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Short communication

A simple and sensitive approach to quantify methyl farnesoate in whole arthropods by matrix-solid phase dispersion and gas chromatography–mass spectrometry

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ABSTRACT

Methyl farnesoate (MF) is an arthropod hormone that plays a key role in the physiology of several arthropods' classes being implicated in biological processes such as molting and reproduction. The development of an analytical technique to quantify the levels of this compound in biological tissues can be of major importance for the field of aquaculture/apiculture conservation and in endocrine disruption studies. Therefore, the aim of this study was to develop a simple and sensitive method to measure native levels of MF in the tissue of three representative species from different arthropods classes with environmental and/or economic importance. Thus, a new approach using whole organisms and the combination of matrix solid-phase dispersion with gas chromatography coupled to mass spectrometry was developed. This method allows quantifying endogenous MF at low levels (LOQs in the 1.2–3.1 ng/g range) in three arthropod species, and could be expanded to additional arthropod classes. The found levels ranged between 2 and 12 ng/g depending on the studied species and gender. The overall recovery of the method was evaluated and ranged between 69 and 96%.

1. Introduction

Methyl Farnesoate (MF), one of the most important hormones in crustaceans and insects, is an isoprenoid structurally similar to insect juvenile hormone III (JHIII). This molecule is biosynthesized through the mevalonate pathway being the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), the rate-limiting step in the production of MF [1]. MF plays central roles in the regulation of crustacean development and reproduction [2,3]. It has been demonstrated that MF is implicated in crustaceans' reproductive maturation by increasing the production of vitellogenin and neonates [4,5], or by stimulation of gonadal development and maturation [4]. These aforementioned studies showed strong and direct evidences that MF is a reproductive hormone in crustaceans. Therefore, by increasing the levels of MF in cultured crustaceans (injecting the MF directly into the animals, or supplementing MF through the food) the productivity of crustacean aquaculture can be improved. Additionally, as MF is one of the major hormones of crustaceans, it is susceptible to be disrupted by environmental chemicals. A potential disturbance of the mevalonate signaling pathway by hypocholesterolaemic pharmaceuticals (statins) in arthropods has been associated with the reduction of reproductive performance in the amphipod *Gammarus locusta* and in the German cockroach *Blatella germanica* [6,7] supporting the view that statins can lead to a reduction of MF levels by inhibiting HMGR. Therefore, the quantification of MF in arthropods can be used as a proxy to evaluate the effects of these endocrine disrupting chemicals.

MF has been measured in different species of crustacean species [8,9] in several tissues such as hepatopancreas and mandibular organ and, specially, in hemolymph [8]. The presence of MF has been also described in insects [8,10], but in this case, the main role of MF is acting as intermediate in the JHIII synthesis that takes place in the *corpus allatum*. Although most of the descriptive works

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had located MF only in this tissue [8], recently, its presence in hemolymph in higher amount has been described [10].

Whereas there are some authors who have measured MF by high performance liquid chromatography (HPLC) using ultraviolet (UV) detection [11,12], the reference technique, due to its higher selectivity is gas chromatography coupled to mass spectrometry (GC–MS) working in selected ion monitoring (SIM) mode [13–15]. Moreover, chemical ionization (CI) has been the most frequently used ionization technique for the analysis of this biomolecule. The use of isobutane, instead of methane, as reagent gas in GC-CI-MS has been described since it allows a softer ionization giving the molecular ion $[M+H]^+$ as the base peak of the spectrum (m/z 251) [10].

In most studies describing the determination of MF in several organisms a previous extraction of hemolymph is mandatory [10,11,16,17]. This step led to tedious and time-consuming protocols since a large amount of hemolymph, and consequently a large number of animals, is needed to proceed with the quantification of MF. Thus, by introducing the use of matrix solid-phase dispersion (MSPD) this previous step can be avoided since the whole organism is used. MSPD is well-known in the environmental field, as extraction technique in the analysis of pollutants in a wide range of biota matrices [18]. This technique encompasses in the same step both extraction and purification simultaneously.

This work describes the development and validation of a MSPD protocol for the direct extraction of MF from three species of crustaceans and insects, including bees that have a key ecological and economic role and have been facing a dramatic decline in several regions [19]. The final protocol can be applied in the quantification of MF in whole animals and can be very useful in conservation biology, ecotoxicological studies of endocrine disrupting chemicals, but also for the field of aquaculture and apiculture.

2. Material & methods

2.1. Reagents and standards

Acetonitrile, ethyl acetate and *n*-hexane for analyses were purchased from Merck (Darmstadt, Germany). Bulk PSA (50 μm particle size) used as sample dispersant and Florisil (60–100 mesh) used as co-sorbent in the MSPD process were provided by Sigma-Aldrich (Milwaukee, WI, USA). Silica gel 60 (0.040–0.063 nm) tested also as co-sorbent was acquired from Merck. Empty solid-phase extraction polypropylene cartridges (15 mL volume) and polyethylene frits (20 μm) were purchased from International Sorbent Technology (Mid Glamorgan, UK). Standards of MF and methyl heptadecanoate-d_{33} (as internal standard, I.S.) were acquired from Echelon Biosciences (Salt Lake City, UT, USA) and Sigma-Aldrich, respectively.

2.2. Samples and sample preparation

The individuals used for method development were provided by CIIMAR (Portugal). The selected species were *Gammarus locusta* (GL) (from CIIMAR culture, derived from field animals collected in Sado estuary, Portugal) and *Artemia franciscana* (AF) (GSL *Artemia* cysts purchased from Ocean Nutrition, CA, USA), two model crustaceans frequently used in ecotoxicological/aquaculture studies. Moreover, the ecological and economic relevant insect *Apis mellifera* (AM) (sampled in the north of Portugal) was also studied. Each sample of GL and AM consisted of 3 individuals. This sample size corresponded to an average weight of 0.302 g in the case of male GL, 0.319 g for AM, and 0.147 g in the case of female GL. The AF samples contained 0.1 g of pooled 48 h nauplii. All the samples were frozen and stored at −80 °C until analysis.

A scheme of the complete extraction protocol is shown in Fig. 1. In the final protocol, the samples were mixed with 0.5 g of PSA and dispersed in a glass mortar, with a pestle, until a visually homogeneous mixture was obtained. Twenty microliters of methyl heptadecanoate-d_{33} as I.S. (50 μg/mL in ethyl acetate) were added. Then, the blend was loaded into a cartridge containing, consecutively, a polyethylene frit, 0.5 g of sodium sulphate anhydrous and 1.5 g of florisil. A second frit was placed over the dispersed sample and compressed. MF was eluted by gravity with 1.5 mL of ethyl acetate. The extracts were evaporated to dryness using a gentle nitrogen stream and reconstituted using 20 μL of ethyl acetate. The final extract was transferred to a 150 μL insert and directly injected in the GC-CI-MS system.

2.3. Instrumentation and determination conditions

MF was determined in a 7890A gas chromatograph combined with a 5975C quadrupole mass spectrometer from Agilent Technologies (Palo Alto, CA, USA), operated in positive CI mode with isobutane as reagent gas [10]. The column, also supplied by Agilent was a HP-5MS (30 m × 0.25 mm i.d., d.f.: 0.25 μm). One microliter of extract or standard was injected in the splitless mode for 1 min. Helium was used as carrier gas at a constant flow rate of 1 mL/min. The temperature set for injector and transfer line was 280 °C, whereas MS source and quadrupole were maintained at 300 °C and 150 °C, respectively. The oven temperature was maintained at 90 °C during 1 min and increased to 280 °C at 10 °C/min (hold for 10 min). An initial solvent delay of 11.5 min was used.

MF was measured in SIM mode using m/z 251 protonated molecular ion for quantification and m/z 219 and 191 as qualifier ions. These main fragment ions represent the loss of CH_3OH (m/z 219) followed by the loss of CO (m/z 191), representatives of a methyl ester. The internal standard methyl heptadecanoate-d_{33} was measured using the m/z 317.5 as quantification ion and 267.5 and 282.5 as qualifier ions.

2.4. Recovery calculation and samples quantification

Calculations of the recovery were made for each type of matrix, male and female GL, AF and AM. Three MSPD replicates of pooled real individuals belonging each class were processed without (unspiked samples) and with addition of MF (spiked samples). A recovery factor was estimated using an internal standard calibration curve by subtracting the mean blank concentration. Then, when quantifying real samples, the concentration was also calculated using the internal standard calibration curve and applying the recovery factor.

3. Results and discussion

3.1. Optimization of MSPD parameters

The optimization of the extraction procedure was carried out using adult females GL, since females were expected to have higher concentrations of natural MF, even presenting lower weight than male individuals. In each test, 3 females were used, thus, the mean sample weight was around 0.1 g. The ratio between sample and dispersant was 1:5. The amount of dispersant was selected according to bibliography recommendations [20] whereas a high amount of clean-up sorbent (1.5 g) was used due to the complexity of the extracts when the whole organism is used. The selection of PSA as dispersant sorbent was made due to its high selectivity when working with complex fatty samples [21]. This material consists of a silica gel base bonded with ethylenediamine-*N*-propyl groups and provides a mixed-mode retention mechanism.