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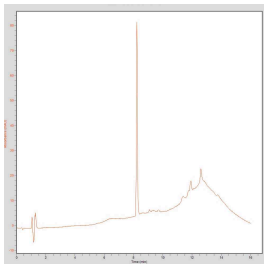
Short HPLC gradient method for 20-Hydroxyecdysone (20E) quantification in malaria vectors V.2



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We use this protocol and it's working

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Abstract

Ecdysteroids are arthropod steroid hormones that are primarily involved in insect moulting. In arthropod vectors, especially in mosquitoes, ecdysteroids of interest include mainly ecdysone (E) and 20-hydroxyecdysone (20E). These two compounds are involved in several important biological processes. Targeting these compounds and their regulatory pathways could lead to the characterisation of novel genetic tools towards implementing new malaria control strategies. To date, there are two main methods for quantifying E and 20E. These methods include an enzymatic method (Enzyme-Linked Immunosorbent Assay (ELISA)) and a chromatographic method (High-Performance Liquid Chromatography (HPLC)). However, for ecdysteroids quantification, the HPLC methods available in literature go from 30 minutes to one hour. Here, we developed a short HPLC gradient method for 20-hydroxyecdysone quantification in the malaria vector *Anopheles gambiae*. This method was developed specifically when sample material is limited as well as to save time and cost.

Guidelines

- A new column has to be equilibrated in the mobile phase for at least 1-2 hours before use and any buffer should be removed from the column daily with 20% methanol or Acetonitrile: 80% HPLC grade water and stored in 80% methanol or Acetonitrile: 20% HPLC grade water.
- The partial loop option should be chosen ("µL-pickup") for sample analysis to inject sample volumes as little as 1µL, resulting in no sample wastage and to avoid large volumes of standard sample preparation.
- All samples and mobile phase buffers should be filtered through a 0.45 um filter prior to being analysed.
- The 20E synthesis in mosquitoes is tightly regulated. To quantify the 20E in non-blood feed mosquitoes, be sure that female mosquitoes have sufficient time for mating to occur.
- All organic mobile phases used for HPLC analysis should be HPLC grade (Sigma Aldridge Inc).
- All other chemicals and standards should be of the highest purity grade (Sigma Aldridge Inc.)

Materials

1. **Reagents**

20-hydroxyecdysone **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H5142-5MG or H5142-10MG

Methanol HPLC **Fisher Scientific Catalog #**9093-03

Acetonitrile HPLC **fisher Catalog #**9012-03

Acetic acid glacial **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**ARK2183

Water MilliQ

2. **Consumables**

- Eppendorf tubes
- 2 mL white HPLC glass vials
- 250 µL conical glass Micro Inserts
- Membrane Filter 0.45 µm pore size
- 2 mL syringe
- Cones tips
- Micropipettes

3. **Equipments**

Equipment	
Flexar LC	NAME
High-Performance Liquid Chrommatography system	TYPE
Perkin Elmer	BRAND
N2910402	SKU

Troubleshooting

Safety warnings

Pump pressure should be closely monitored: an increasing pressure toward the limit of the system is an indication of mobile phase leaking (very low pressure) or blockage in the system (very high pressure).

Before start

- The HPLC system pump should be purged prior to usage to remove air bubbles.
- The autosampler (if available) should be flushed to clean the injection system.
- The column should be equilibrated with the mobile phases (starting conditions) for at least 30 min prior to initiating analyses.

Instrument

1 Analytical HPLC separations were performed on the Flexar LC system (Perkin Elmer) with a UV/Vis Detector and a Phenomenex Kinetex RP C18–5 µm column (4.6 × 150 mm).
The detection wavelength was set at 245 nm.
The flow rate was 1 mL/minute.
The mobile phase included gradient elution with Methanol:Acetonitrile (85-15) and 0.5% acetic acid-Water for 16 minutes (See the chromatographic conditions).
The injection volume was 1 µL.

Chromatographic conditions

2 Column: Phenomenex, Kinetex® 5 µm C18, 100 Å, 150 × 4.6 mm
Column temp: 20°C
Mobile phase A: 85:15:Methanol:acetonitrile (v:v)
Mobile phase B: 0.5% acetic acid: 95% HPLC grade water (v:v)
Flow rate: 1 mL/min
Detector: UV at 245 nm
Injection volume: 1 µL
Run time: 16 min
Gradient profile: (ramps are linear)

	A	B	C	D
			% Mobile phases	
	Time (min)	**Flow** (ml/min)	**A** (MeOH-ACN)	**B** H2O – 0.5% acetic acid
	0.5	1	10	90
	10	1	70	30
	6	1	10	90

Note

If available on the HPLC system, consider choosing the µL-pickup injection mode.

Mobile phase preparation

3 Mobile phase A: 85:15 Methanol:acetonitrile (v:v)
Mobile phase B: 0.5% acetic acid: water (v:v)

Preparation of mobile phase A

Mobile phase A was prepared by mixing methanol and acetonitrile to have a ratio of 85:15.
For example, to prepare 1 L , combine 850 mL of methanol, 150 mL of acetonitrile and mix well.

Preparation of mobile phase B

Mobile phase B was prepared by adding glacial acetic acid to water at a ratio of 0.5% glacial acetic acid.
For example, to prepare 1 L , combine 5 mL of glacial acetic acid and 995 mL of MilliQ water and mix.

Note

The solutions preparation may be scaled up as necessary.

Standard samples preparation

4 The standard samples used were prepared from the 20-hydroxyecdysone (20E) stock solution at (5 µg/µL).
All samples were prepared in methanol to a final volume of 500 µL.
Make three injections for each standard sample.

	A	B	C	D
	Concentration (µg/µL)	**Final volume (µL)**	**Volume of 20E solution (5 µg/µL) in µL**	**Volume of methanol to be added (µL)**
	2.5	500	250	250
	1.25	500	250	250
	0.625	500	250	250
	0.3125	500	250	250
	0.156	500	250	250
	0.078	500	7.8	492.2
	0.0097	500	0.97	499.03

Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ)

5 Example of calculation

Concentration (µg/µL)	Concentration as % of analyte target	Peak Area (mean of three injections)	Peak Area Standard Deviation	Peak Area RSD (%)
0.009765	0.10	24518.79	324.9409	1.33
0.078	0.78	102442.4	2838.319	2.77
0.15625	1.56	213946.2	7341.033	3.43
0.3125	3.13	366212.8	7090.87	1.94
0.625	6.25	790091.8	58677.44	7.43
1.25	12.50	1557900	26350.44	1.69
2.5	25.00	2776984	9269.747	0.33
Linearity range			0.009 to 2.5 µg/µL	
Equation for regression line: Y = 1118158*X + 45412			Correlation coefficient (R^2): 0.9964	

Equation for regression line: Y = 1118158*X + 45412		Correlation coefficient (R^2): 0.9964
SE of intercept		32934
SD of intercept	SE of intercept*sqrt(N)	87135.17368
LOQ	10*(SD of intercept/slope)	0.779
LOD	3.3*(SD of intercept/slope)	0.257

Note

In the "SD of intercept" calculation formula, "N" represents the total number of the standard samples.

Total ecdysteroid extraction and 20E quantification

6

Here, female mosquitoes of *Anopheles gambiae* (COGS strain) were used.

Total ecdysteroid was extracted using a mix of 95% methanol and 10% Ethanol (v:v).

1. Homogenize 20 adult females (4 days old) in 250 µL of mL of 95% methanol+10% Ethanol in 1.5 mL Eppendorf tubes using plastic pestles.
2. Vortex and centrifuge for 4 min at 5000 rpm
3. Transfer the supernatant to another Eppendorf tube
4. Homogenize the remaining pellet in 250 µL of 95% methanol +10% Ethanol
5. Vortex and centrifuge for 4 min at 5000 rpm
6. Poole the supernatant with the first one.
7. Filter the solution before injection.

6.1 **HPLC analysis of the extracts**

Inject the sample using the same method as with the standard samples.

Note

For the HPLC analysis, it was observed that with the 1 µL injection volume the interpolated X value (the concentration of the sample) falls outside the output range of X for the fitted curve. We then recommend analysing the mosquito extracts with at least 20 µL injection volume and reporting the calculated concentration to 1 µL injection by dividing the calculated value by a factor of 20.