Quantification of ecdysteroids and retinoic acids in whole daphnids by liquid chromatography-triple quadrupole mass spectrometry

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Abstract

Quantification of ecdysteroids and retinoic acids at picograms per individual is typically achieved with radioimmunoassay methods. However, those methods cannot identify individual types of ecdysteroids or provide an absolute concentration, which poses problems for comparative assays such as the metabolic profiling approach for toxicity testing. The method described in the present paper, based on liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry, was developed to allow the quantification in whole daphnids extracts of ecdysteroids (20-hydroxyecdysone, ecdysone, ponasterone A) and retinoic acid (sum of isomers). This approach avoids having to perform the difficult task of sampling the haemolymph on small organism (<5 mm). Recoveries, evaluated at three concentrations in matrix blank fortified samples, ranged from 83 to 119% for ecdysteroids and from 144 to 155% for retinoic acids. Precision (2.4 to 14.2%) and accuracy (-41.7 to 14.5%) were reproducible and stable over three quality controls concentrations. The described liquid chromatography-triple quadrupole mass spectrometry method achieved quantification limits ranging from 210 to 380 pg mL^{-1} for ecdysteroids and 5 ng mL^{-1} for retinoic acids in spiked matrix blanks. 20-hydroxyecdysone was quantified in *D. magna* adults (19 ± 8 pg ind^{-1}) and juveniles (3.6 ± 1.0 pg ind^{-1}), but was below the limit of quantification in neonates (≈0.19 pg ind^{-1}). Ecdysone was also detected in adult specimens (≈1.8 pg ind^{-1}).

1. Introduction

Contaminants of emerging concern such as pharmaceuticals, personal care products, flame retardants and plasticizers are transported into the aquatic environment mainly through municipal sewage. Although removal of these substances occurs in wastewater treatment plants, these facilities were not designed to eliminate those types of contaminants and therefore contaminants of emerging concern are continually released into the aquatic environment [1]. Effects of single contaminants of emerging concern on aquatic species at environmental concentrations (<100 ng L^{-1}) have been reported for a few compounds such as 17α-ethinylestradiol [2], ibuprofen and ciprofloxacin [3], however anthropogenic introduction of thousands of these compounds at nanogram-per-liter concentrations into surface waters still poses an unknown risk to the aquatic environment.

Current regulatory toxicity assessment of effluents and receiving waters that include endpoints such as mortality, behavioural effects and reproductive dysfunction are limited and are not able to detect differences between a control group and individuals exposed to contaminants of emerging concern at concentrations <1µg L^{-1} [4]. Thus, more sensitive bioassays are needed to detect subtle changes in aquatic species caused by prolonged exposure to trace amounts of mixtures of these compounds.

A metabolic profiling approach to toxicity testing may bridge this gap between environmental levels of contaminants of emerging concern and bioassay toxicity levels. Among the different organisms suitable for a metabolomic bioassay, the water flea *Daphnia magna* was chosen because it is already extensively used in laboratories, reproduces very quickly, and is relatively easy to culture. An adult *D. magna*, is able to reproduce every 3-4 days to an average of 6-10 neonates per clutch via cyclical parthenogenesis resulting in a clonal female population [5,6]. Studies have shown that ecdysteroids and terpenoids are potential candidates for a targeted metabolic bioassay since they are suspected to be involved in moulting, reproduction and stress response in *D. magna* and other crustaceans [7-10]. Therefore, they could be potential biomarkers for the detection of subtle toxic effects of contaminants of emerging concern in this organism. Quantification of ecdysteroids at the picogram-per-individual level in small organisms is usually done using radioimmunoassay methods [11,12], which although very sensitive, have several limitations: they cannot distinguish individual types of ecdysteroids, provide semi-quantitative values representing the sum of all cross-reacting substances (often called 'ecdysteroids equivalent') and do not account for the ecdysteroids that do not bind to available antisera due to different antisera specificity profile. As a result, data obtained using radioimmunoassay methods cannot be compared [13]. The relatively poor selectivity of radioimmunoassay methods quickly led to the development of selective gas chromatography-mass spectrometry methods using *N*-trimethylsilylimidazole derivatization [14,15]. Those methods required derivatization for 30 min to 60 hours followed by purification by thin-layer chromatography before analysis by gas chromatography–mass spectrometry. Interestingly, significant structural information can be obtained using those methods by comparing hydroxyl groups' reactivity; however since ecdysteroid stability during derivatization is unknown such long derivatization step makes routine application difficult and could affect method reproducibility.

More recently, methods focusing on the profiling and characterization of ecdysteroids have used liquid chromatography-triple quadrupole mass spectrometry with great success. A method using that technique achieved the detection of 20 pg per injection [16]. Further improvement was later done by another group using nano-liquid chromatography-quadrupole-linear ion trap mass spectrometry and achieving the detection of 4.81 pg per injection in *Drosophila melanogaster* larvae extracts [17]. A method of characterization and detection of ecdysteroids with liquid chromatography-tandem mass spectrometry using derivatization of ecdysteroids has also been published by Lavrynenko et al. (2013) in *D. melanogaster* with a detection limit of 10 pg per injection [18]. However, quantification methods of ecdysteroids using methods other than radioimmunoassay in small crustacean or insect such as *D.magna* or *Drosophila melanogaster* could not be found in scientific literature.

To the best of our knowledge, quantification of ecdysteroids including a complete validation and determination of the analytical precision and accuracy has been achieved for the *Bombyx mori* (silkworm) only [19] and no study has reported the quantification of retinoic acids in any crustacean or insect. Straightforward application of the method developed for silkworm was not possible due to different sample preparation requirements, the absence of optimization for retinoic acids in addition to ecdysteroids as well as the difference in analytical instruments at our disposal.

Our objective was to achieve detection of three ecdysteroids (20-hydroxyecdysone, ecdysone, ponasterone A) and two retinoic acids (9-cis-retinoic acid and all trans-retinoic acids) (Supplementary material, Fig.S1) at low pictogram levels in whole *D. magna* samples by optimizing analyte extraction, derivatization, and chromatographic separation. Such a method will allow the monitoring of the concentrations of these key metabolites in *D. magna* in order to study at the molecular level the effect on *D. magna* of exposure to mixtures of contaminants of emerging concern.

2. Material and methods

2.1. Reagents and chemicals

20-Hydroxyecdysone, ponasterone A, makisterone A, ecdysone, 9-cis retinoic acid (9-cis) and all-trans retinoic acid (all > 95% purity) were obtained from Santa Cruz Biotech (Dallas, TX, USA). Acitretin (> 95% purity) was purchased from Cedarlane (Burlington, Ontario). Makisterone A and acitretin were used as internal standards (ISTD) for ecdysteroids and retinoic acids quantification respectively. Additional purification of makisterone A to remove 20-hydroxyecdysone and ecdysone impurities was necessary and was done using ultra-performance liquid chromatography (UPLC) separation (Supplementary material, Fig. S2). Water, methanol, acetonitrile, methyl tert-butyl ether and liquid-chromatography mass spectrometry mobile phase additives formic acid, ammonium acetate and acetic acid are LC or LC-MS grade and were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The derivatization reagent hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) was purchased from Sigma Aldrich (ReagentPlus, purity > 99%). Frozen daphnids (Hikari Bio-Pure, Hayward, CA, USA), used as a matrix blank to prepare quality control samples, were purchased from a local aquarium store (Aquatica, Montreal, QC, Canada). Stock solutions were prepared at 0.1 mg mL^{-1} in methanol and stored at

-20°C. Working solutions were prepared in 1 % formic acid in methanol and stored at -20°C. A new aqueous solution of the derivatization agent was prepared fresh before each experiment.

2.2. Culture of Daphnia magna

Daphnids are cultured and maintained in ISO Standard Freshwater [20] at 25°C under a 16 h: 8 h light: dark photoperiod. Cultures are maintained at a density of 40 organisms per liter of culture medium. Culture medium is renewed once a week and daphnids are feed daily using a concentrated algal suspension of *P. subcapitata*. Daily rations were calculated to obtain 0.1-0.2 mg of organic carbon per daphnid per day using a nomograph plotting optical density versus total organic carbon in accordance with OECD 211 [21]. Algal cultures were grown in Bold's modified medium.

2.3. Sample preparation

2.3.1. Extraction and derivatization

Water fleas (*D.magna*) are sorted by size by filtering through a series of sieves (300µm, 560µm, 900µm). Adults are collected on the 900 µm sieve, juveniles on the 560 µm sieve and neonates on the bottom sieve (300 µm). Between 25 (adults) and 100 (juveniles and neonates) *D. magna* individuals are collected on a 250 µm tissue strainer (Pierce, Thermo Scientific), washed with deionized 18MΩ H_2O and sonicated in an ultrasonic bath (VWR symphony, model 97043-964) for 15 min in a volume of 1 mL 1% formic acid in methanol inside a 50 mL Falcon tube (Corning Life Sciences). Then, an 800 µL aliquot is evaporated to dryness under a $N_{2(g)}$ flow. Derivatization of ecdysteroids is done at 70°C for 90 min using 1 mL of a 100 mg mL^{-1} hydroxylamine hydrochloride aqueous solution. Analytes are then extracted from the aqueous phase using 2 × 1.5 mL of methyl tert-butyl ether. The organic phase is transferred in another glass tube, evaporated to dryness and reconstituted in 150 µL of methanol.

2.4. Quantitative analysis by ultra-performance liquid chromatography-triple quadrupole mass spectrometry

UPLC was performed on an Acquity system from Waters Corp. using a solid-core particle column. Experimental conditions of the chromatographic method are summarized in Table 1.

The UPLC system was coupled to a triple quadrupole mass spectrometer (Quattro Premier, Waters Inc.) equipped with an electrospray ionization (ESI) source. The ESI source was operated in the positive mode from 0 to 10 min and in the negative mode from 10 to 30 min. Data acquisition was performed in the selective reaction monitoring (SRM) mode. The method developed used electrospray ionization instead of atmospheric pressure chemical ionization as it is more commonly used in laboratories. Mass spectrometry parameters are summarized in Table 2.

Data processing was done using built-in software QuanLynx. Smoothing was done using the mean smoothing method. Quantification of analytes was done using calibration curves obtained by least squares linear regression of the ratio of the area of the analyte and the internal standard as a function of analyte concentration; no weighting function was used. Peak areas of retinoic acids and acitretin isomers were summed. Calibration was done using pure solution standards.

2.5. Method validation

2.5.1. Matrix effects, recovery and process efficiency

Matrix effects were evaluated using two different techniques : postextraction addition and postcolumn infusion as described by Taylor [22]. Briefly, postextraction addition consists in spiking the analytes to pure solvent solutions and to extracted matrix blanks and measuring the resulting signals. The ratio between the mean peak area of the postextraction addition samples and the mean peak area of a pure solution give us the matrix effects. Extracts of frozen daphnids were used as matrix blank quality control (QC) samples and were fortified at 285, 475, 713 pg mL^{-1} for 20E, at 318, 530, 795 pg mL^{-1} for E, at 411, 685, 1028 pg mL^{-1} for PonA, and at 7.7, 12.8, 19.2 ng mL^{-1} for retinoic acids in order to evaluate matrix effects at low (QC_{LOW}), medium (QC_{MED}) and high (QC_{HIGH}) levels. Determination of matrix effects by postcolumn infusion consists in injecting a non-fortified matrix blank and adding to the column effluent a solution containing the analytes using a tee connector and a syringe pump. This technique thus allows us to determine the presence of matrix effects during the whole chromatographic run. Recovery was calculated as the ratio between preaddition extraction and the mean peak area of the postextraction addition solution.

2.5.2. Accuracy, precision, lower limit of quantification and limit of detection

Accuracy and precision (intra and interday) were calculated using the QC samples described previously. Accuracy is expressed as the mean relative error (bias) and precision as the coefficient of variation (CV%) of 5 injections done the same day (intraday) or 5 injections done in 5 different days (interday). Acceptable lower limit of quantification (LLOQ) is defined according to U.S. Food and Drug Administration (FDA) guidelines [23] as the lowest analyte concentration giving an accuracy within ±20% (bias), inter-assay precision (CV%) ≤20% and minimum signal-to-noise ratio (S/N) >8. The limit of detection (LOD) is determined as the concentration achieving a minimum S/N > 3.

2.5.3. Stability

Short-term stability of analytes at -20° C in methanolic extracts was evaluated with matrix fortified frozen daphnids using the QCs described previously. Each experiment was performed in duplicate.

3. Results and discussion

3.1. Optimization of sample preparation

Initial sensitivity and LLOQ assessment of the triple quadrupole mass analyzer (Quattro Premier, Waters Corp.) for the targeted compounds proved insufficient (Fig.S3, Supplementary material) for the quantification of the low level of ecdysteroids in *D.magna*. Consequently, derivatization was needed to improve sensitivity. Several derivatization agents were evaluated and our selection processes was guided by three criteria: 1) the derivatization agent is able to react with the majority of known ecdysteroids; 2) the derivatization reaction should not add bulky groups to the molecular structure of the ecdysteroids that could shift retention times considerably, given that chromatographic separation of ecdysteroids had already been achieved successfully; and 3) the derivatization reaction should not cause the elimination of possible conjugated forms [24]. Taking into consideration criterion #1 we aimed for the derivatization of the 6-keto group, the most conserved group among ecdysteroids [25], to enhance ionization. Among the derivatization agents used for ketone derivatization we selected hydroxylamine, which yields a ketoxime. Other sterol ketone derivatization agents used for mass spectrometry detection [26] such as 1-(carboxymethyl)pyridinium chloride hydrazide (Girard P) , 1-(carboxymethyl)trimethylammonium chloride hydrazide (Girard T), 2-hydrazino-1-methylpyridine (HMP) or 2-hydrazinopyridine (HP), were eliminated due to one or more unmet criteria.

Experiments showed that optimal pH and reaction time for the derivatization of ecdysteroids with hydroxylamine were different than those normally used for mammalian 3-keto sterols [27]. Derivatization reaction (Supplementary material, Fig.S4) was ultimately successful at lower pH (a pH of 2.7 was used instead of 10), lower temperatures (70°C instead of 90°C) and longer derivatization time (90 min instead of 30 min) (Fig. 1). As expected, chromatographic separation was maintained (Supplementary material, Fig.S5) and it was achieved faster than for Girard P or Girard T derivatized ecdysteroids [18].

Elimination of a water molecule was observed for all the derivatized ecdysteroids. We suspect that this is not an ion-source fragment but rather an elimination in solution due to the acidic conditions used. Reaction yield was also evaluated to be >97% by quantifying remaining underivatized ecdysteroids form in a fortified matrix blank (Fig. S6, Supplementary Information). Confirmation of the proposed structure of the ecdysteroid 6-ketoximes was not performed due to the limited amount of ecdysteroids available. However, previously published specific loss of the 14-hydroxyl group yielding a double bond between C14 and C15 is the most probable explanation for this water loss [18,28].

Retinoic acids isomerization occurs upon exposure to ambient light and can be delayed by using amber glass vials. However, this additional step was not taken for method validation as retinoic acids were never detected in daphnids. As a result, the sum of the different isomers of retinoic acids areas was used for quantitative analysis.

3.2. Optimization of liquid chromatography

Adequate chromatographic resolution of the target analytes can be achieved with classic porous C_{18} UPLC columns; however liquid chromatography optimization experiments showed that UPLC columns containing solid-core particles were able to achieve better chromatographic resolution for target retinoic acids (Fig. 2). Different combination of solvents (acetonitrile, methanol, isopropanol, H_2O) and additives (formic acid, acetic acid) were tested in order to achieve optimal separation and signal intensity for target analytes. As shown in Fig. 3, optimal mobile phase additive is 0.1% acetic acid. We observed that this mobile phase additive achieves the highest signal and signal-to-noise ratio for all analytes, compared to the other conditions tested, when coupled with polarity switch and hydroxylamine derivatization. This figure also shows the most abundant ion detected for each mobile phase additives tested in both negative and positive mode. Interestingly, underivatized ecdysteroids form negatively charged acetate adducts when acetic acid and ammonium acetate is used. In some cases, these acetate adducts achieve higher signal intensities than the protonated molecule $[M+H]^+$ and the formate adducts.

3.3. Method validation

3.3.1. Matrix effects, recovery and stability.

Matrix effects were assessed using two methods appropriate for liquid chromatography-mass spectrometry methods: postcolumn infusion and postextraction addition. Postcolumn infusion method was used as a qualitative determination of matrix effects over the entire chromatographic run during method development. As shown in the chromatograms presented in Supplementary material (Fig. S7), signal suppression for the chosen mobile phases (0.1% acetic acid) is low or the first 10 min of the chromatographic run; and increases significantly after. However, further improvements to reduce ionization suppression of retinoic acids could not be achieved without modifications to sample preparation and or chromatographic modification resulting in ecdysteroids and retinoic acids loss and/or ionization reduction. Quantitative measurement of matrix effects using the post addition method, reported in Table 3, shows that low (<11%) signal enhancement was observed for 20E and slight signal suppression was observed for E (>-10.1%). However significant ME are observed for Pon A (-17.2 to -13.1%.) and RA (-68.1 to -66.1%). In order to obtain the true value of ponasterone A and retinoic acids, the detected concentration can be corrected using accuracy bias corresponding to the closest QC samples. The accuracy bias can be used for that purpose since it quantifies the effect of matrix effects and sample preparation on the signal of the analyte. For example, if ponasterone A is detected in samples at 500 pg mL^{-1}, a correction factor of -30.5% (interday accuracy bias for ponasterone A) would be applied, giving a true concentration of 719 pg mL^{-1}. Surprisingly, ponasterone A signal suppression was higher than what was evaluated with post column infusion method (-10%). Although very useful for method development, post column infusion is not a substitute to post addition method when determining matrix effects.

Relative recovery for targeted analytes ranged from 83 to 155 %. Higher recoveries for retinoic acids cannot be fully explained but met FDA validation criteria since they remained consistent, precise, and reproducible over three QC concentrations [23]. Short term stability of methanolic extracts was evaluated at -20 °C, results are presented in Table 5. Daphnids methanolic extracts

were stable for 24h; extracts kept for more than 24h at -20°C resulted in higher bias for 20-hydroxyecdysone and ponasterone A.

3.3.1. Accuracy and precision and lower limit of quantification (LLOQ)

Accuracy and precision were evaluated using fortified matrix blanks QC samples and reported in Table 3. Analytical precision of all QCs (2.4 to 14.2%) were below acceptable limits (<15%) according to FDA. Accuracy for 20-hydroxyecdysone and ecdysone met FDA validation criteria. However, a negative bias was observed for ponasterone A and retinoic acids and this bias is attributed to higher matrix effect for these analytes. The negative bias between pure solution standard and quality control prepared using frozen daphnids matrix blank was reproducible and can be easily corrected mathematically to obtain actual ponasterone A and retinoic acids levels in daphnids. As matrix blank QC samples may not be readily available for other species, the use of pure solution standards as calibrants was preferred in order for the method to be potentially applied to other similar small crustaceans and arthropods with minimal partial validation.

Results for the determination of LOD and LLOQ are shown in Table 4. LOD between 0.04 and 0.22 pg per injection for ecdysteroids and 10 pg per injection for retinoic acids achieved with the present method are lower than previously published methods using LC-MS [17]. LLOQ is similar to what is obtained with radioimmunoassay methods, which typical range is between 100 to 3500 pg [13]. However, method specificity is greatly improved as identification of individual type of ecdysteroid is now possible using both retention time and MS detection. More importantly, results are no longer dependent on the antisera used and correction using cross-reaction factors is not needed. Therefore, data obtained with the liquid chromatography-triple quadrupole mass spectrometry method developed can be more easily compared.

3.4. Analysis of ecdysteroids and retinoic acids in D. magna cultures

The developed method was applied to the analysis of 20-hydroxyecdysone, ecdysone, ponasterone A and retinoic acids in laboratory-cultured daphnids. Reported concentrations are presented in Table 6. Detection of 20-hydroxyecdysone in *D. magna* neonates, juveniles and adults was achieved (representative chromatograms are presented in Fig. 4). While detection of 20-hydroxyecdysone was possible at all development stages of *D. magna*, quantification was only possible in specimens larger than 560 µm (juveniles and adults). These results are of the same order of magnitude than previous studies on the presence of ecdysteroids in adult daphnids [10], which reported ecdysteroids equivalents between 5 to 10 pg per individual to 250 pg per individual using radioimmunoassay. Neither makisterone A (internal standard), ponasterone A or retinoic acids were detected in daphnids extracts.

4. Conclusion

Sample preparation and chromatographic separation were optimized to allow quantification of picogram per individual levels of ecdysteroids and retinoic acids in *D. magna* using liquid chromatography-triple quadrupole mass spectrometry. Such method is particularly useful in small organisms where hemolymph extraction is difficult. The method presented fulfilled standard analytical validation criteria in terms of precision (<15%), accuracy (<±15%), for 20-

hydroxyecdysone and ecdysone. Although the negative accuracy observed for ponasterone A and retinoic acids is higher than FDA acceptance criteria, this negative bias was reproducible, stable over three QCs concentrations and can be explained by matrix effects. To the best of the authors' knowledge these are the first results reported on the use of a quantitative approach for the analysis of complex small organisms without hemolymph sampling. The applicability of the method was demonstrated by analysis of *D. magna* extracts in adults, juvenile and neonate daphnids. 20-hydroxyecdysone could be quantified in both adults and juveniles but was below LLOQ in neonates; ecdysone was only detectable in adult specimens. Further work is required to focus on the evaluation of daphnids ecdysteroids levels in toxicity response upon exposure to environmental concentrations of mixtures of contaminants of emerging concern.

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Figures

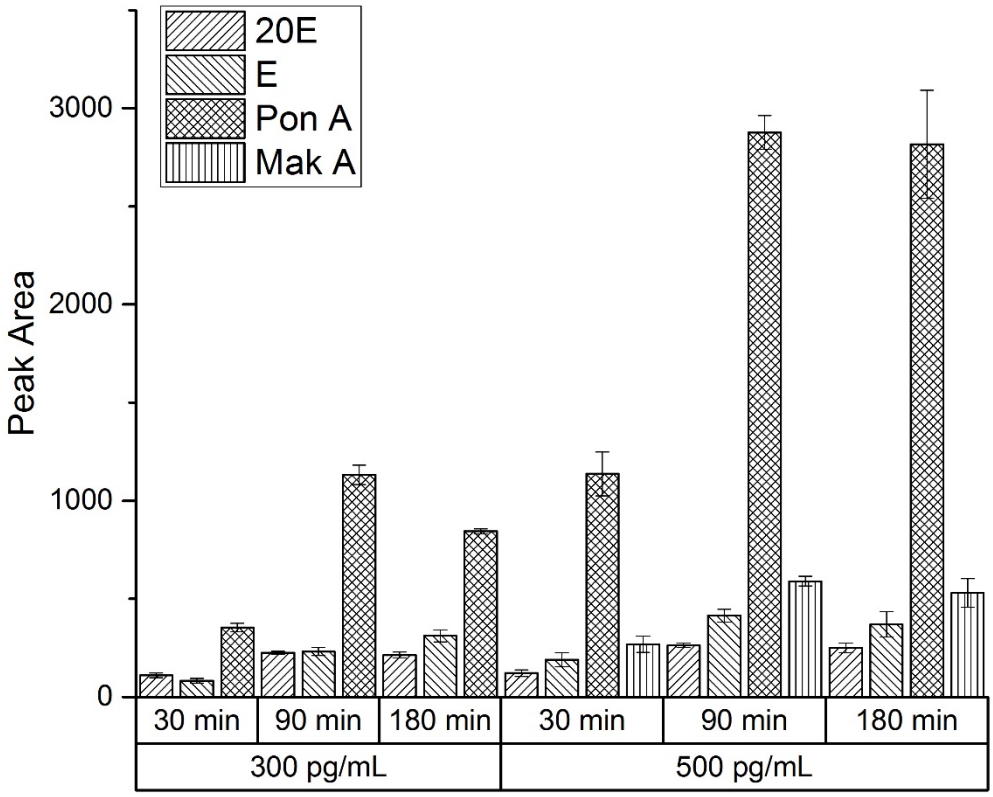

Fig. 1. Derivatization of 300 pg and 500 pg mL^{-1} of 6-keto ecdysteroids (20E: 20-hydroxyecdysone; E: ecdysone; Pon A: ponasterone A; Mak A: makisterone A) in frozen daphnids matrix using hydroxylamine hydrochloride 100 mg mL^{-1}. Total length of error bars represents two standard deviations of 5 replicates.

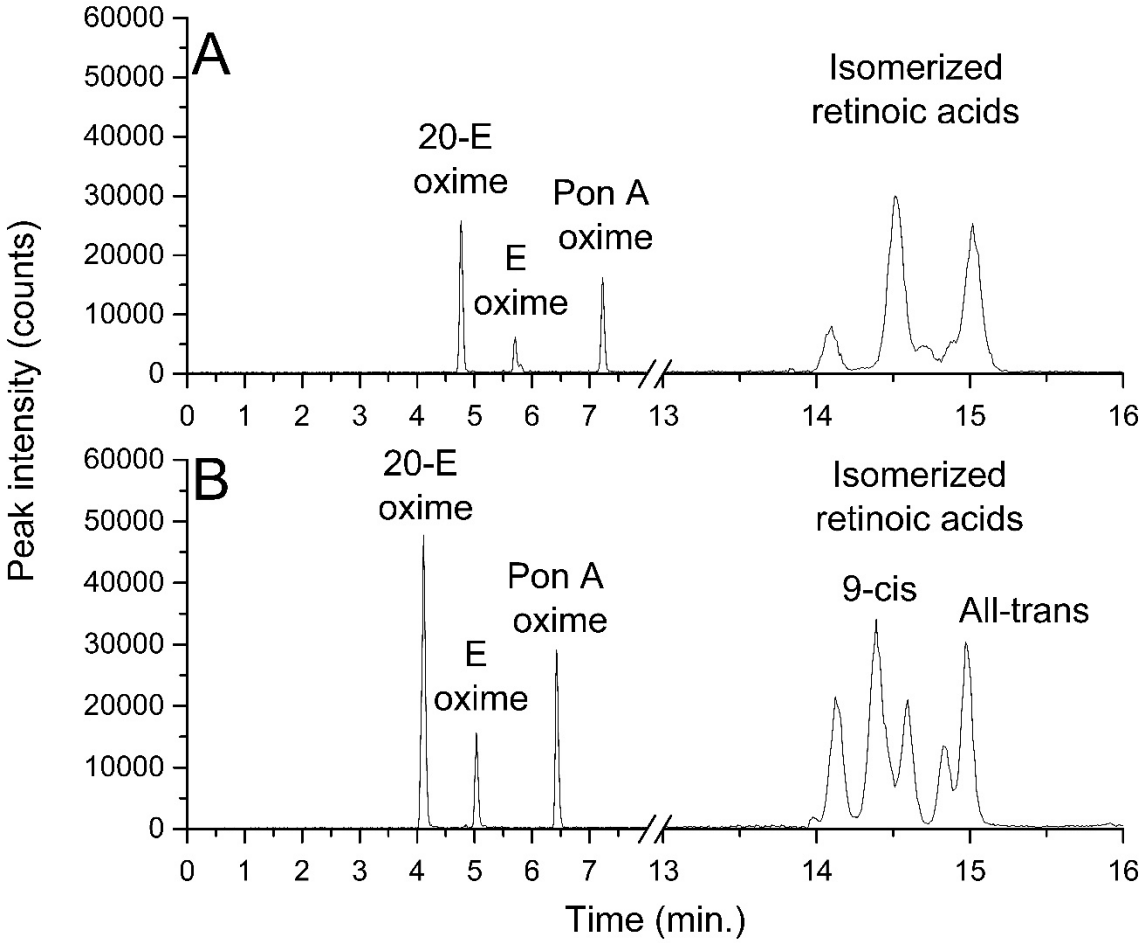

Fig. 2. Chromatographic comparison between porous C_{18} column (A) and solid-core C_{18} column (B) using identical chromatographic gradient. 20-E: 20-hydroxyecdysone; E: ecdysone; Pon A:

ponasterone A.

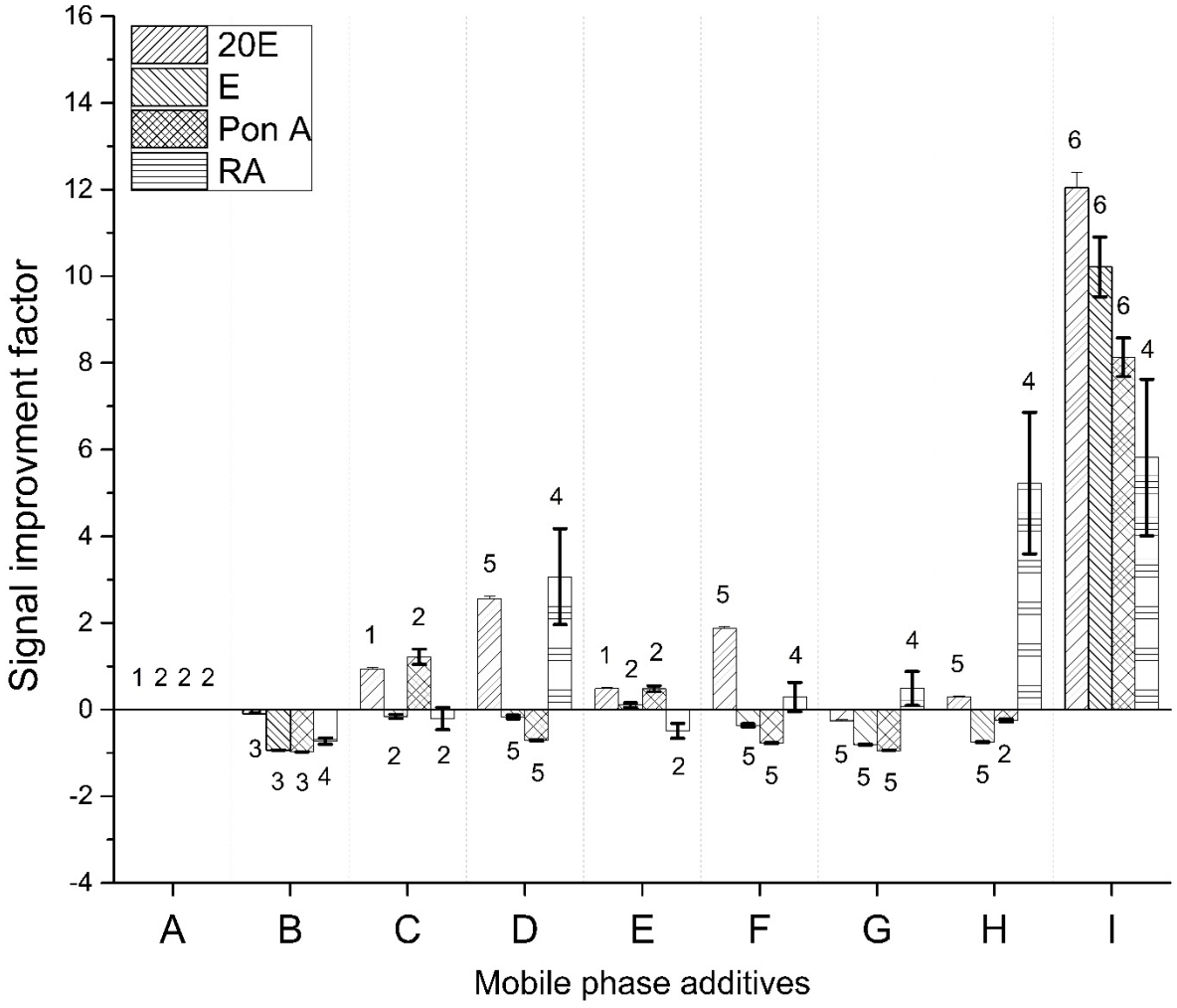

Fig. 3. Optimization of mobile phase additives and ionization conditions. A: ESI + 0.1% formic acid; B : ESI - 0.1% formic acid; C : ESI+ 0.1% acetic acid; D : ESI - 0.1% acetic acid; E : ESI + 0.5% acetic acid; F : ESI - 0.5% acetic acid ; G :ESI - 5 mM ammonium acetate; H: ESI +/- 0.1% acetic acid polarity switch; I: ESI +/- 0.1% acetic acid polarity switch and derivatization. Bar labels identifies the most abundant adducts: 1: $[M-2H_2O]^+$ 2: $[M+H]^+$ 3: $[M+HCOO]^-$ 4: $[M-H]^-$ 5: $[M+CH_3COO]^-$ 6: $[M+NOH-H_2O]^+$. Total length of error bars represents two standard deviations of 3 replicates. 20-E: 20-hydroxyecdysone; E: ecdysone; Pon A: ponasterone A; RA: retinoic acids.

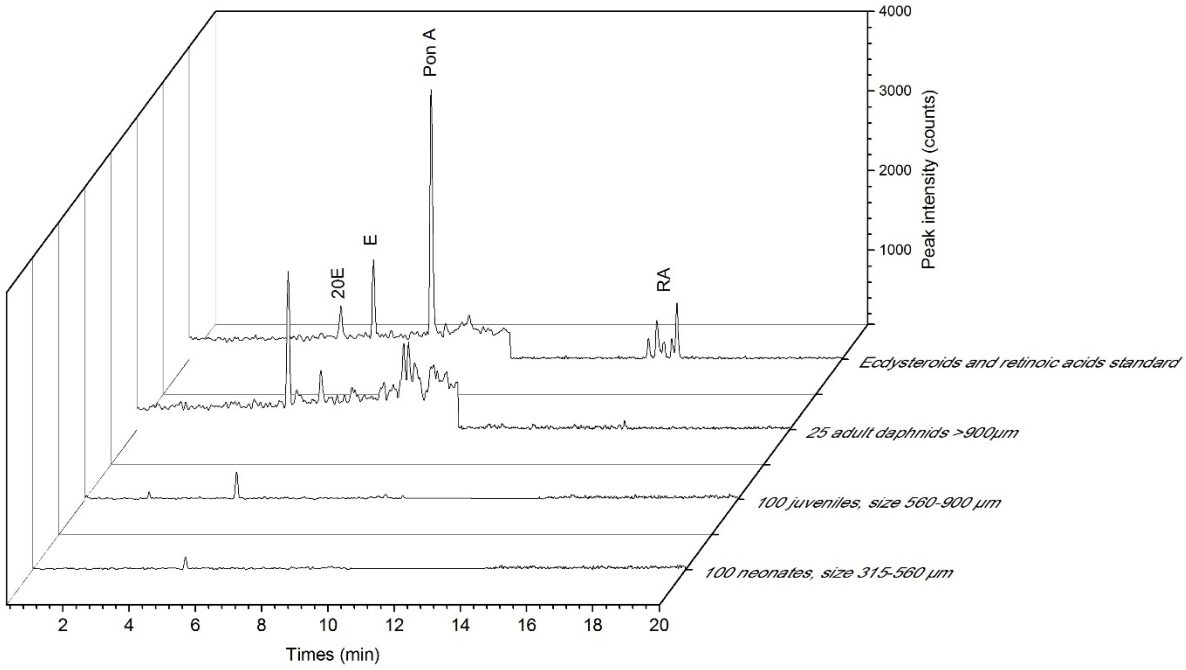

Fig. 4. Representative chromatograms of ecdysteroids (20-E: 20-hydroxyecdysone; E: ecdysone; Pon A: ponasterone A) and retinoic acids (RA) levels in *D. magna*.

Tables

Table 1. UPLC method parameters for the quantification of ecdysteroids and retinoic acids.

Parameter	UPLC
Column	Acquity UPLC Cortecs (C_{18}^{+}), 2.1 × 50 mm, 1.6 µm
Flow rate	0.5 mL min^{-1}
Column temperature	30 °C
Mobile phases	A: 0.1 % AA in H_2O
	B: 0.1 % AA in MeOH: ACN (3:2)
Gradient	0-7.9 min : 5-55% B
	7.9-8 min : 55-65% B
	8-13 min : 65-72% B
	13-20 min : 72-100% B
	20-22 min : 100% B
	22-30 min : 5% B
Wash solvents	Strong: MeOH
	Weak: H_2O 0.1% AA
Injection volume	10 µL in partial loop mode
Injection loop volume	20 µL
Autosampler temperature	20 °C

Table 2. ESI source and mass spectrometer parameters for the quantification of ecdysteroids and retinoic acids using the SRM mode.

Parameter	20E	E	Pon A	Mak A (ISTD)	RA	Acitretin (ISTD)
Retention time (min)	4.9	5.9	7.6	5.6	14.0-15.1	11.6-13.0
Ionization mode	ESI +	ESI +	ESI +	ESI +	ESI -	ESI -
Capillary voltage (kV)	2.5	2.5	2.5	2.5	3.0	3.0
Cone voltage (V)	50	50	50	50	35	35
Source temperature (°C)	120	120	120	120	120	120
Desolvation temp. (°C)	450	450	450	450	450	450
Cone gas flow (L/Hr)	50	50	50	50	50	50
Desolvation gas flow (L/Hr)	700	700	700	700	700	700
Collision pressure (mbar)	3.0	3.0	3.0	3.0	3.0	3.0
Dwell time (s)	0.2	0.2	0.2	0.2	0.2	0.2
Collision energy (eV)	30	20	30	30	15	15
Precursor ion (*m/z*)	478.4	462.4	462.4	492.4	299.3	325.2
Product ion (*m/z*)	316.3	444.4	316.3	316.3	255.3	265.9
Smoothing iteration	2	2	2	2	2	2
Smoothing width	2	2	2	2	2	2

Table 3. Matrix effects, relative recovery, intra- and interday accuracy and precision for low, medium and high QC samples.

Compound	Postextraction addition (*n* = 6)		Intraday (*n* = 5) QC_{LOW}; QC_{MED}; QC_{HIGH}		Interday (*n* = 5)	
	Matrix effects %	Relative recovery %	Precision CV%	Accuracy Bias%	Precision CV%	Accuracy Bias%
20E	11.0; 3.7; 2.6	92; 104; 104	6.2; 6.2; 10.5	14.5; 6.2; 10.8	8.7; 8.9; 2.0	11.7; 10.8; 8.7
E	-0.3; -7.8; -10.1	119; 106; 108	3.7; 3.5; 2.4	3.8; -4.4; 1.3	8.5; 4.4; 6.0	5.5; -2.0; -4.4
Pon A	-17.2; -19.3; -13.1	83; 90; 86	14.2; 12.5; 14.1	-27.4; -30.5; -24.8	12.8; 9.2; 13.7	-27.6; -26.7; -27.5
RA	-66.1; -67.0; -68.1	144; 155; 153	10.8; 10.3; 10.3	-36.5; -41.7; -37.3	11.7; 9.4; 12.5	-41.1; -40.2; -38.8

Table 4. Linearity of calibration curves, limits of quantification and detection for ecdysteroids and retinoic acids.

Compound	LLOQ	LOD		R^2	Calibration range
	pg mL^{-1}	pg mL^{-1}	pg per injection		pg mL^{-1}
20E	230	15	0.15	0.992	230 - 1140
E	210	22	0.22	0.997	210 - 1270
Pon A	380	4	0.04	0.991	380 - 1640
RA	5000	1000	10	0.999	5000 - 31000

Table 5. Stability, quantified as bias percentage at -20°C for low, medium and high QC samples.

Compound	Bias (%)			
	QC_{LOW}; QC_{MED}; QC_{HIGH}			
	24 h	48h	72h	96h
20E	11.8; 3.1; 3.4	25.7; 9.5; 15.0	4.7; 12.6; 17.5	13.1; 0.1; 2.7
E	-2.3; 4.2; 7.5	7.5; -5.8; -4.1	5.5; 0.4; -1.3	2.3; -2.1; -3.2
Pon A	-30.3; -26.5; -22.3	-22.3; -30.6; -31.9	-29.3; -37.0; -38.3	-14.1; -16.7; -13.8
RA	-46.0; -39.1; -34.9	-32.3; -41.8; -35.5	-42.7; -47.3; -46.6	-38.8; -40.2; -37.8

Table 5. Levels of ecdysteroids and retinoic acids in laboratory-cultured *D. magna*.

Concentrations are reported in pg per individual and were not corrected for accuracy bias.

Compound	Laboratory-cultured daphnids extraction (n = 3)		
	100 neonates, size 315-560 µm	100 juveniles , size 560-900 µm	25 adults, size >900µm
	pg ind^{-1}	pg ind^{-1}	pg ind^{-1}
20E	(0.19)	3.6 ± 1.0	19 ± 8
E	< LOD	< LOD	(1.8)
Pon A	< LOD	< LOD	< LOD
RA	< LOD	< LOD	< LOD

Concentrations reported in parentheses are higher than LOD but below LOQ.