

Short Communication

## Quantification of Melittin in Iranian Honey Bee (*Apis mellifera meda*) Venom by Liquid Chromatography-electrospray Ionization-ion Trap Tandem Mass Spectrometry (LC-ESI-IT-MS/MS)

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### ABSTRACT

The current research aimed to quantify melittin (MEL) in Iranian honey bee (*Apis mellifera meda*) venom. To this end, a liquid chromatography-electrospray ionization-ion trap tandem mass spectrometry (LC-ESI-IT-MS/MS) approach was employed. Melittin is the main toxic peptide of honey bee venom with various biological and pharmacological activities. It was extracted with pure water from the bee venom samples. The analyses were performed on XBridge BEH300 C4 column using a gradient method with the mobile phase consisting of ultrapure water and acetonitrile (containing 0.1% formic acid). Signals of the melittin were recorded with the selected reaction monitoring (SRM) mode, which is a quantitative approach capable of quantifying analyte peptides with high sensitivity and. The mass spectrum of MEL was obtained in the positive ion mode and the quantification analysis was performed using precursor to product ion transition of m/z 570.2/669.9. This method demonstrated good linearity ( $R^2 > 0.997$ ) in the range of 1-100  $\mu\text{g mL}^{-1}$ , with a limit of quantification (LOQ) of 1.0  $\mu\text{g mL}^{-1}$ . The content of MEL in Iranian honey bee venom accounts for 43-55% of total dry weight. This method can be used to evaluate the quality and authenticity of bee venom samples for different therapeutic applications of MEL.

**Keywords:** *Apis mellifera meda*, Bee venom, Melittin, Peptide, LC-ESI-IT-MS/MS

### Quantification de la Mélittine dans le Venin des Abeilles Irlandaises (*Apis mellifera meda*) par Chromatographie en Phase Liquide- spectrométrie de Masse en Tandem avec Piège à L'ionisation-ion par Électronébuliseur (LC-ESI-IT-MS/MS)

**Résumé:** Cette recherche visait à quantifier la mélittine (MEL) dans le venin des abeilles irlandaises (*Apis mellifera meda*). À cette effet, une approche basée sur une chromatographie en phase liquide- spectrométrie de masse en tandem avec piège à l'ionisation-ion par électronébuliseur (LC-ESI-IT-MS/MS) a été employée. La mélittine est le principal peptide toxique du venin d'abeille mellifère ayant diverses activités biologiques et pharmacologiques. Elle a été extraite à partir des échantillons de venin d'abeille avec de l'eau pure. Les analyses ont été effectuées sur une colonne XBridge BEH300 C4 en utilisant une méthode de gradient avec une phase mobile comprenant de l'eau ultrapure et de l'acétonitrile (contenant 0,1% d'acide formique). Les spectres de la mélittine ont été enregistrés avec le mode de surveillance de la réaction sélectionné (SRM), qui est une approche quantitative capable de quantifier les peptides d'analyte avec une sensibilité élevée. Le spectre de masse de la MEL a été obtenu en mode ion positif et l'analyse de quantification a été effectuée utilisant un précurseur pour

produire une transition ionique de  $m/z$  570,2/669,9. Cette méthode a démontré une bonne linéarité ( $R^2 > 0,997$ ) dans la gamme de 1-100  $\mu\text{g mL}^{-1}$ , avec une limite de quantification (LOQ) de 1,0  $\mu\text{g mL}^{-1}$ . Le contenu de la MEL dans le venin d'abeille iranien compte 43 –55% du poids sec total. Cette méthode peut être utilisée pour évaluer la qualité et l'authenticité d'échantillons de venin d'abeille pour différentes applications thérapeutiques de la MEL.

**Mots-clés:** *Apis mellifera meda*, Venin d'abeille, Mélittine, Peptide, LC-ESI-IT-MS/MS

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## INTRODUCTION

*Apis mellifera meda* is a name first given by Skorikov in 1929 to a honey bee existing in the North Persia, Lencoron (on the Caspian Sea coast) (Ruttner et al., 1985). It is a subspecies of western honey bee that is widely distributed across Iran, northern Iraq, Syria, and the southeastern of Turkey (Ruttner et al., 1985; Eimanifar et al., 2017). Bee venom is a natural defense tool against predators, intruders, and for colony defense. This biotoxin contains various pharmacologically and enzymatically active components, such as melittin, apamin, mast cell deregulating peptide, phospholipase A, hyaluronidase, noradrenaline, histamine, and other smaller molecules (Oršolić, 2012). In traditional medicine, bee venom has been used to treat such diseases as rheumatoid arthritis, musculoskeletal pain, back pain, sciatica, and skin diseases. In addition, in recent years, it is widely used in the treatment of cancerous tumors, multiple sclerosis (MS), lupus, and Parkinson's disease (Son et al., 2007; Moreno et al., 2014). Melittin ( $\text{C}_{131}\text{H}_{229}\text{N}_{39}\text{O}_{31}$ ) is confirmed to be the principal component which constitutes approximately 50% of the venom dry weight and the major pain-producing substance of bee venom. It is a water-soluble amphipathic lytic peptide which consists of 26 amino acids with a molecular weight of 2846 Da. This peptide has a broad spectrum of various biological activities, such as antifungal, antibacterial, antiviral, anti-inflammatory, and anti-cancer activity on several types of cancer cells (Gajski and Garaj-Vrhovac, 2013; Moreno and Giralt, 2015). Several analytical methods have been used for the determination of melittin in bee

venom. They include capillary electrophoresis (Pacáková et al., 1995), high-performance liquid chromatography with ultraviolet detector (Szókán et al., 1994), diode array detector (Kokot and Matysiak, 2009; Haghi et al., 2013), fluorescence detector (Dong et al., 2015), mass spectrometry detector using triple quadrupole system (Zhou et al., 2010), and matrix-assisted laser desorption/ionization-time Of flight (MALDI-TOF), and nanoelectrospray ionization quadrupole time-of-flight(ESI-QqTOF) mass spectrometry (Matysiak et al., 2011). In the current study, melittin was extracted with pure water from bee venom samples and LC/ESI-MS/MS method coupled with ion trap mass spectrometer was described for the quantification of melittin (MEL) in Iranian honey bee (*Apis mellifera meda*) venom. The main analytical problem in complex biological matrices (i.e., coelution of interfering compounds) was resolved by  $\text{MS}^2$  mode since it facilitates further confirmatory data with molecular mass and characteristic structural fragmentation.

## MATERIAL AND METHODS

**Materials.** LC-MS grade acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany). Melittin (purity  $\geq 97\%$ ) was provided by Sigma-Aldrich (Steinheim, Germany). Samples of *Apis mellifera meda* venom were collected from northeastern Iran in summer.

**Instrumentation.** The analysis was performed using an Agilent Ion Trap LC/MS system consisting of an Agilent 1200 series LC system equipped with a micro vacuum degasser, a binary pump SL, an autosampler, a thermostatted column compartment (Agilent

Technologies, USA), a 6330 ion trap mass spectrometer, and an electrospray ionization (ESI) interface (Agilent Technologies, Germany). The instrument was controlled and processed by ChemStation data processing software (version B.01.03, SR1/SR2/714, 2007). The separation was carried out using XBridge BEH300 C4 column (150 mm×2.1 mm, 3.5  $\mu$ m; Waters, Ireland). The mobile phase consisted of solvent A: acetonitrile (containing 0.1% formic acid) and solvent B: ultra-pure water (containing 0.1% formic acid). This phase was used under gradient conditions as follows: 0 min, 78% B; 0-3 min, from 78% to 0% B; 3-6 min, holding at 0% B. Thereafter, the initial condition (78% B) was restored until 6.1 min and retained until 15 min. The temperature of the autosampler during the operation was held at 4 °C. The symmetrical peak was obtained at a column temperature of 30 °C and a flow rate of 0.2 mLmin<sup>-1</sup> with a sample injection volume of 1  $\mu$ L. Ionization of the melittin was performed in positive ion mode with a nebulizer gas pressure of 20 psi, a drying gas flow of 10 Lmin<sup>-1</sup>, and a drying gas temperature of 350 °C. The capillary voltage was adjusted at -4100 V. The detection of the ions was carried out in SRM mode with the precursor to product ion transitions of m/z 570/669.

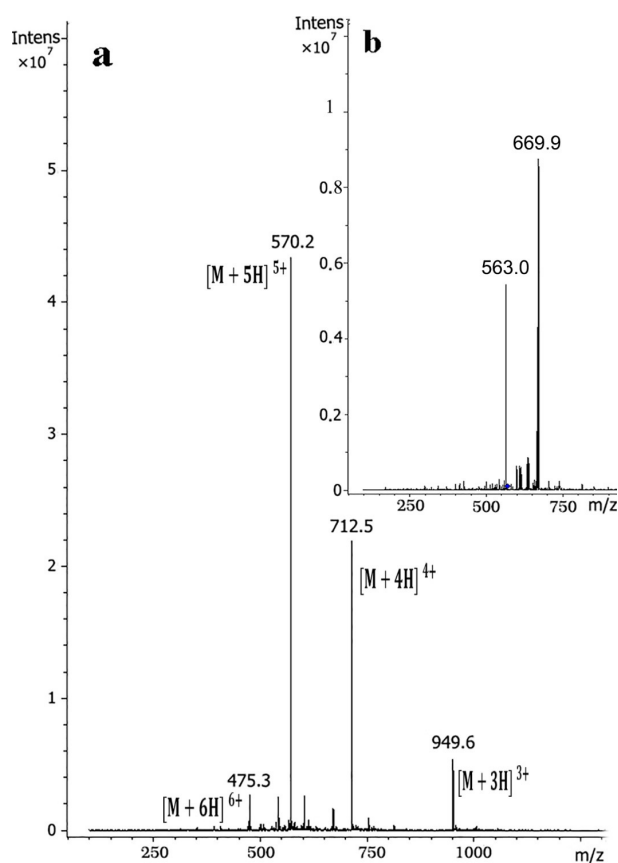
**Preparation of standard solutions.** The stock standard solution of MEL (1000  $\mu$ g mL<sup>-1</sup>) was prepared in ultrapure water, aliquoted and stored at -20 °C. The working solutions were prepared by appropriate dilution of stock solution using ultrapure water.

**Preparation of bee venom samples.** The freeze-dried venom samples (2 mg) were dissolved in 1 mL of ultrapure water and thoroughly mixed by a vortex shaker for 2 min. The resultant solutions were centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatants were diluted with ultrapure water and were used for analysis.

## RESULTS AND DISCUSSION

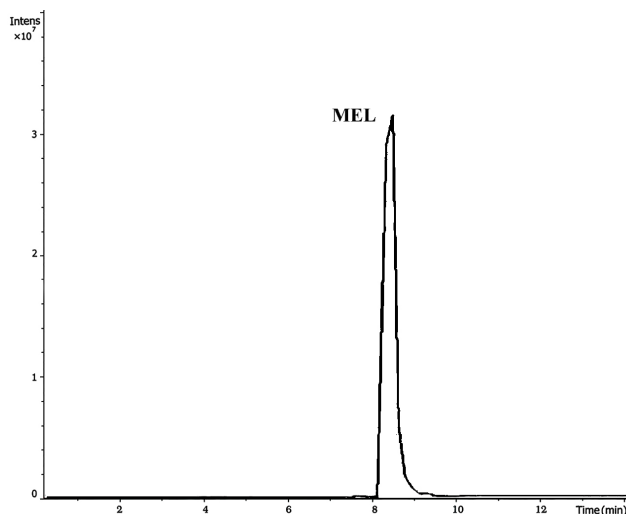
**Optimization of MS/MS conditions.** Melittin is a polar compound that takes protons and forms positive

molecular ions in the acidic solution. Therefore, the positive ion mode was selected for mass detection. The mass spectrometric parameters optimized by direct infusion of MEL standard solution were as follows: Oct 1 DC voltage 6.66 V, Trap Drive voltage 58.33 V, Cap Exit voltage 145.83 V. The full scan and product ion mass spectra of MEL were initially obtained (Figure 1) and the [M+5H]<sup>5+</sup> ion with m/z 570.2 was selected as the precursor ion due to its high abundance.



**Figure 1.** (a) full scan mass spectrum of melittin, (b) product ion spectrum of the mass-selected [M+5H]<sup>5+</sup> ion of m/z 570.2.

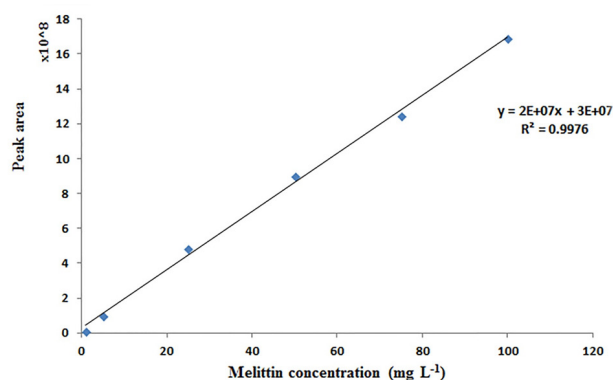
The major product ion at m/z 669.9 ( $y_{24}^{4+}$ ) created by the loss of two amino acid residues from the N-terminal side of MEL was chosen for the selected reaction monitoring (SRM) transition (m/z 570.2/669.9). The fragmentation amplitude was optimized at 0.92. Representative SRM chromatogram of MEL from the LC/ESI-MS/MS analysis is depicted in Figure 2a.



**Figure 2a.** Representative selected reaction monitoring chromatogram of melittin.

**Method validation.** Validation of the method was investigated in terms of linearity, the limit of quantification (LOQ), intraday and interday precision, recovery, and matrix effect. The calibration curve was obtained by plotting the peak area of MEL against the corresponding concentration in water (Figure 2b). It demonstrated good linearity ( $R^2 > 0.997$ ) over a wide concentration range ( $1-100 \text{ mg } \mu\text{L}^{-1}$ ) with LOQ of  $1.0 \text{ } \mu\text{g ml}^{-1}$ , based on a signal-to-noise ratio of 10. The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day) and reported as relative standard deviation (RSD). The intra-day and inter-day precisions were obtained at  $<8.5\%$  and  $<11.4\%$ , respectively, in venom samples at spiked concentrations of MEL ( $1, 10, \text{ and } 100 \text{ } \mu\text{g ml}^{-1}$ ). The recovery and matrix effects were evaluated by spiking bee venom samples with MEL at three different concentrations ( $1, 10, \text{ and } 100 \text{ mg } \mu\text{L}^{-1}$ ). The recovery of  $76-105\%$  was obtained by comparing the peak areas of MEL in venom samples that were spiked before extraction procedure (B) with those of the venom samples that were spiked after extraction procedure (A), [ $\text{Recovery} = (\text{B}/\text{A}) \times 100$ ]. With a similar equation, the matrix effect was calculated by the

comparison of the peak areas of MEL in ultrapure water (W) with those of the venom samples that were spiked with MEL at the same concentration (V), [ $\text{Matrix effect} = (\text{V}/\text{W}) \times 100$ ]. The mean matrix effect was calculated at  $89\%$  for six experiments. The ratio of less than  $100\%$  for matrix effect indicates a phenomenon called ion suppression which was caused by co-eluting matrix compounds in electrospray ionization source.



**Figure 2b.** Calibration curve of standard solutions of melittin in water

**Method application.** The validated method was used to determine the amount of melittin in *Apis mellifera meda* venom samples. Quantitation was performed using standard solutions of MEL in ultrapure water for external calibration. The obtained results reported that the average content of melittin in Iranian honey bee venom accounted for  $43-55\%$  of dry weight. It was consistent with melittin content in the venom of other species of *Apis mellifera* (Zhou et al., 2010). Nonetheless, melittin content in the bee venom can vary according to the season, climate, bee age, and feeding factors (Dong et al., 2015).

#### Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

#### Conflict of Interest

The authors declare that they have no conflict of interest.

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