**	697.3441	**558.5293*#**	462.2451	344.1306#
	1152.5043	**931.5659#**	**695.8517*#**	555.2606	460.9195	340.2060
	1149.0310	920.9678	690.3624	551.2502	457.2611	335.1890
	1148.9313	903.4464	689.3043	**546.9496*#**	452.2278#	332.1783
	1147.5789†*#	**896.5312*#**	678.3747	538.3171	449.2831	329.1211
	1139.1251*#	882.9268	676.3392	**537.8019#**	**448.7692†#***	318.1629
	1129.2561	879.4233	672.5185	535.2555	444.2195#	**299.5152*#**

†Ions used in Targeted MS/MS Qualitative Analysis, *Ions with high intensity response, # Ions reported in previous literature

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Moreover, Table II summarizes the key peptide markers identified in this study, specifically highlighting the peptides unique to ricin, as well as those could be used to distinguish ricin D from E type. These peptide markers provide crucial evidence for confirming the presence of ricin in the extract.

Table II

Trypsin digest peptides identified from A chain and B chain for ricin identification extracted from Algerian cultivar.

N⁰	Tryptic fragment	Precursor ion (m/z)	Amino acid sequence
01	**T20B***	**2277.2431**$^{1+}$ **1139.1251**$^{2+}$ **759.7525**$^{3+}$	QIILYPLHGDPNQIWLPLF
02	**T18B***	**1862.0018**$^{1+}$ **931.5659**$^{2+}$	NDGTILNLYSGLVLDVR
03	T15B	**575.3399**$^{1+}$	ETVVK
04	T13B	**1116.0512**$^{2+}$ **558.5293**$^{4+}$ **744.3699**$^{3+}$	AEQQWALYADGSIRPQQNR
05	**T14B-CAM***	**1294.5692**$^{1+}$ **647.7882**$^{2+}$	DNCLTSDSNIR
06	T11B-glyc	**997.7684**$^{3+}$	WQIWDNGTIINPR
07	T10B-SS	**983.1031**$^{3+}$ **737.5791**^{4}+	CLTTYGYSPGVYVMIYDCNTAATDATR
08	T10B-CAM	**1021.7893**^{3}+	CLTTYGYSPGVYVMIYDCNTAATDATR
09	**T6B***	**1390.6961**$^{1+}$ **348.4295**$^{4+}$ **695.8517**$^{2+}$	SNTDANQLWTLK
10	**T5B-CAM***	**1584.7741**$^{1+}$ **792.8906**$^{2+}$	FHNGNAIQLWPCK
11	T3B-CAM	**932.4618**$^{1+}$	NGLCVDVR
12	T14B-SS-T16B	**1205.5579**$^{2+}$ **804.0410**$^{3+}$	DNCLTSDSNIRILSCGPASSGQR
13	T3B-SS-T5B	**1200.5886**$^{2+}$ **600.7979**$^{4+}$	NGLCVDVRFHNGNAIQLWPCK
14	T7A	**896.5312**$^{1+}$ **299.5152**$^{3+}$ **448.7692**$^{2+}$	VGLPINQR
15	T6A	**537.8019**$^{2+}$	HEIPVLPNR
16	T8A	**1603.8584**$^{2+}$	FILVELSNHAELSVTLALDVTNAYVVGYR
17	T9A	**1103.1728**$^{3+}$	AGNSAYFFHPDNQEDAEAITHLFTDVQNR
18	T10A	**1310.5800**$^{1+}$ **655.7936**$^{2+}$	YTFAFGGNYDR
19	T11A	**507.2905**$^{2+}$	LEQLAGNLR
20	T12A	**1147.5789**$^{3+}$ **860.9359**$^{4+}$	ENIELGNGPLEEAISALYYYSTGGTQLPTLAR
21	T13A-CAM	**819.9207**$^{2+}$ **546.9496**$^{3+}$	SFIICIQMISEAAR

* Ions could be used to distinguish Ricin D sequence.

C. Method Validation Using Specific Peptide Targets

The working standard solution used for method validation was prepared by diluting a certified solution by the OPCW. To determine the limit of detection (LOD), low-level matrix-matched calibration standards were used for each peptide. The LOD was defined as the concentration that produced a signal-to-noise ratio (S/N) ≥ 3. Table III presents the LODs for the tryptic peptides, based on triplicate injections. The table lists the average signal-to-noise values for each peptide along with their corresponding standard deviations and relative standard deviations (RSD). The LODs ranged from 0.27 µg/mL to 2.65 µg/mL.

Table III

Analytical performance parameters for determining the LODs using Target Peptide Analysis method.

Tryptic peptides	S/N [1]	S[2]	RSD% [3]	LOD[4] (µg/ml)
$T7_A$	93,067	0,839	0,901	0,270 ± 0,839[a]
$T10_{B\text{-}SS}$	95,733	1,762	1,840	0,552 ± 1,762[b]
$T12_A$	14,833	0,404	2,725	0,81 7 ± 0,404[c]
$T13_B$	8,433	0,723	8,578	2,573 ± 0,723[d]
$T14_B$-SS-$T16_B$	23,233	2,055	8,845	2,654 ± 2,055[d]

[1]Signal to noise ratio, [2]standard deviation, [3]Relative standard deviation, [4]Limit of detection measurements, values are given as mean ± standard deviation (n = 3). Different letters in the same column indicate significantly different ($p < 0.05$).

Peptide identification confirmed by comparing the retention time of each analyte with that of the reference standard. A deviation in retention time of no more than 2% from the standard was accepted. Table IV summarizes the results of the repeatability and reproducibility experiments, with RSD% values ranging from 0.116% to 1.956%. To establish a 95.45% confidence level, the expanded uncertainty for the upper and lower control limits (UCL, LCL) was calculated using a coverage factor (k = 2).

Table IV

Intra-day repeatability and inter-day reproducibility of target peptides analytical method

Tryptic peptides	Retention time (min)	Intra-day repeatability			Inter-day reproducibility		
		RSD [1] (%)	UCL[2] (min)	LCL[2] (min)	RSD (%)	UCL (min)	LCL (min)
$T7_A$	5,163 [a]	1,589	5,327	4,999	1,956	5,344	4,941
$T10_{B\text{-}SS}$	8,486 [b]	0,325	8,541	8,431	0,954	8,764	8,436
$T12_A$	4,667 [c]	0,685	4,731	4,603	1,515	4,516	4,798
$T13_B$	5,713 [d]	1,219	5,852	5,574	1,885	5,441	5,867
$T14_B$-SS-$T16_B$	4,163 [e]	0,116	4,173	4,153	0,405	4,120	4,188

[1]Relative standard deviation, [2]Upper Control Limit, [3]Lower Control Limit, values are given as mean (n=9 for Injection, n=3 for Operators), Different letters in the same column indicate significantly different ($p < 0.05$).

The mass spectrometer operated in targeted mode, analyzing the specific peptides. For the repeatability and reproducibility studies, the most intense peptide ion was used. In accordance with OPCW criteria, the relative intensities of the detected ions (expressed as a percentage of the most intense ion or transition) were compared to those of the standard at comparable concentrations, under identical conditions. The tolerances are indicated in Table V The average ion ratios for the ion transitions were examined across nine injections (n=9) for each type of study and are summarized in Tables V and VI.

Table V

Intra-day repeatability of $T7_A$ target peptide ions transitions.

		Intra-day reproducibility	
Ions/transitions	Ion Ratio† (%)	Deviation* (%)	Tolerance# (%)
m/z 270.17	30.41 [a]	3.49	7.60
m/z 314.18	36.90 [b]	1.81	9.22
m/z 797.45	5.87 [c]	0.34	2.93

†Ion intensity (abundance) in the mass spectrum; intensity compared to that of the most abundant ion in the mass spectrum to obtain ion ratio, *Difference in peak area ion ratios between sample and standard, #Compare with the relative ion intensity of the standard: >50%: ±20%; >20% to 50%: ±25%; >10% to 20%: ±30%; ≤10%: ±50%, values are given as mean (n=9 for Injection, n=1 for Operators) Different letters in the same column indicate significantly different ($p < 0.05$).

Table VI

Inter-day reproducibility of **$T7_A$** target peptide ions transitions.

		Intra-day reproducibility	
Ions/transitions	Ion Ratio† (%)	Deviation* (%)	Tolerance# (%)
m/z 270.17	35.45 [a]	2.52	8.86
m/z 314.18	55.31 [b]	2.37	11.06
m/z 797.45	5.55 [c]	0.91	2.77

†Ion intensity (abundance) in the mass spectrum; intensity compared to that of the most abundant ion in the mass spectrum to obtain ion ratio, *Difference in peak area ion ratios between sample and standard, #Compare with the relative ion intensity of the standard: >50%: ±20%; >20% to 50%: ±25%; >10% to 20%: ±30%; ≤10%: ±50%, values are given as mean (n=9 for Injection, n=3 for Operators), Different letters in the same column indicate significantly different ($p < 0.05$).

Accuracy was evaluated by analyzing the target peptide T7A. The errors, listed in Table VII represent the average mass errors, measured in ppm.

Table VII

Intra-day repeatability and inter-day reproducibility of $T7_A$ target peptide calculated mass.

	Intra-day repeatability		Inter-day reproducibility	
peptide	Calculated[2] (m/z)	Accuracy[3] (ppm)	Calculated[2] (m/z)	Accuracy[3] (ppm)
T7A peptide (448.7692 m/z) [1]	448,76930	2.67	448.76810	2.45
	448,76930	2.67	448.76850	1.55
	448,76970	3.56	448.76870	1.11
	448,76760	1.11	448.76850	1.55
	448,76790	0.44	448.76940	0.44
	448,76630	4.01	448.76860	1.33
	448,76710	2.22	448.76890	0.66
	448,76850	0.89	448.76860	1.33
	448,76920	2.45	448.76820	2.22

[1]Theoretical mass of T7A tryptic peptide. [2] Calculated masses of T7A tryptic peptide, [3]Mass error presented in part per million, (n=9 for Injection, n=1 for Operators).

Selectivity was evaluated by comparing chromatograms from the negative control and a 2-ppm spiked sample, both in the presence of potential interfering compounds. As shown in Fig. 5, no significant interference was observed for the target peptides T7A, T10B-SS, T13B, T14B-SS-T16B, and T12A, with signal-to-noise ratios consistently above 3. This confirms the method's ability to selectively detect the ricin prepared from the Algerian cultivar.

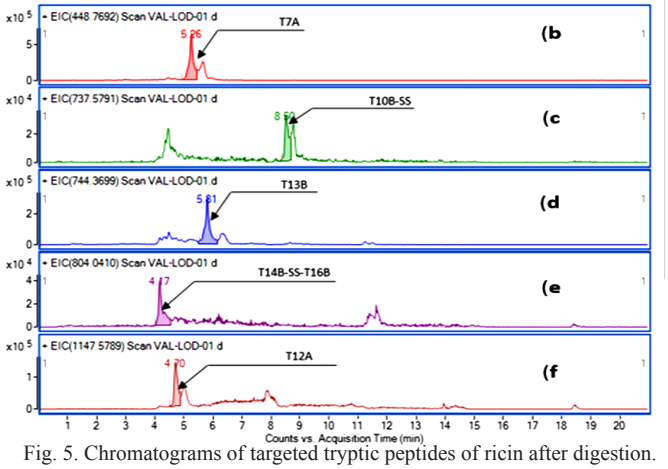


Fig. 5. Chromatograms of targeted tryptic peptides of ricin after digestion. Extracted ion chromatograms of tryptic peptides b) T7A. c) T10B-SS. d) T13B. e) T14B-SS-T16B. f) T12A.

CI. DISCUSSION

A. Peptides identification of Auto MS/MS Analysis for ricin *identification extracted from Algerian cultivar*

Several peptides were detected in the extract preparation analyzed in this study. Thirty-seven peptides, previously reported in the literature as prototypic peptides for ricin detection [35][36], were identified. The peptides detected at high intensity and suitable for identification were consistent with those proposed in earlier studies. The diagnostic peptides with high identification potential observed in this analysis included T7, T8, T9, T10, T11, T12, and T13A-CAM from the A chain, and T20, T18, T15, T13, T14, T11, T10, T6, T5, and T3 from the B chain. Additionally, the disulfide-linked peptides T14B-SS-T16B and T3B-SS-T5B were identified.

Tryptic peptides unique to ricin isoforms D and E were also screened. It was found that only peptides related to ricin D were present. In the D isoform of ricin, the amino acid sequence of the A chain is identical to that of ricin E, with differences between the two isoforms located in the B chain. Previously reported tryptic peptides that can distinguish these isoforms were also identified [5][37][38].

The disulfide-linked peptide T14B-SS-T16B was identified with a high electrospray response, consistent with previous studies using electrospray ionization. In addition to these ricin-specific peptides, other tryptic peptides were detected. Future work will focus on identifying marker peptides related to the specific *Ricinus communis* cultivar used in this study, as well as peptides related to other proteins present in the extract.

B. Method validation based on specific peptide targets

The limit of detection (LOD) was calculated based on the intensity ratio of each target peptide, using a signal-to-noise ratio (S/N) ≥ 3. The LOD varied between peptides, with the target peptide T14B-SS-T16B having the highest LOD at 2.65 µg/mL. As a result, this value was adopted as the LOD for our study. When preparing and injecting a dilution of spiked standard at a concentration of 2 ppm, the signal intensity of the target peptide remained consistent comparative with previous studies, where the LOD was determined to be 5 µg/mL. and interestingly, the signals decreased when injecting 5 µg/mL, attributed to thermal degradation of the peptides in the ionization chamber [39]. A case did not appear in our study and could also be due to the use of the Agilent Jet Stream electrospray ionization source, which operates with a low voltage and temperature, allowing us to maintain the 2.65 µg/mL LOD.

The identification of ricin at a concentration of 2.65 µg/mL in the crude extract was confirmed based on the expected retention time and intensity ratios of the five selected tryptic peptides. The assay's reproducibility and repeatability were evaluated by calculating the relative standard deviation (RSD) of multiple injections, with RSD values of less than 1.589% for repeatability and 1.956% for reproducibility. The results, presented in Table IV show consistency between different injections and operators for all target peptides. The control limits, set at a 95.45% confidence level, indicate that all measurements fell within the upper (UCL) and lower control limits (LCL), demonstrating good reproducibility across instruments and operators for retention time.

Tables V and VI show the identification of three precursor-product ion transitions of the T7A target peptide, which was selected for initial identification. The peak ratios of the T7A tryptic peptide, along with the ratio between all defined transitions, were calculated with their corresponding standard deviations. The precision values for peak ratios and intensities were found to be within the deviation tolerance for transition ratios and consistent with the average ion ratios obtained from positive control samples. These results align with the OPCW guidelines for ricin identification using precursor-product ion transitions.

The mass errors for the T7A tryptic peptide, listed in Table VII represent the average absolute values of individual peptide mass errors. The accurate mass was measured within 2.45 ppm, centered around the peptide mass without any mass correction or internal standard. Due to the consistently high mass accuracy of the tryptic peptides, a narrow mass window of 2 ppm could be used without any signal loss, which could be advantageous for more complex samples.

Selectivity was evaluated by comparing chromatograms obtained after the addition of interfering compounds from the crude extract preparation (Fig. 5) with those of the reference materials at a concentration level of 2 ppm. No significant matrix interferences were detected, except for a minor peak shape variation observed for the T10B-SS tryptic peptide, which led to the selection of an alternative ion transition for subsequent analyses. For all target peptides, the S/N ratio was above 3, demonstrating that the method is robust for qualitative confirmation at the LOD level.

CII. CONCLUSION

This study presents the development and validation of a method for the unequivocal identification of ricin from an Algerian *Ricinus communis* cultivar using LC-HR-MS and (LC-MS/MS) analysis. The method demonstrated limit of detection (LOD) values ranging from 0.27 µg/mL to 2.65 µg/mL, which are higher than those achieved by methods utilizing antibody-based affinity capture. This is because our approach did not involve affinity capture to enhance sensitivity by concentrating ricin prior to analysis, as is common in LC-MS methods that combine antibody-based techniques. Instead, our method focused on direct detection through specific tryptic peptides such as T7A, T12A, and T14B-SS-T16B, providing high specificity for ricin detection.

While the LODs presented here may not reach the sensitivity required for detecting ricin in ultra-trace amounts, they are fully suitable for the intended forensic application, where the primary goal is the unambiguous identification of ricin in a local environment. The reproducibility and robustness of the method were confirmed through consistent retention times and ion ratios, ensuring reliable detection across multiple analyses.

It was found that only peptides related to ricin D were present in the Algerian cultivar studied, Future work will focus on identifying marker peptides related to the specific Algerian *Ricinus communis* cultivar to distinguish it from other cultivars.

APPENDIX

Supplementary data can be found at the end of the article.

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APPENDIX



Fig 1S. Algerian *Ricinus communis* seeds sourced from Mila.

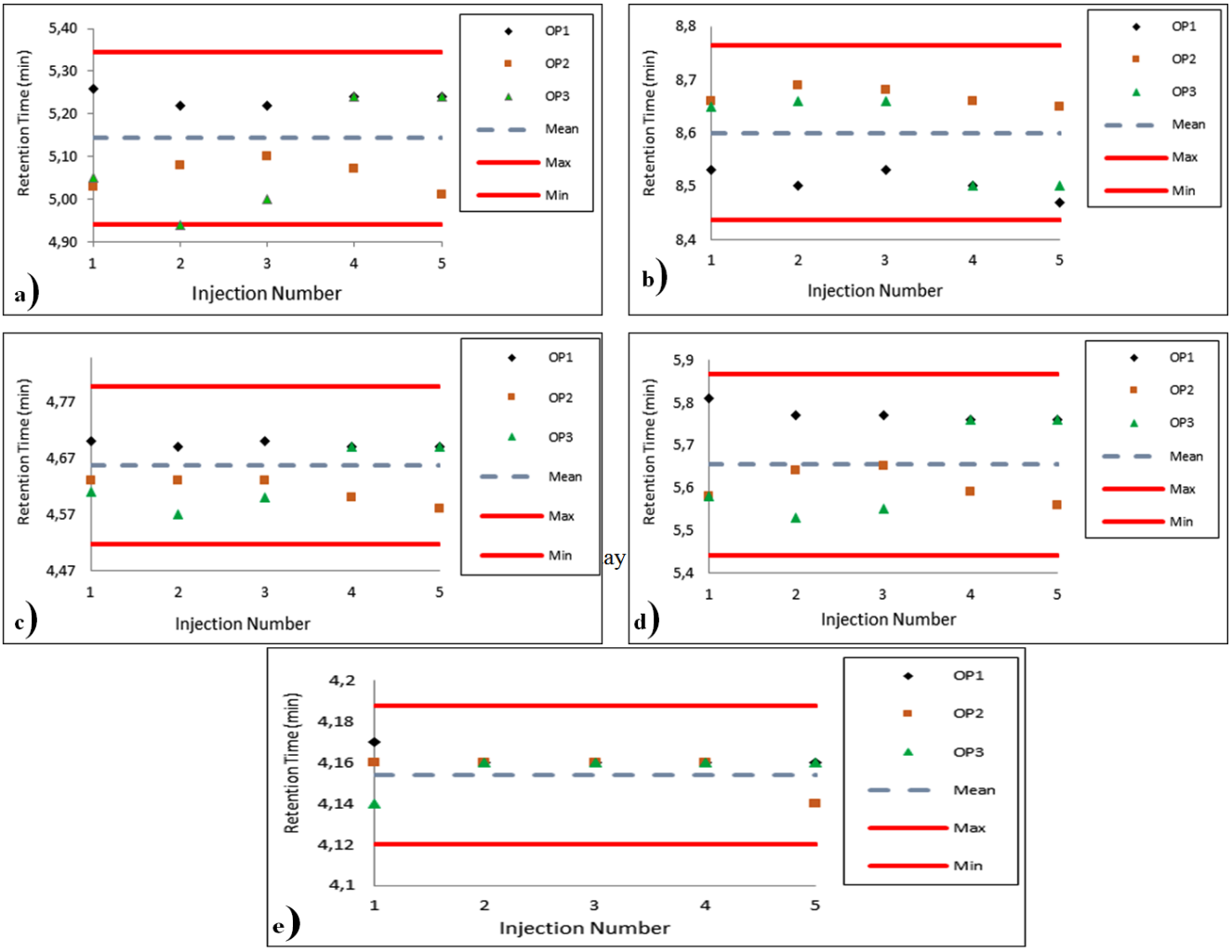


Fig 2S. Inter-day reproducibility.

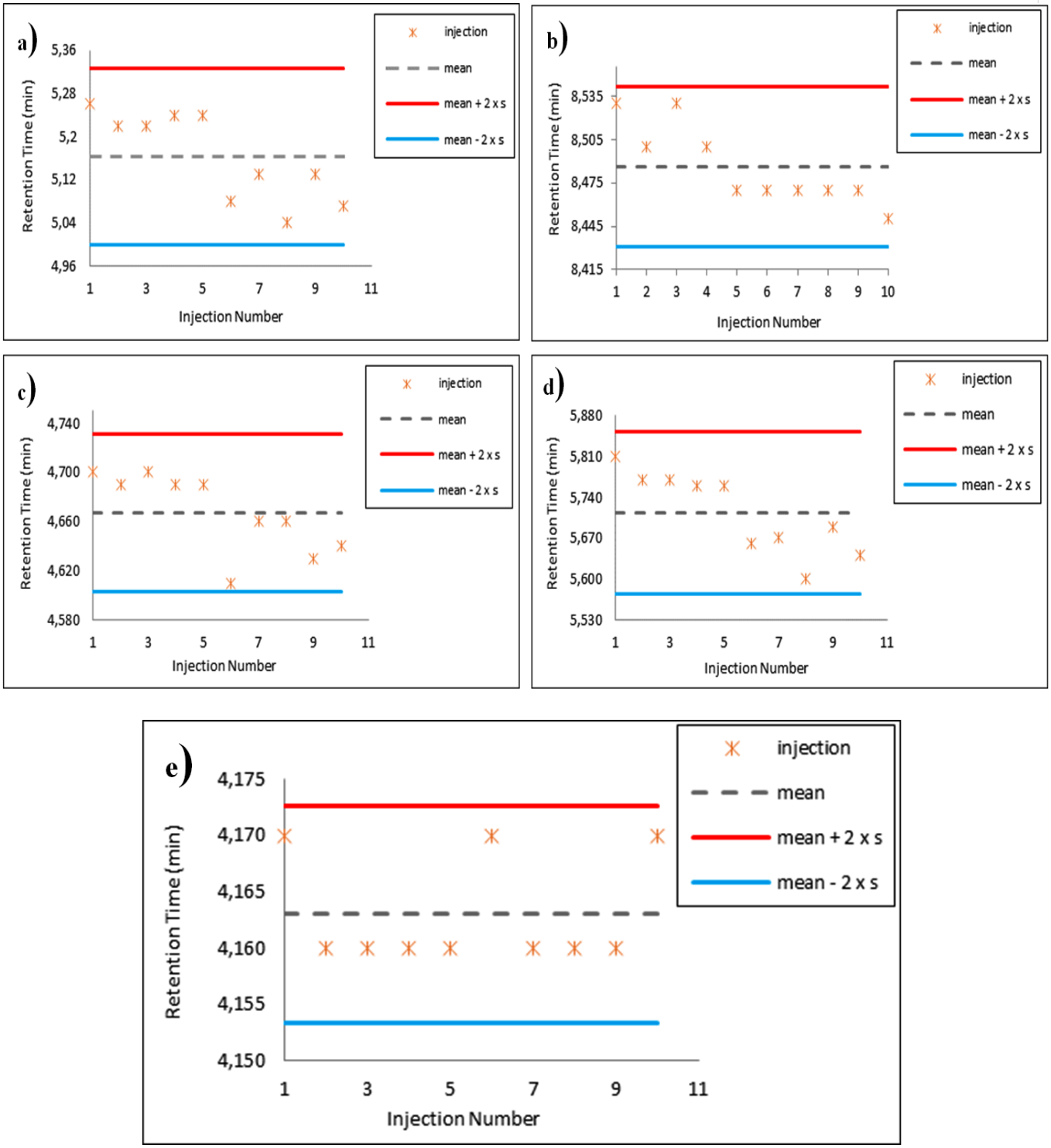


Fig 3S. Intra-day repeatability.