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Quantitative determination of 20-hydroxyecdysone in methanolic extract of twigs from *Vitex polygama* Cham.

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Abstract

20-Hydroxyecdysone (20E) is effective in stimulating protein synthesis, therefore, it has been largely used as anabolic agent in several commercial formulas. Phytochemical study of methanolic extract of twigs from *Vitex polygama*, used in traditional Brazilian medicine as emenagogue, yielded a large quantity of 20E. This finding led us to developing and validating a simple and reliable method to determine 20E in the surveyed extract. Chromatographic separation of 20E was achieved on a phenyl-hexyl-based column using reversed elution mode. Extract was cleaned-up by solid phase extraction employing C_{18} cartridge, and an absolute recovery of 97% was acquired. External standard and standard addition calibration graphs were obtained and good linearity was accomplished ($r > 0.999$ for both curves). The limit of quantification and detection were determined. The results for accuracy fell within the −5 to +7% range.
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Keywords: Tarumã; Verbenaceae; Polypodine A; External standard; Standard addition; Solid-phase extraction

1. Introduction

Vitex polygama Cham. (Verbenaceae), commonly called “Maria-Preta, Tarumã and Velame-do-Campo”, is a widespread and well-known Brazilian species which occurs as trees or shrubs, mainly in Minas Gerais, Espírito Santo, Rio de Janeiro and São Paulo States [1]. Twigs and fruit are used in folk medicine as emenagogue and diuretic [2]. In our phytochemical investigation of methanolic extract of twigs, among several ecdysteroids we isolated and characterized 20-hydroxyecdysone (20E; Fig. 1). It is known that 20E is able to exert stimulatory effect on protein synthesis and increase metabolic activity [3], therefore, it is extensively used as anabolic agent in more than 180 commercial pharmacological preparations [4]. In contrast to anabolic vertebrate steroid hormones, 20E is not associated with the adverse androgenic, antigonadotropic or thymolytic side effects and cannot be readily detected by normal anti-doping assays [3]. Moreover, 20E has a number of pharmacological and physiological effects such as anti-arrhythmic, analgesic, antioxidant, anti-inflammatory, and antidiabetic among others [3].

For analytical purposes, ecdysteroids can be analyzed by high-performance liquid chromatography. Normal elution mode employing silica as stationary phase and mixtures of dichloromethane and alcohol as mobile phase are preferred [5,6]. Addition of water makes the resolution better but increases the time for equilibrium [7]. Both cyclohexane- and *iso*-octane-containing mixtures can also be used and help to improve the detection [7].

Elution in the reversed-phase mode has also been widely employed for the analysis of ecdysteroids, covering their whole polarity range [6,8,9]. Octadecyl silica has been the most used stationary phase, using aqueous mixtures with either methanol or acetonitrile, usually with the addition of 0.1–0.01% trifluoroacetic acid [7]. Generally, methanol–0.1 M triethanolamine phosphate at pH 1.8 is used to ameliorate poor reversed phase separation of 20-hydroxyecdysone [10].

Solid-phase extraction (SPE) has been performed for sample clean-up procedure employing either normal-phase,

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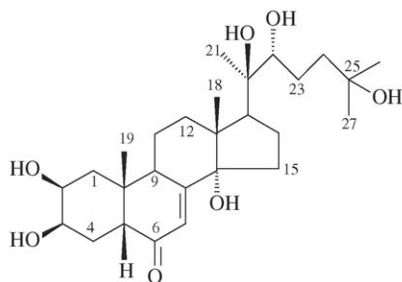

Fig. 1. Structure of 20-hydroxyecdysone.

reversed-phase or immobilized phenylboronic acid (PBA) phase [6,11,12]. Among these phases, PBA adsorption process is able to distinguish between ecdysone and 20-hydroxyecdysone-type compounds with the combined use of acid solutions, alkaline and acid buffers, and pH control [6].

In this paper, we present the validation of an HPLC method for analysis and quantification of 20E in methanolic extract of twigs from *V. polygama* making use of a phenyl-hexyl based column on reversed elution mode with sample clean-up performed by SPE using C_{18} cartridge, without employment of acid or buffer in the process.

2. Experimental

2.1. Chemicals and materials

Methanol (J.T. Baker, Philipsburg, PA, USA) used for the mobile phase was HPLC grade. All other solvent used in extraction and isolation were of analytical grade (Mallincrodt Baker, SA, Xalostoc, Mexico). Per-deuterated pyridine (C_5D_5N) was from Aldrich Chemical Company, Inc. (Milwaukee, Wis, USA) or Merck-Schuchardt (8011 Hohenbrunn bei München). Water was purified with Millipore Milli-Q apparatus (Millipore, São Paulo, SP, Brazil). Column chromatography was performed with either Kieselgel 60 (230–400 mesh, Merck KGaA, 64271, Darmstadt, Germany) or Sephadex LH-20 (25–100 μm, Pharmacie Fine Chemical Co. Ltd., Uppsala, Sweden). Solid phase extraction cartridge used was Bond Elut C_{18} 1 mL supplied by Varian (Harbor City, CA, USA).

2.2. Instrumentation

The HPLC system consisted of two Shimadzu LC-10AD pumps (Kyoto, Japan), an SUS mixer, an SIL-10AF auto sample injector, an SPD-10A UV–vis detector and an SPD-10AVP photodiode array detector. A CBM-10A interface was applied for both detectors. Data acquisition was done on CLASS LC10 software.

A stainless-steel column (150 mm × 4.6 mm I.D.) was home-packed with Luna® phenyl-hexyl-based silica (Phenomenex, Torrance, CA, USA; 10 μm particle and 100 Å pore sizes) by the ascending slurry method, using methanol for the preparation of the slurry (50 mL) and for the packing, which was carried out at a pressure of 7500 psi [13], and employed for the quantification analysis of 20E. A commercial phenyl-hexyl column (Phenomenex, Torrance, CA, USA, 250 mm × 4.6 mm I.D., 5 μm) was employed for the screening analysis of methanolic extract.

SPE clean-up was performed on a vacuum manifold processor (Varian, Harbor City, CA, USA) using a vacuum bomb TE-058 (Tecnal, Piracicaba, SP, Brazil).

1D and 2D NMR spectra were recorded on a Bruker Avance DRX-400 spectrometer (Karlsruhe, Germany) (^{1}H, 400 MHz; ^{13}C, 100 MHz). Low resolution electrospray mass spectra were carried out on a Micromass Quattro LC-triple quadrupole instrument (Manchester, UK), which was operated in the negative ion mode, scanning from *m/z* 100–700. The mass conditions were optimized by direct injection. The source voltage was 3.90 kV; capillary voltage, 26–28 V; extractor, 4 V; RF lens, 0.23 V; capillary temperature, 130 °C; desolvation temperature, 300 °C. Data acquisition and processing were carried using the MassLynx 3.0 software supplied with the instrument. Elemental analysis was obtained on a Fisions Instrument, EA1108-CHNS-O (Milan, Italy).

2.3. Chromatographic method development and validation

The final chromatographic system was chosen after a two-step screening analysis. First, the crude methanolic extract solution (10 mg crude extract in 1 mL MeOH) was submitted to the HPLC analysis employing a phenyl-hexyl 5 μm column (250 mm × 4.6 mm I.D.) using a gradient elution with methanol (B) in water (A) (5 a 100% B in 30 min, Δ% B = 3.17). Second, the preceding solution was cleaned-up by SPE and analyzed through the 10 μm phenyl-hexyl silica column (150 mm × 4.6 mm) under gradient elution −35 a 100% of B in 21 min. The injected volume was 10 μL, the flow rate of 1 mL min^{-1}, and the detection was acquired with DAD (200–400 nm) for both analyses.

The optimal conditions consisted of an isocratic solvent system containing 40% MeOH in water, delivered to the phenyl-hexyl 10 μm column (150 mm × 4.6 mm I.D.) at a flow rate of 1 mL min^{-1} over a run time of 20 min. The eluant was monitored at 254 nm detection wavelength under ambient temperature. The injected volume was 20 μL.

The method was validated by a set of parameters whose are in compliance with the recommendations as defined by ISO 17025 and Agência Nacional de Vigilância Sanitária (ANVISA) [14,15]. The accuracy was evaluated by back calculation and also tested using two blinded unknown concentrations, which were prepared by a different analyst. The samples were prepared in triplicate. The method selectivity was determined by comparison of chromatograms of the extract and standard 20E solutions, recorded with a photodiode array UV–vis detector. Identification was achieved by comparing the retention time of peaks. Limit of detection (LOD) was performed using the signal-to-noise ratio = 3/1 criterion. Limit of quantification (LOQ) was established analyzing spiked standard samples with serial diluted solutions, and was considered as the concentration at which the method is able to quantify the substance into 20% variability.

2.4. Sample preparation

2.4.1. Calibration solutions for the external standard curve

A stock solution of standard 20-hydroxyecdysone ($1000\,\mu g\,mL^{-1}$) was prepared in MeOH and stored at 4 °C. Aliquots of 100, 75, 50, 40, 30, 20 and 10 μL were placed in a 7.5 cm × 0.8 cm glass tubes and the solvent was evaporated to dryness at room temperature under a gentle stream of compressed air. The dry residues were reconstituted with 1000 μL of a 15% methanol aqueous solution. After vortex mixing for 10 s, solutions of 100, 75, 50, 40, 30, 20 and 10 $\mu g\,mL^{-1}$ were obtained. Aliquots of 50 μL from those solutions were passed through a C_{18} cartridge, which had been conditioned with 4 mL of MeOH followed by 4 mL of water, both by vacuum. The SPE sorbent was then washed with 4 mL of a 15% methanol aqueous solution. After drying, the analyte concentrated on the sorbent bed was desorbed with 1 mL of MeOH. The resultant solutions had the solvent evaporated. The dry residues were redissolved in 1000 μL of MeOH and vortex mixed for 10 s, affording calibration solutions of 5.0, 3.75, 2.5, 2.0, 1.5, 1.0 and 0.5 $\mu g\,mL^{-1}$. These samples were prepared in triplicate to determine the calibration curve, which was constructed by plotting the peak area against the concentration.

2.4.2. Calibration solutions for the standard addition curve

A stock solution of the extract ($500\,\mu g\,mL^{-1}$) was obtained by dissolving 2.0 mg of crude methanolic extract in 4 mL of MeOH through ultra-sonic mixing for 15 s. Fifty microliters of that solution were added to 50 μL of calibration standard solutions at the following concentrations 75, 50, 40, 30 and 20 $\mu g\,mL^{-1}$, respectively, into glass tubes and vortex mixed for 10 s. The solvent was evaporated to dryness and the dry residues were reconstituted with 1000 μL of a 15% methanol aqueous solution, vortex mixed for 10 s, to obtain standard solutions at a final concentration of 3.75, 2.5, 2.0, 1.5 and 1.0 $\mu g\,mL^{-1}$, respectively, added to crude extract solution. Later, the resultant solutions were submitted to the SPE clean-up procedure described in Section 2.4.1. An SPE-extract sample without addition of standard was obtained to consider the influence of matrix. The samples were prepared in triplicate to construct the standard addition curve by reporting peak areas as a function of analyte concentrations. The obtained calibration solutions were transferred into an autosample vial and a 20 μL aliquot was injected onto the LC column.

2.4.3. Control standard solutions

Aliquots of the stock solution of standard 20E ($1000\,\mu g\,mL^{-1}$) of 11, 60 and 90 μL were placed in a 7.5 cm × 0.8 cm glass tubes. Later, the same procedure described in Section 2.4.1 was carried out and solutions of 0.55, 3.0 and 4.5 $\mu g\,mL^{-1}$ were acquired to determine the relative recovery and evaluate the within- and between-day variability. The peak area ratios of five-extracted control solutions at each concentration were compared with those five not extracted samples to derive a percent recovery. Five samples of each concentration were prepared and analyzed on three non-consecutive days.

The absolute recovery was calculated by comparing the amount of 20E obtained through the parameters a and b from the standard addition curve equation and the amount of 20E acquired by external standard curve equation.

2.5. Plant material

V. polygama Cham. (Verbenaceae) twigs were collected in Poços de Caldas, Minas Gerais State, Brazil, in April, 2004. The plant was identified by Dr. Fátima Regina Salimena-Pires (UFJF). A voucher specimen is deposited in the Herbarium of the Universidade Federal de Juiz de Fora (CESJ), Minas Gerais State, Brazil.

2.6. Extraction and isolation of 20-hydroxyecdysone (20E) external standard

The air-dried powdered twigs (1.4 kg) of *V. polygama* were extracted with methanol through percolation processing at room temperature. Crude methanolic extract (69.2 g) was obtained after filtration and evaporation of the organic solvent under vacuum at 40 °C. A portion of the resultant methanolic extract (17.6 g) was dissolved in H_2O/MeOH 1:1 (v/v) and fractionated by liquid–liquid partition using CH_2Cl_2 and ethyl acetate affording the correspondent residues. 1.9 g of the ethyl acetate residue (EV) was submitted to column chromatography (3.0 cm × 46.0 cm) using Sephadex® LH-20 and MeOH as eluent, affording twenty-three fractions of 50 mL each. The similar fractions were joined, yielding seven one (EV1 to EV7). Fraction EV3 (551.0 mg) was rechromatographed over silica gel (2.5 cm × 16.0 cm) using gradient elution from EtOAc/CH_2Cl_2 9:1 to MeOH. Sixty fractions of 50 mL each were collected and pooled into 17 fractions (EV3A to EV3Q). Fraction EV3E (140.5 mg) was purified by preparative thin-layer chromatography on laboratory-prepared plates (20.0 cm × 20.0 cm; 3.0 mm thickness; 30.0 mg of fraction in 3.0 mL MeOH) covered with silica gel 60 F_{254}, employing double development with CH_2Cl_2/acetone 1:1 as mobile phase and UV detection, yielding 20-hydroxyecdysone (white powder, 77.7 mg), Polypodine B (white powder, 11.0 mg) and Stachysterone B (white powder, 14.3 mg). This procedure was repeated with isolated 20E to obtain a completely pure compound. 20E and the two other obtained ecdysteroids were characterized by 1D and 2D NMR, mass and microanalysis data, whose were in agreement with literature [16–18].

3. Results and discussion

3.1. Method development and solid phase extraction

To obtain initial information about the compounds in the crude methanolic extract of twigs from *V. polygama* as well as adjust the optimum conditions for quantification of 20E, an LC–DAD (200–400 nm) analysis was carried out using a 5 μm phenyl-hexyl silica column (250 mm × 4.6 mm I.D.) and a gradient elution of methanol (B) in water (A)—5 a 100% of B in 30 min [19] (Fig. 2c). Based on the chromatogram results, a

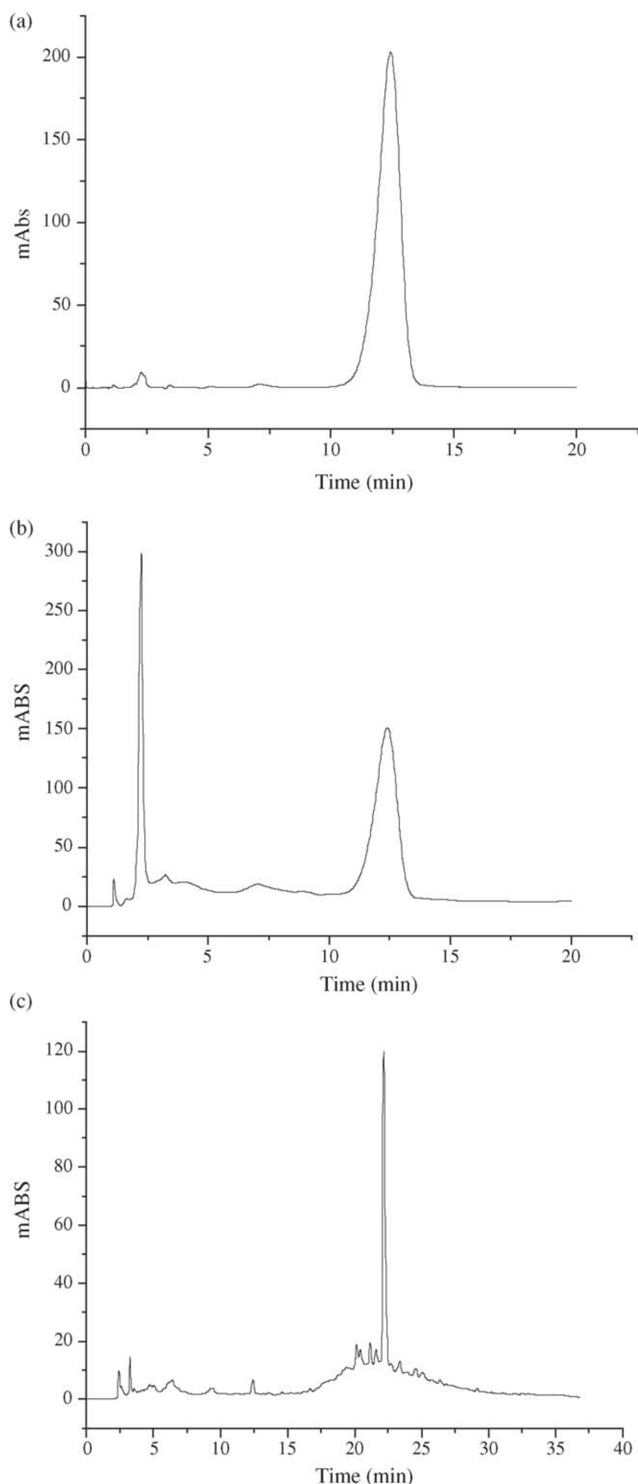

Fig. 2. Chromatograms of the analysis of 20-hydroxyecdysone in methanolic extract of twigs from *V. polygama*: (a) standard (1 mg mL^{-1}; injected volume 10 μL; optimal conditions Section 2.3); (b) SPE extract (10 mg mL^{-1}; injected volume 10 μL; optimal conditions Section 2.3); (c) crude extract (10 mg mL^{-1}; injected volume 10 μL; screening analysis conditions Section 2.3).

sample clean-up procedure was carried out through a C_{18} cartridge. This was selected rather than the suggested PBA one [6], considering the fact that only 20-hydroxyecdysone-type compounds were characterized and detected in both the current and earlier [2] studies of the surveyed extract, thus needing no selective retention and saving time and reagents. In addition, C_{18} cartridges are being successfully used to separate the two types of ecdysteroids in extracts containing mixtures of them [11]. The better composition of the wash solvent (water/MeOH 85:15) was selected after several tested mixtures. The crude methanolic extract solution obtained after SPE clean-up (SPE extract) was evaluated under gradient elution—35 a 100% of B in 21 min, using now a 10 μm phenyl-hexyl silica column (150 mm × 4.6 mm) to estimate subsequent conditions for the method development. Based on the retention time of the analyte and the concentration of B, an isocratic solvent system containing 40% of methanol in water was chosen for the quantification of 20E chromatographic band. Fig. 2 shows the chromatograms for the standard 20E (a) and the SPE extract (b) solutions.

3.2. Method validation

In view of the fact that SPE was employed for sample clean-up, the use of external calibrators was preferred to internal ones. External standard calibration curve obtained was validated by the standard addition method. This method of calibration is particularly important to evaluate the matrix interference, the recovery in the clean-up procedure, and recommended for conditions which the matrix without the analyte cannot be used for preparing the calibration solutions [20]. Besides the parallelism occurred between the regression lines from both curves showed the selectivity of the method. Regression analysis of the least-square line for data acquired from the external standard

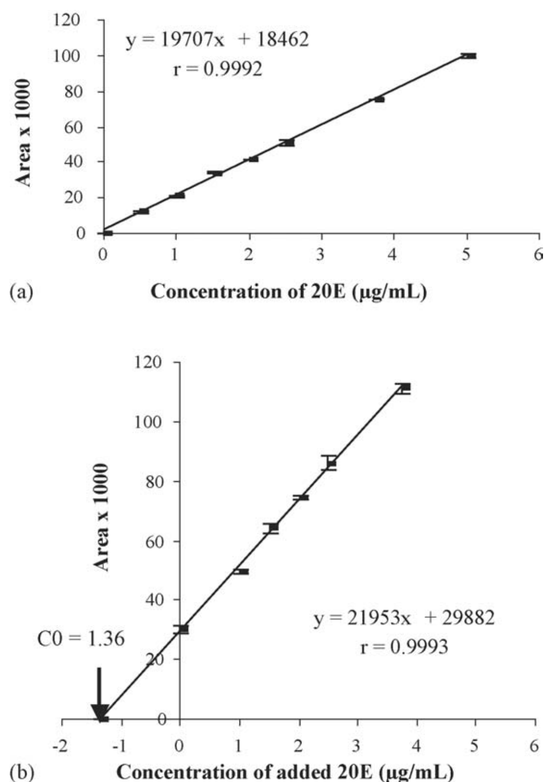

Fig. 3. Mean calibration curves for 20-hydroxyecdysone (mean ± 3S.D., $n = 3$): (a) external standard; (b) standard addition.

Table 1
Precision and accuracy data of 20-hydroxyecdysone, inter-day ($n = 5$)

Concentration ($\mu g\, mL^{-1}$)	1st day		2nd day		3rd day		Pooled ($n = 3$)	
	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
0.55	1.76	97.5	1.73	97.1	1.18	95.0	1.56	96.5
3.0	1.22	100.2	1.05	101.4	1.16	99.4	1.14	100.3
4.5	0.91	106.5	0.62	107.2	0.52	103.4	0.68	105.7

calibration curve showed good linearity at a 0.5–5.0 $\mu g\, mL^{-1}$ concentration range with a correlation coefficient above 0.999. The standard addition curve revealed a good linearity too, with a similar correlation coefficient. The calibration curves are shown in Fig. 3.

The intra- and inter-day precisions were assessed using the data of three quality controls analyzed over a 3-day period. The results are given in Table 1 and are expressed as coefficients of variation (CV, %). The accuracy evaluated from back calculation was expressed as the percent deviation between amount found and amount added of standard at the three concentrations examined. The two blinded concentration solutions, at the concentrations of 3.5 and 1.35 $\mu g\, mL^{-1}$, presented CV of 1.56 and 0.32% with accuracy of 102.8 and 103.6%, respectively.

The LOQ for 20E was 400 $ng\, mL^{-1}$, presenting a CV of 0.25% and accuracy of 81.4%, while the LOD was determined at 180 $ng\, mL^{-1}$.

A percentage relative recovery of 100 ± 4 ($n = 5$) for the standard was acquired. The absolute recovery of 20-hydroxyecdysone was found to be 97%, as determined by comparing the obtained analyte concentration through external standard curve with that one originated from the parameters obtained with the addition standard curve equation. Moreover, our analysis revealed that the content of 20E in methanolic extract of twigs from *V. polygama* is equivalent to 0.27% dry twigs weight (2528 $\mu g\, g^{-1}$), which is a relatively high concentration in comparison with other plants, e.g., *Leuzea carthamoides* ethanolic extract of roots (0.4% of dry matter), that have been commercialized [3,4].

4. Conclusion

The good linearity, precision, accuracy, sensitivity, selectivity, and small analysis time obtained under the established conditions confirm the suitability of the method as a routine and simple procedure for quantifying 20-hydroxyecdysone in methanolic extract of twigs from *V. polygama*. The sample preparation by means of a SPE clean-up on an octadecyl sorbent enabled the efficient removal of interfering compounds. The quantitative proportion of the analyte found in the extract also suggests the potential application of *V. polygama* in commercial formulas or as source of pure 20-hydroxyecdysone.

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