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Determination of melatonin in *Acyrthosiphon pisum* aphids by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Melatonin is a hormone mainly involved in the regulation of circadian and seasonal rhythms in both invertebrates and vertebrates. Despite the identification of melatonin in many insects, its involvement in the insect seasonal response remains unclear. A liquid chromatography tandem mass spectrometry (LC–MS/MS) method has been developed for melatonin analysis in aphids (*Acyrthosiphon pisum*) for the first time. After comparing two different procedures and five extraction solvents, a sample preparation procedure with a mixture of methanol/water (50:50) was selected for melatonin extraction. The method was validated by analyzing melatonin recovery at three spiked concentrations (5, 50 and 100 pg/mg) and showed satisfactory recoveries (75–110%), and good repeatability, expressed as relative standard deviation (<10%). Limits of detection (LOD) and quantitation (LOQ) were 1 pg/mg and 5 pg/mg, respectively. Eight concentration levels were used for constructing the calibration curves which showed good linearity between LOQ and 200 times LOQ. The validated method was successfully applied to 26 aphid samples demonstrating its usefulness for melatonin determination in insects. This is -to our knowledge- the first identification of melatonin in aphids by LC–MS/MS.

1. Introduction

Melatonin (MEL) or 5-methoxy-N-acetyltryptamine (Fig. 1) is an indole hormone synthesized from L-tryptophan present in most organisms from bacteria to mammals (Tilden et al., 1997; Tomita et al., 2003; Ikegami and Yoshimura, 2012). In vertebrates, MEL is the main hormone secreted by the pineal gland and it is involved in the regulation of multiple physiological processes, including circadian rhythms, neuroendocrine signaling, cardiovascular and immune functions, and thermoregulation (Harasimowicz et al., 2012) being the conveyance of light/dark information generally considered its most prominent function. MEL synthesis is regulated by the circadian pacemaker, therefore MEL levels oscillate in a daily (circadian) manner, with the highest plasma levels occurring during the night (hence the nickname of hormone of darkness) (Hardeland, 2008; Hiragaki et al., 2015). In addition, because of the shorter days, MEL accumulates to higher levels during winter than during the long days of summer. As a result, at least in vertebrates, the synthesis of MEL is a key element in the regulation of both circadian and seasonal rhythms observed in the physiology and behavior of many organisms (Tilden et al., 1997; Hardeland, 2008; Ikegami and Yoshimura, 2012). The latter include adaptive responses observed in many photoperiodic organisms such as reproduction or migration limited to a particular season (Ikegami and Yoshimura, 2012).

While the presence of MEL has been demonstrated in different invertebrates including *Cnidaria* (Peres et al., 2014), *Nematoda* (Migliori et al., 2012), *Mollusca* (Muñoz et al., 2011), *Crustacea* (Aguzzi et al., 2009), and *Insecta* (Vivien-Roels and Pévet, 1993) among others, contrary to vertebrates, its involvement in the control of seasonal rhythms in these organisms, such as the onset and termination of diapause in insects, is not fully established (Ichihara et al., 2001).

Aphids (Hemiptera: Aphididae) were the first animals described as photoperiodic. These insects show a strong seasonal response, namely the development of sexual morphs triggered by the shortening of photoperiod (i.e. the decrease in day-length typical of winter). Although MEL was preliminarily shown to induce a partial seasonal response in aphids under non-inducing conditions (i.e. under long days) (Gao and Hardie, 1997), a definitive role for MEL in the process has not been yet demonstrated (Barberà et al., 2013). Preparation and accurate quantitation of MEL is a prerequisite to study the role (if any) of MEL in seasonal processes in invertebrates. The determination of MEL at very low concentration levels and in complex biological matrices needs specific and

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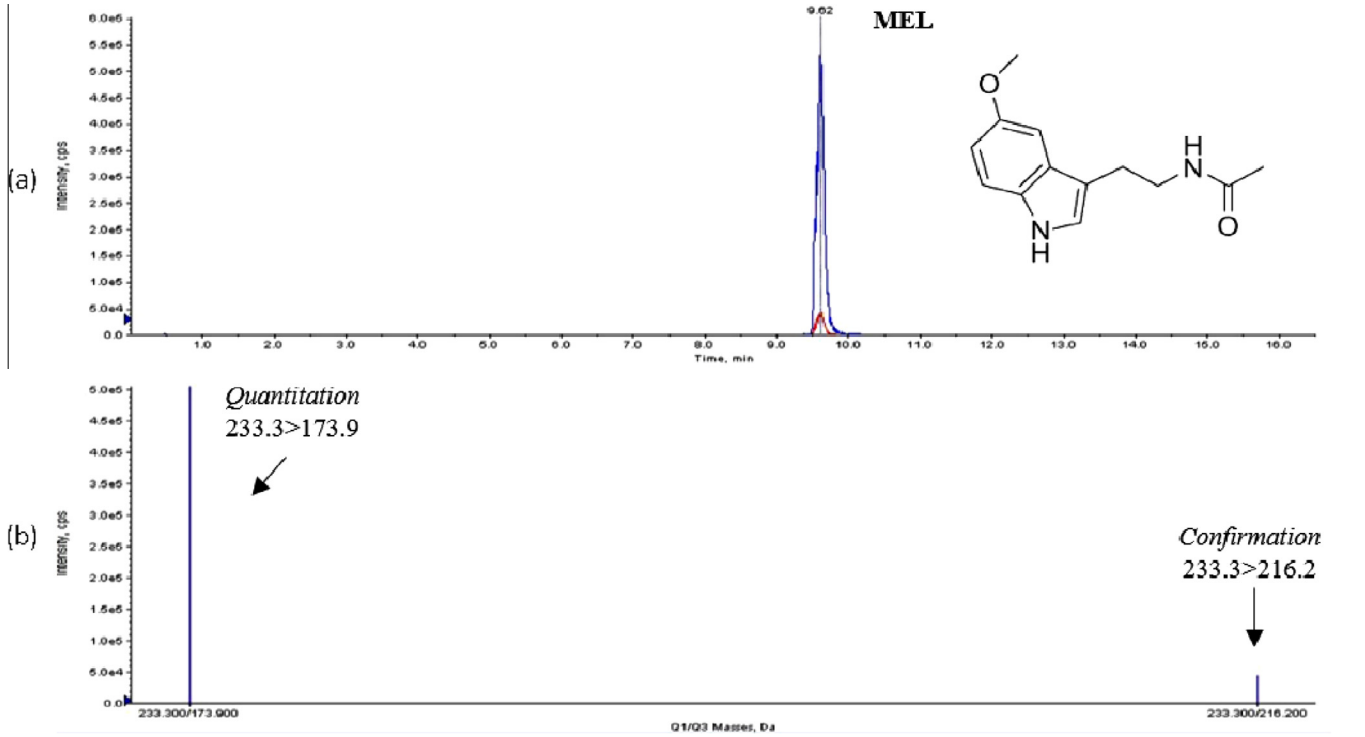

Fig. 1. (a) Total ion current chromatogram and chemical structure of MEL and (b) signal of the ion transitions used for the quantitation and confirmation in insect sample corresponding to 50 pg/mg.

sensitive methods (Poboz et al., 2005). Within the reported methods for MEL quantitation from different matrix, the use of antisera immunoassays such as radioimmunoassay (RIA) or enzyme-linked immunoassay (ELISA) remain the most popular methods for MEL quantitation in invertebrates (Yang et al., 2007; Suzuki et al., 2008). Only few studies were developed employing liquid chromatography (LC) coupled to the fluorometric (FLD) (Itoh and Sumi, 1998), electrochemical (ED) (Vieira et al., 2005), or ultraviolet (UV) (Tilden et al., 1997) detection for the identification and quantitation of MEL from insects. LC tandem mass spectrometry (MS/MS) is currently the gold standard for chemical identification when nuclear magnetic resonance (NMR) is not available, however, no LC–MS/MS method -recognized to combine selectivity, sensitivity, and high precision- has been developed until now for the analysis of MEL in insects.

The aim of this study was to develop a sensitive and specific LC–MS/MS method for MEL identification and quantitation in aphids performing a simple and generic preparation procedure.

2. Material and methods

2.1. Chemical and reagents

Methanol and ethanol were purchased from Fisher Scientific (Madrid, Spain). Deionized water (<18 MΩ cm^{-1} resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., CT, USA). Standard solution stock (purity: ⩾98%) of MEL (mw: 232.28) were purchased from Sigma Aldrich (Madrid, Spain).

2.2. Preparation of standard solutions

The stock solutions were daily prepared in darkness by dissolving 50 mg of MEL in 1 ml of pure methanol in amber glass vials, obtaining a 50 mg/ml (50,000 mg/L) solution. Standard solutions were daily prepared by dilution of the stock solutions with methanol in amber glass vials obtaining 500, 50, 10 and 1 μg/L solutions. All standards were kept at −20 °C before HPLC–MS/MS analysis.

2.3. Aphids

All aphids used corresponded to parthenogenetic females of the *Acyrthosiphon pisum* YR2 strain, long-established in our laboratory. Aphids were collected from the Vicia fabae plants where they were reared at 18 °C under long day conditions (i.e. 16 h light, 8 h dark) and sampled between ZT6 and ZT18. Aphids were taken directly from the stocks without regard to their developmental synchronization and sacrificed by immersion in liquid nitrogen.

2.4. Sample preparation and extraction

MEL extraction from insects was carried out on ice under dark conditions to prevent compound degradation. 100 mg of aphids were introduced into 2 ml tubes (wrapped in foil) and 0.5 ml of the mixture methanol/water (50–50 (v/v) were added. Samples were completely grounded using an Ultraturrax T8 IKA (Staufen, Germany) during 1 min and centrifuged at 14,000 rpm at 4 °C for 10 min. (Centrifuge 5810R, Eppendorf, Germany). The supernatant fraction was filtered through a 0.22 μm filter Phenomenex (Madrid, Spain) and 10 μl were injected into LC–MS/MS system.

2.5. LC–MS/MS analysis

Detection and quantitation of MEL was performed with 3200 QTrap® LC/MS/MS System (Applied Biosystems, Foster City, CA) equipped with a Turbo V® ionspray ESI source coupled to Agilent 1200 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA). Chromatographic separation of MEL was performed with a reversed-phase analytical column (Gemini® C18 column, 3-μm