Analysis of neurotransmitters in *Daphnia magna* affected by neuroactive pharmaceuticals using liquid chromatography-high resolution mass spectrometry

Cristian Gómez-Canela[a,c*], Xavier Rovira-García[b], Fernando Martínez-Jerónimo[d],Rosa María Marcé[a], Carlos Barata[b]

[a]Department of Analytical Chemistry and Organic Chemistry, Campus Sescelades, Faculty of Chemistry, Universitat Rovira i Virgili, Marcel·lí Domingo s/n, Tarragona, Spain.

[b]Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona 18-26, 08034, Barcelona, Catalonia, Spain.

[d]Instituto Politécnico Nacional, Laboratorio de Hidrobiología Experimental, México City, México.

[c]Current address:
Department of Analytical Chemistry and Applied (Chromatography section), School of Engineering, Institut Químic de Sarrià-Universitat Ramon Llull, Via Agusta 390, 08017 Barcelona, Spain (present address).

*Corresponding author:
Email: cristian.gomez@iqs.edu (C. Gómez-Canela)
Tel: +34 93 267 23 43

Abstract

Neurotransmission plays an essential role during the central nervous system (CNS) development. A few number of studies about the neurotransmitters changes by the effect of pharmaceuticals in aquatic organisms have been reported in the last years. There are evidences that the aquatic ecotoxicological model organism *Daphnia magna* shares with vertebrates several of the neurotransmitters targeted by antidepressant and other neuro-active drugs. So, a method based on liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) has been applied for the first time to *Daphnia magna* and validated to study the levels of 41 neurotransmitters under the effect of four different neuro-active pharmaceuticals (sertraline, venlafaxine, duloxetine and fluoxetine). In addition, the performance of LC-HRMS was studied in terms of linearity, sensitivity, intra- and inter-day precision, and overall robustness. The developed analytical method using LC-HRMS is a new tool for neurotoxicology research using the *Daphnia magna* model. The results show a general increasing or decreasing in the concentrations of the neurotransmitters after the exposure of the pharmaceuticals studied respect to controls.

Capsule

This is the first study that analyse the changes in the neurotransmitter profiles using liquid chromatography coupled to high resolution mass spectrometry.

Keywords: *Daphnia magna*; LC-Orbitrap; pharmaceuticals; neurotransmitters.

INTRODUCTION

Neurotransmitters are endogenous chemicals that enable neurotransmission. It is a type of chemical messenger which transmits signals across a chemical synapse, such as a neuromuscular junction, from one neuron (nerve cell) to another "target" neuron, muscle cell, or gland cell (Lodish et al., 2000). In this line, neurotransmitters plays an essential role during the central nervous system (CNS) development (Horzmann and Freeman, 2016; Kurian et al., 2011). Thus, there is a growing interest to investigate the effect of neuro-active contaminants on neurotransmitter dysregulation and how such effect alter the structure and function of the CNS in different biological models (Faria et al., 2018; Gómez-Canela et al., 2018; Ren et al., 2015). Of particular interest are antidepressant drugs belonging to selective serotonin and serotonin-norepinephrine reuptake inhibitors (SSRI, SNRI), which act increasing the levels of serotonine and norepinephrine in the CNS, and are broadly used in the first world (HAINER et al., 2006). Consequently chemical residues of SSRIs such as fluoxetine and sertraline and of SNRIs such as venlafaxine and duloxetine are conspicuous of most surface waters (Coelho et al., 2019; Reis-Santos et al., 2018).

The crustacean *Daphnia magna* (*D. magna*) may be a suitable non-vertebrate model to study the toxicological consequences of contaminant mediated disruptive effects on neurotransmitters. D. magna is one of the organisms most used in Aquatic toxicology (Barata et al., 1998; Miner et al., 2012). *D. magna* is a small planktonic invertebrate crustacean that can be found in lakes and shallow ponds rich in organic matter sediment distributed throughout the northern hemisphere. Therefore, it is an important herbivore and at the same time the prey for a huge number of predators (fishes, amphibians, and invertebrates). Moreover, their genome has been recently coded, being quite similar to the vertebrate genome (Colbourne et al., 2005). D. magna has a high number of neurotransmitters in common with many vertebrates (Dircksen et al., 2011; McCoole et

al., 2011) that are affected by the action of neuroactive pharmaceuticals (Rivetti et al., 2016). Indeed inmunohistological studies have reported that the SSRI fluoxetine increases the activity of serotonin in the D. magna brain (Campos et al., 2016). In this study we selected four pharmaceuticals that share similar modes of action and are widely used worldwide to treat depressive disorders (Abreu et al., 2016; Fong and Ford, 2014; Schultz et al., 2010).

The analysis of neurotransmitters requires a sample pretreatment, extraction method and an accurate and reliable instrumental method. Most of the analytical methods recently published for the analysis of neurotransmitters in aquatic organisms have been using liquid chromatography coupled to triple quadrupole (QqQ) mass spectrometers (LC-MS/MS) (Gómez-Canela et al., 2018; Santos-Fandila et al., 2013; Tufi et al., 2015). Triple quadrupole mass spectrometry is particularly fitted for the analysis of target compounds because its extraordinary sensitivity and selectivity (Gómez-Canela et al., 2013). The limitation of this technique is the possibility to have false positive due to the low resolution of the mass spectrometer. Therefore, the arrival of the high-resolution mass spectrometry (HRMS) got over the limitations commented before. Orbitrap, which is an ion trap mass analyzer, can operate in full-scan mode in a single run and the medium high resolution is around 100,000 FWHM (full width at half maximum) at *m/z* 200 and high mass accuracy < 2 ppm using internal standard and within 5 ppm with external calibration (Hogenboom et al., 2009). Moreover, using HRMS a theoretically unlimited number of analytes can be extracted from the full scan data without any compromise regarding the resulting sensitivity. Furthermore, HRMS permits the elucidation of elemental composition of analytes based on exact masses and isotopic patterns (Kaufmann et al., 2010). Thus, the aim of this study has been to develop a multiresidue method for the analysis of 43 neurotransmitters and related metabolites in *D. magna* using LC-HRMS after the exposure to neuroactive pharmaceuticals.

EXPERIMENTAL

Chemicals and materials

All neurotransmitter standards, including the isotope labelled compounds used for the optimization of the analytical method were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Santa Cruz Biotechnology (Dallas, TX, USA). All target compounds and molecular formulas are shown in Table 1. Methanol (MeOH), water (H_2O), and acetone HPLC grade were supplied from Merck (Darmstadt, Germany). Pharmaceuticals used

for the expositions (sertraline, venlafaxine, duloxetine and fluoxetine) were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) and formic acid (FA) were supplied by Fischer Scientific (Loughborough, UK). Ultra-pure water was obtained through Millipore Milli-Q purification system (Millipore, Bedford, MA, USA). Initially, all pure standards were prepared at 1000 ng μL^{-1} in MeOH or milliQ water depending on their solubility. Once prepared in silanized amber vials (2 mL), the standards were kept and at –80 °C in the dark to prevent possible degradation.

Aquatic organism husbandry

D. magna was kept in the laboratory at 20 °C, in ASTM medium water (reconstituted hard water formulation), adding 4 mL L^{-1} of marine microalgae extract (as a medium conditioner). *D. magna* fed on the microalga *Chlorella vulgaris* at concentrations of 400,000 cells mL^{-1}. The hatchery for the production of neonates (juveniles under 24 h), were kept in tanks of 1.5 to 2.5 L, with a density of 5 females L^{-1}. The culture medium and the food were completely renewed 3 times per week.

Under adequate conditions, *D. magna* reproduces asexually by parthenogenesis, having several litters throughout its life cycle. Once the sexual maturation is reached, the first litter of neonates is released (around the age of 9-10 days). From then, they continue to produce offspring, which are released from the incubator chamber of the female every 3 days (approx.). The organisms used for toxicity tests were those produced from the third litter. Breeding tanks of females were kept for a maximum period of 30 days (although their longevity is longer), so they have staggered cultures, with a gap of 1 to 2 weeks, to replace those that once they reach 30 days old were discarded.

Exposures and sample collection

Chronic, toxicologically relevant concentrations of sertraline (0.1 and 1 μg L^{-1}), venlafaxine (0.1 and 1 μg L^{-1}), duloxetine (0.1 and 1 μg L^{-1}) and fluoxetine (1 μg L^{-1}) were studied in *D. magna*. At those concentrations, the studied species reveal reproductive changes (Campos et al., 2016; Rivetti et al., 2016). These drugs have been detected in the natural aquatic environment at low concentrations (ng L^{-1}; μg L^{-1}) (Schreiber and Szewzyk, 2008). Exposures were initiated with new born neonates (<24 h old) in groups of five individuals in 100 mL of ASTM medium plus food (5x 10^5 cells of *Chlorella vulgaris*) and lasted four days. At the end of exposures animals were collected, pooled in a 2 mL

Eppendorf, frozen with N_2 and preserved at -80ºC until processed for analysis. In addition the body length of least 10 animals from each treatment was measured. Body length measurements were performed from the top of the head to the base of the spine using a Nikon stereoscope microscope (SMZ 150, Nikon, Barcelona, Spain) and the ImageJ software (http://rsb.info.nih.gov/ij/) (Glazier, 1998).

Sample extraction

Pools of 5 exposed or control crustaceans (*D. magna*) were spiked with 500 ng of isotope labelled solution (Glycine-2-^{13}C, L-Aspartic acid-^{15}N, Serotonin-d_4, Dopamine-1,1,2,2-d_4, GABA-d_6, Acetylcholine-d_9, L-Glutamic-^{15}N acid. Choline-1-^{13}C, L-Phenylalanine-1-^{13}C and Taurine-^{15}N) as internal standard (internal standard mixture, ISM, in the next sections). Then, 500 µL of ACN (+0.1% formic acid + 0.02% ascorbic acid) were added and shaken using a vortex mixer. Three stainless steel beads (3 mm diameter) were placed in each sample and were homogenized using a bead mill homogenizer (TissueLyser LT, Qiagen) at 50 oscillations per min during 60 s. After that, samples were centrifuged 10 min at maximum speed at 4ºC. The supernatant was transferred to a new centrifuge tube and evaporated to complete dryness under mild nitrogen. Then, the samples were resuspended in 100 µL of ACN:H2O (50:50). Finally, the supernatant was filtered using 0.20 µM PTFE filters (DISMIC -13 JP, Advantec®) and kept in amber chromatographic vials at -80 ºC until LC-HRMS analysis. More details about the extraction protocol are included in a previous published paper about the characterization of neurotransmitter profiles in *D. magna* juveniles exposed to environmental concentrations of antidepressants, anxiolytic and antihypertensive drugs (Rivetti et al., 2019).

LC-HRMS analysis

An LC-Orbitrap-MS (Exactive) from Thermo Fischer Scientific (Bremen, Germany) equipped with a heated electrospray ionization (H-ESI) source was used. The system was equipped with a HTC PAL autosampler and a Surveyor MS Plus pump. Synergi Polar-RP 80 Å column (250 mm× 4.6 mm i.d., particle size 4 µM, Phenomenex, Torrance, USA) was chosen following a previous published paper about the analysis of neurotransmitters in zebrafish larvae using an LC-MS/MS instrument (Gómez-Canela et al., 2018). The mobile phase composition consisted of binary mixtures with 0.1% of formic acid in water (A) and 0.1% formic acid in MeOH (B). Gradient elution started at 95% A and 5% B in the first 2 min and increased to 30% B in 5 min. Then, gradient

increased to 95% B in the next 13 min, and held for 5 min. Initial conditions to stabilize the system were attained in 5 min being 30 min the total run time. The flow rate was set at 600 µL min^{-1} and 10 µL was injected. Neurotransmitters were measured under both positive and negative electrospray ionization (ESI+/ESI-). In the case of the metabolites detected by positive and negative ionization, the mode with better MS signal intensity for each compounds was used. Full scan acquisition over a *m/z* 50-600 was performed at 70,000 FWHM and spray voltage at 3.00 kV, capillary voltage at 25 V, skimmer voltage at 16 V and tube lens voltage at 55 V were used. Sheath gas flow rate at 35 arbitrary units (au), auxiliary gas flow rate at 10 au and capillary temperature at 300 ºC were chosen. High energy Collision Dissociation (HCD) voltage at 25 V was optimized to obtain the most intense fragments. All Orbitrap conditions are displayed in Table SI1 (*Supplementary information*). The data were acquired and processed using the Thermo Xcalibur 2.1 software package. Solvent blanks did not contain any of the investigated metabolites, indicating no carryover effect in any of the LC-HRMS runs.

Quality assurance

Table 2 displays the quality parameters obtained by LC-HRMS. The linearity of response was evaluated over a concentration range from 0.005 to 5 ng μL^{-1}, using eight calibration points. Internal standard calibration was used to correct the analyte MS response and to ensure exact quantification performance. A mix of ten isotope labelled compounds, including L-aspartic acid-^{15}N, glycine-2-^{13}C, Serotonin-d_4-hydrochloride, dopamine-1,1,2,2-d_4 hydrochloride, GABA-d_6, acetylcholine-d_9, L-glutamic-^{15}N acid, choline chloride-1-^{13}C, L-phenylalanine-1-^{13}C and taurine-^{15}N, were used as internal standard (IS) for extraction and analytical quality control. With LC-HRMS, good determination coefficients ($R^2 > 0.99$) were obtained for all compounds with good linearity from 0.005 to 5 ng μL^{-1} in most cases. Instrumental Detection Limit (IDL) was calculated as the concentration that yielded an S/N ratio higher than or equal to three using the lowest concentration standard solution at 2.5 ng/mL and method detection limit (MDL) was calculated in the same way, using *D. magna* samples spiked at 200 ng. Intra-day precision was assessed by five consecutive injections of 1 ng μL^{-1} standard solution, and inter-day precision was determined by measuring the same standard solution during four

different days. Solvent blanks did not contain any of the investigated analytes, indicating no carryover effect during the LC-HRMS runs.

On the other hand, recovery studies were performed with five replicates, using *D. magna* samples spiked at 200 ng with the neurotransmitter standard mixture and 500 ng of the ISM. Five replicates of a pool with five *D. magna* were analyzed first, subtracting the possible traces of target compounds. In addition, the matrix effect (ME) was assessed by comparing the peak area of analyte from the spiked zebrafish larvae with the peak area of the analyte from the standard solution at the same concentration in methanol (N = 5), following the equation:

$$ME = \frac{(A - B)}{C}\ x\ 100$$

where A is the peak area of each analyte from spiked *D. magna* samples; B is the peak area of each analyte from non-spiked *D. magna*; and C is the peak area of each analyte in methanol. ME values below 100% suggest signal suppression and ME values above 100% suggest signal enhancement.

RESULTS AND DISCUSSION

Optimization of the LC-HRMS conditions

As commented before, chromatographic conditions used were those reported in Gómez-Canela et al. study (Gómez-Canela et al., 2018). Optimization of the ionization source conditions was performed using LC-Orbitrap. Flow Injection Analysis (FIA) experiments were performed at a capillary voltage from 20 to 80 V, skimmer voltage from 10 to 40 V, and tube lens voltage from 60 to 200 V. The best sensitivity (highest response) was obtained at a capillary voltage of 25 V, skimmer voltage of 16 V and tube lens voltage of 55 V that produced the most intense protonated molecule at high resolution (70,000 FWHM). Sheath gas flow rate was optimized from 35 to 55 au, auxiliary gas flow rate (from 2 to 20 au) and capillary temperature (>225 ˚C). These conditions were optimized taking into account the best results for all target compounds. HCD voltages were tested at 15, 25 and 45 eV, obtaining the most intense fragments at 25 eV. Tables SI1 displays the final Orbitrap ionization parameters. On the other hand, Synergy Polar-RP 80 Å column was used to obtain the best separation of all target compounds in a total run analysis of 30 minutes. Figure 1 shows the LC-HRMS extracted ion chromatogram of 43 target compounds from a full scan LC-Orbitrap-MS spectrum

using a 5 ppm extraction window. Compounds with the same exact mass could be identified by their different retention time (R_t), were injected individually at the same conditions. Thus, octopamine and dopamine ($[M+H]^+$= 154.0863) eluted at 5.53 and 7.33 min, respectively. Epinephrine and normetanephrine ($[M+H]^+$= 184.0968) at 5.59 and 6.27 min; and betaine and valine ($[M+H]^+$= 118.0863) at 5.21 and 5.47 min, respectively. Therefore, one or two fragments were identified for each compound using HCD cell at 25 eV, as indicated in Table 3. Directive 2002/657/CE, indicates the performance criteria and other requirements for mass spectrometric detection (Official Journal of the European Communities, 2002). To comply with this Directive, the identification criteria required that the accurate mass measurements of the molecular and the product ions should present an error <5 ppm and when possible; the isotopic pattern should fit better than 90% to be accepted as a positive sample. To comply with the requirements of the Directive 2002/657/CE, a molecular and two fragment ions for R > 20,000 earn 2 and 2.5 IPs, respectively, which reinforces the robustness of the identification criteria (IPs ≥ 4) for HRMS. In some cases, more than two HCD ions were formed, which can be used for identification criteria and provide 2n identification points, being “n” the number of ions at high resolution.

Method validation

Table 2 shows the performance parameters obtained by LC-Orbitrap-MS. In order to correct the MS responses and ensure exact quantification performance, internal standard calibration was used. Acceptable correlation coefficients (R^2) were over 0.99 for all target compounds in a range from 0.005 to 5 ng μL^{-1} in most of the cases. Linearity and response factors are displayed in Table 2. IDL values ranged between 0.2 pg (betaine) and 156.74 pg (CDP-Choline). On the other hand, MDL values of *D. magna* were from 0.03 pg daphnia^{-1} (choline, valine and BW284c51) to 4.75 pg daphnia^{-1} (L-DOPAC) (Table 2). Regarding intra- and inter-day precision, values differed significantly depending on the compound. Intra-day precision (n=5) ranged from 0.29 to 26.26% and inter-day precision (n=5) ranged from 1.3 to 40.9% at 1 ng μL^{-1} level, indicating a robust method response (Table 2).

Method performance was tested using five replicates of a pool with 5 daphnids at 100 ng (low concentration) and 200 ng (high concentration) with the mix of the target neurotransmitters and 500 ng of the ISM in order to assess the efficiency of the extraction

method. Good recovery values (between 75±12%-124±8%) were obtained for all target compounds except for acetylcholine and guanidine, 55±9% and 67±3% respectively, and cytidine-5'-diphosphocholine that was not recovered. Matrix effect was also evaluated obtaining signal suppression for 8 neurotransmitters (acetylcholine, histidine, 5-hydroxy-L-tryptophan, octopamine, norepinephrine, taurine, L-arginine and guanosine). However, only phosphocholine, guanidine and phenylalanine had ME above 100% giving a signal enhancement. The rest of them had good matrix effect values in the range of 60±8% to 111±13% (Table 2). Thus, quantification with the internal standard procedure seems adequate to avoid over- or underestimation of the calculated concentration.

Changes in the neurochemical profile of Daphnia magna

The body length of the Daphnia individuals at the end of exposures was 2.22±0.39 mm (Mean±SD, N =83) and did not vary significantly across treatments (ANOVA results, $F_{1,75}$ =1.85, P>0.05). Raw data (mean ± SE) is depicted in Table SI3 whereas significant and other important results relative to unexposed controls are shown in Figures 2-6. Results obtained indicated significant (P<0.05) differences in 24 out of the 34 metabolites quantified across treatments (Table SI4). There is quite a lot of inconsistency between drugs acting similarly (Figures 2-6). For example, sertraline and fluoxetine are selective serotonin reuptake inhibitors that bind to the serotonin transporter and hence inhibit the re-uptake of serotonin, increasing its accumulation in the synaptic spaces. In humans, SSRI by increasing serotonin levels in the brain diminished depression disorders, whereas in *D. magna* it increases reproduction and carbohydrate metabolism (Campos et al., 2016; Jordão et al., 2016). Unfortunately, our analytical method did not allow detection of serotonin but instead two serotoninergic metabolites: its precursor tryptophan and its major degradation product 5-HIAA. Sertraline and fluoxetine had opposed patterns for 5-HIAA increasing and decreasing it, respectively (Figure 2). Interestingly in rats similar opposed effects of sertraline and fluoxetine in the concentration of 5-HIAA have been reported (Harkin et al., 1995; Kudrin et al., 2010), which may be related to the fact that both compounds despite of having a similar primary mode show different bioaccumulation patterns and probably different secondary mode of action. In fish, sertraline bioaccumulated in neural and other tissues to a greater extent than fluoxetine (Chen et al., 2017; David et al., 2018), and there is reported evidence indication that sertraline is more toxic than fluoxetine in fish and other aquatic organisms (Alsop and Wilson, 2019; Sehonova et al., 2018).

Venlafaxine and duloxetine are antidepressants of the selective serotonin-norepinephrine reuptake inhibitor (SNRI) class. This means they increase the concentrations of the neurotransmitters serotonin and norepinephrine in the body and the brain. Unfortunately, norepinephrine could not be detected in *Daphnia* by the method used. Nevertheless, our results showed quite similar concentration patterns for 5-HIAA and epinephrine among the studied SNRI (Figure 2). None of them changed significantly the levels of 5-HIAA, although both compounds reduced it and only venlafaxine increased epinephrine probably since the concentrations used (100 and 1000 ng/L) were too low for duloxetine to affect its target neurotransmitter (Figure 2). These results are in line with reported studies in mammalian models (Chappell et al., 2014; Fuller et al., 1994; Vasudev et al., 2016).

The observed effects on the remaining neurotransmitter pathways are quite variable, which is a consequence that the studied drugs only affected them as secondary and unknown mechanisms of action in *Daphnia*. As expected, none of the studied compounds affected ACh, which is the target of organophosphorus and neonicotinoid pesticides in *Daphnia* (Figure 3). However, the concentrations of ACh precursors such as choline, betaine, CHOP and GPCh varied across the studied compounds (Figure 3). Choline and betaine decreased upon exposure to most compounds but fluoxetine, and CHOP and GPCh increased at low concentrations of venlafaxine and GPCh, respectively. Guanidine increased upon exposure to fluoxetine. It is difficult to provide a conclusive argument to the disparity of responses observed for the cholinergic related metabolites since many of them are also precursors of lipids and the studied drugs in general increased reproduction (Campos et al., 2012b). *D. magna* females provision large quantities of lipids to their eggs thus it is possible that by doing so lipid precursors such as choline, CHOP and GPCh diminished (Jordão et al., 2015).

GABA is the principal neurotransmitter with inhibitory effects in the central nervous system. GABA was enhanced upon exposure to low levels of sertraline but not at high levels neither by any other tested chemicals (Figure 4). Although these results are quite inconsistent there is reported information indication that SSRI can enhance GABA (Bhagwagar et al., 2004).

Tyrosine and phenylalanine were enhanced by fluoxetine, and dopamine concentrations decreased upon exposure to sertraline (Figure 4). Tyrosine and phenylalanine are amino acid involved in many metabolic pathways, thus it is difficult to establish a mechanistic functional relationship for fluoxetine. Reported studies in *Daphnia*, however, found that fluoxetine increased catabolic activity (Campos et al., 2012a), which may favour the accumulation of these amino acid as by-products of degradation of proteins. Effect of sertraline on dopamine contradict reported studies in

rats that found the opposite pattern (Kitaichi et al., 2010). Glutamatergic and glycinergic metabolites were consistently decreased upon exposure to the tested chemicals (Figure 5). There is ample information that there is a cross-talk among serotonergic, glutamatergic and glycinergic signaling pathways and that SSRI and SNRIs may interact with those pathways (Aprison et al., 1996; Dale et al., 2015; Musazzi et al., 2013; Ortiz et al., 2016; Pehrson and Sanchez, 2014; Rodrigues et al., 2015). Despite that *Daphnia* has an active histaminergic system (McCoole et al., 2011), any of the metabolites involved in this system were affected by the studied compounds (Table SI3). Among other metabolites related with the neurotransmitter system, L-arginine and aspartic acid decreased upon exposure to all the studied compounds, whereas fluoxetine enhanced the level of taurine and methionine. Again, it is difficult to establish a mechanistic relationship between observed changes in these metabolites and neurotoxicological effects since these amino acids are also involved in other metabolic functions (Figure 6). However, there is information reporting that fluoxetine can enhanced the levels of taurine and that low levels of L-arginine and aspartic acid in human plasma are related with depressive disorders (Colmenares-Aguilar et al., 2017; Lu et al., 2014).

CONCLUSIONS

A comprehensive optimization of a multiresidue method for the analysis of 43 neurotransmitters and related metabolites from different biochemical classes in *D. magna* using LC-Orbitrap-MS has been performed. The method has been validated in terms of reproducibility, sensitivity and mass accuracy. Moreover, a study of the fragmentation pattern was done in order to confirm the target compounds in the samples. Forty-one neurotransmitters were recovered with good values (between 75±12%-124±8%) with the exception of acetylcholine and guanidine, 55±9% and 67±3% respectively, and cytidine-5'-diphosphocholine that was not recovered. Moreover, matrix effect has been evaluated for all the neurotransmitters studied, obtaining high values of signal suppression for some compounds such as acetylcholine, histidine, 5-hydroxy-L-tryptophan, octopamine, norepinephrine, taurine, citrulline and guanosine. This analytical technique is adequate for the multi-group analysis of neurotransmitters and offers advantages with respect to previous reported methods: 43 compounds can be analyzed in a single injection; the compounds are unequivocally identified using high-resolution mass spectrometry and subsequently the excellent sensitivity of LC-Orbitrap-MS is reported.

Results demonstrated that the pharmaceuticals exposed to *D. magna* affected to the neurotransmitter system increasing or decreasing their concentrations respect to controls. In *D. magna* most tested pharmaceuticals altered neurotransmitters per se or their associated metabolites at environmental concentrations (0.1 ng L^{-1}). Thus, it can be concluded that pharmaceuticals are an important cause of concern in the aquatic environment. Further studies are needed in order to know more information about these organic pollutants in terms of human risk.

Acknowledgments

Cristian Gómez-Canela acknowledge "Ministerio de Ciencia e Innovación" for the "Juan de la Cierva" program. This work was supported by the Spanish Government project CTM2017-83242-R.

REFERENCES

Abreu, M.S., Giacomini, A.C.V., Gusso, D., Rosa, J.G.S., Koakoski, G., Kalichak, F., Idalêncio, R., Oliveira, T.A., Barcellos, H.H.A., Bonan, C.D., Barcellos, L.J.G., 2016. Acute exposure to waterborne psychoactive drugs attract zebrafish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 179, 37-43.

Alsop, D., Wilson, J.Y., 2019. Waterborne pharmaceutical uptake and toxicity is modified by pH and dissolved organic carbon in zebrafish. Aquatic Toxicology, 11-18.

Aprison, M.H., Galvez-Ruano, E., Lipkowitz, K.B., 1996. Comparison of binding mechanisms at cholinergic, serotonergic, glycinergic and GABAergic receptors. Journal of Neuroscience Research 43, 127-136.

Barata, C., Baird, D.J., Markich, S.J., 1998. Influence of genetic and environmental factors on the tolerance of Daphnia magna Straus to essential and non-essential metals. Aquatic Toxicology 42, 115-137.

Bhagwagar, Z., Wylezinska, M., Taylor, M., Jezzard, P., Matthews, P.M., Cowen, P.J., 2004. Increased Brain GABA Concentrations Following Acute Administration of a Selective Serotonin Reuptake Inhibitor. American Journal of Psychiatry 161, 368-370.

Campos, B., Piña, B., Barata, C., 2012a. Mechanisms of action of selective serotonin reuptake inhibitors in *Daphnia magna*. Environmental Science and Technology 46, 2943-2950.

Campos, B., Piña, B., Fernández-Sanjuán, M., Lacorte, S., Barata, C., 2012b. Enhanced offspring production in *Daphnia magna* clones exposed to serotonin reuptake inhibitors and 4-nonylphenol. Stage- and food-dependent effects. Aquatic Toxicology 109, 100-110.

Campos, B., Rivetti, C., Kress, T., Barata, C., Dircksen, H., 2016. Depressing Antidepressant: Fluoxetine Affects Serotonin Neurons Causing Adverse Reproductive Responses in *Daphnia magna*. Environmental Science and Technology 50, 6000-6007.

Coelho, M.M., Lado Ribeiro, A.R., Sousa, J.C.G., Ribeiro, C., Fernandes, C., Silva, A.M.T., Tiritan, M.E., 2019. Dual enantioselective LC–MS/MS method to analyse chiral drugs in surface water: Monitoring in Douro River estuary. Journal of Pharmaceutical and Biomedical Analysis 170, 89-101.

417 Colbourne, J.K., Singan, V.R., Gilbert, D.G., 2005. wFleaBase: The Daphnia genome database.
418 BMC Bioinformatics 6.
419 Colmenares-Aguilar, M.G., Urbina, M., Obregón, F., Fazzino, F., Lima, L., 2017. Fluoxetine
420 Treatment in Rats Increases the Rate of Taurine Transport in Mononuclear Cells.
421 NeuroImmunoModulation 24, 132-142.
422 Chappell, J.C., Eisenhofer, G., Owens, M.J., Haber, H., Lachno, D.R., Dean, R.A., Knadler, M.P.,
423 Nemeroff, C.B., Mitchell, M.I., Detke, M.J., Iyengar, S., Pangallo, B., Lobo, E.D., 2014. Effects of
424 duloxetine on norepinephrine and serotonin transporter activity in healthy subjects. Journal of
425 Clinical Psychopharmacology 34, 9-16.
426 Chen, F., Gong, Z., Kelly, B.C., 2017. Bioaccumulation Behavior of Pharmaceuticals and Personal
427 Care Products in Adult Zebrafish (Danio rerio): Influence of Physical-Chemical Properties and
428 Biotransformation. Environmental Science and Technology 51, 11085-11095.
429 Dale, E., Bang-Andersen, B., Sánchez, C., 2015. Emerging mechanisms and treatments for
430 depression beyond SSRIs and SNRIs. Biochemical Pharmacology 95, 81-97.
431 David, A., Lange, A., Tyler, C.R., Hill, E.M., 2018. Concentrating mixtures of neuroactive
432 pharmaceuticals and altered neurotransmitter levels in the brain of fish exposed to a
433 wastewater effluent. Science of the Total Environment 621, 782-790.
434 Dircksen, H., Neupert, S., Predel, R., Verleyen, P., Huybrechts, J., Strauss, J., Hauser, F.,
435 Stafflinger, E., Schneider, M., Pauwels, K., Schoofs, L., Grimmelikhuijzen, C.J.P., 2011. Genomics,
436 transcriptomics, and peptidomics of *Daphnia pulex* neuropeptides and protein hormones.
437 Journal of Proteome Research 10, 4478-4504.
438 Faria, M., Ziv, T., Gómez-Canela, C., Ben-Lulu, S., Prats, E., Novoa-Luna, K.A., Admon, A., Piña, B.,
439 Tauler, R., Gómez-Oliván, L.M., Raldúa, D., 2018. Acrylamide acute neurotoxicity in adult
440 zebrafish. Scientific Reports 8.
441 Fong, P.P., Ford, A.T., 2014. The biological effects of antidepressants on the molluscs and
442 crustaceans: A review. Aquatic Toxicology 151, 4-13.
443 Fuller, R.W., Hemrick-Luecke, S.K., Snoddy, H.D., 1994. Effects of duloxetine, an antidepressant
444 drug candidate on concentrations of monoamines and their metabolites in rats and mice.
445 Journal of Pharmacology and Experimental Therapeutics 269, 132-136.
446 Glazier, D.S., 1998. Does body storage act as a food-availability cue for adaptive adjustment of
447 egg size and number in Daphnia magna? Freshwater Biology 40, 87-92.
448 Gómez-Canela, C., Cortés-Francisco, N., Ventura, F., Caixach, J., Lacorte, S., 2013. Liquid
449 chromatography coupled to tandem mass spectrometry and high resolution mass spectrometry
450 as analytical tools to characterize multi-class cytostatic compounds. Journal of Chromatography
451 A 1276, 78-94.
452 Gómez-Canela, C., Tornero-Cañadas, D., Prats, E., Piña, B., Tauler, R., Raldúa, D., 2018.
453 Comprehensive characterization of neurochemicals in three zebrafish chemical models of
454 human acute organophosphorus poisoning using liquid chromatography-tandem mass
455 spectrometry. Analytical and Bioanalytical Chemistry 410, 1735-1748.
456 HAINER, V., KABRNOVA, K., ALDHOON, B., KUNESOVA, M., WAGENKNECHT, M., 2006. Serotonin
457 and Norepinephrine Reuptake Inhibition and Eating Behavior. Annals of the New York Academy
458 of Sciences 1083, 252-269.
459 Harkin, A.J., Kelly, J.P., Leonard, B.E., 1995. The comparative effects of long-term administration
460 of sertraline, paroxetine, citalopram, fluoxetine and amitriptyline on monoamine content in rat
461 brain. Journal of Serotonin Research 2, 181-190.
462 Hogenboom, A.C., van Leerdam, J.A., de Voogt, P., 2009. Accurate mass screening and
463 identification of emerging contaminants in environmental samples by liquid chromatography-
464 hybrid linear ion trap Orbitrap mass spectrometry. Journal of Chromatography A 1216, 510-519.
465 Horzmann, K.A., Freeman, J.L., 2016. Zebrafish Get Connected: Investigating Neurotransmission
466 Targets and Alterations in Chemical Toxicity. Toxics 4, 19.

Jordão, R., Campos, B., Piña, B., Tauler, R., Soares, A.M., Barata, C., 2016. Mechanisms of Action of Compounds That Enhance Storage Lipid Accumulation in Daphnia magna. Environmental science & technology 50, 13565-13573.
Jordão, R., Casas, J., Fabrias, G., Campos, B., Piña, B., Lemos, M.F.L., Soares, A.M.V.M., Tauler, R., Barata, C., 2015. Obesogens beyond vertebrates: Lipid perturbation by tributyltin in the crustacean *Daphnia magna*. Environmental Health Perspectives 123, 813-819.
Kaufmann, A., Butcher, P., Maden, K., Walker, S., Widmer, M., 2010. Comprehensive comparison of liquid chromatography selectivity as provided by two types of liquid chromatography detectors (high resolution mass spectrometry and tandem mass spectrometry): "Where is the crossover point?". Analytica Chimica Acta 673, 60-72.
Kitaichi, Y., Inoue, T., Nakagawa, S., Boku, S., Kakuta, A., Izumi, T., Koyama, T., 2010. Sertraline increases extracellular levels not only of serotonin, but also of dopamine in the nucleus accumbens and striatum of rats. European Journal of Pharmacology 647, 90-96.
Kudrin, V.S., Mosin, V.M., Klodt, P.M., Narkevich, V.B., Molodavkin, G.M., Voronina, T.A., 2010. Studying effects of amitriptyline, fluoxetine, and tianeptine on the content of monoamines and their metabolites in wistar rat brain structures. Eksperimental'naya i Klinicheskaya Farmakologiya 73, 7-10.
Kurian, M.A., Gissen, P., Smith, M., Heales, S.J., Clayton, P.T., 2011. The monoamine neurotransmitter disorders: an expanding range of neurological syndromes. The Lancet Neurology 10, 721-733.
Lodish, H., Berk, A., Zipursky, S.e.a., 2000. Section 21.4. Neurotransmitters, Synapses, and Impulse Transmission., in: Freeman, W.H. (Ed.), Molecular Cell Biology, 4th edition ed, New York.
Lu, Y.-R., Fu, X.-Y., Shi, L.-G., Jiang, Y., Wu, J.-L., Weng, X.-J., Wang, Z.-P., Wu, X.-Y., Lin, Z., Liu, W.-B., Li, H.-C., Luo, J.-H., Bao, A.-M., 2014. Decreased plasma neuroactive amino acids and increased nitric oxide levels in melancholic major depressive disorder. BMC Psychiatry 14, 123-123.
McCoole, M.D., Baer, K.N., Christie, A.E., 2011. Histaminergic signaling in the central nervous system of Daphnia and a role for it in the control of phototactic behavior. Journal of Experimental Biology 214, 1773-1782.
Miner, B.E., de Meester, L., Pfrender, M.E., Lampert, W., Hairston Jr, N.G., 2012. Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. Proceedings of the Royal Society B: Biological Sciences 279, 1873-1882.
Musazzi, L., Treccani, G., Mallei, A., Popoli, M., 2013. The action of antidepressants on the glutamate system: Regulation of glutamate release and glutamate receptors. Biological Psychiatry 73, 1180-1188.
Official Journal of the European Communities, O., 2002. Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, p. 29.
Ortiz, A.E., Gassó, P., Mas, S., Falcon, C., Bargalló, N., Lafuente, A., Lázaro, L., 2016. Association between genetic variants of serotonergic and glutamatergic pathways and the concentration of neurometabolites of the anterior cingulate cortex in paediatric patients with obsessive–compulsive disorder. World Journal of Biological Psychiatry 17, 394-404.
Pehrson, A.L., Sanchez, C., 2014. Serotonergic modulation of glutamate neurotransmission as a strategy for treating depression and cognitive dysfunction. CNS Spectrums 19, 121-133.
Reis-Santos, P., Pais, M., Duarte, B., Caçador, I., Freitas, A., Vila Pouca, A.S., Barbosa, J., Leston, S., Rosa, J., Ramos, F., Cabral, H.N., Gillanders, B.M., Fonseca, V.F., 2018. Screening of human and veterinary pharmaceuticals in estuarine waters: A baseline assessment for the Tejo estuary. Marine Pollution Bulletin 135, 1079-1084.
Ren, Z., Zhang, X., Wang, X., Qi, P., Zhang, B., Zeng, Y., Fu, R., Miao, M., 2015. AChE inhibition: One dominant factor for swimming behavior changes of Daphnia magna under DDVP exposure. Chemosphere 120, 252-257.

Rivetti, C., Campos, B., Barata, C., 2016. Low environmental levels of neuro-active pharmaceuticals alter phototactic behaviour and reproduction in *Daphnia magna*. Aquatic Toxicology 170, 289-296.
Rivetti, C., Climent, E., Gómez-Canela, C., Barata, C., 2019. Characterization of Neurotransmitter Profiles in Daphnia magna juveniles exposed to environmental concentrations of antidepressants, anxiolytic and antihypertensive drugs using liquid chromatography-tandem mass spectrometry. Analytical and Bioanalytical Chemistry, submitted.
Rodrigues, D.O., Bristot, I.J., Klamt, F., Frizzo, M.E., 2015. Sertraline reduces glutamate uptake in human platelets. NeuroToxicology 51, 192-197.
Santos-Fandila, A., Zafra-Gómez, A., Barranco, A., Navalón, A., Rueda, R., Ramírez, M., 2013. Quantitative determination of neurotransmitters, metabolites and derivates in microdialysates by UHPLC–tandem mass spectrometry. Talanta 114, 79-89.
Schreiber, F., Szewzyk, U., 2008. Environmentally relevant concentrations of pharmaceuticals influence the initial adhesion of bacteria. Aquatic Toxicology 87, 227-233.
Schultz, M.M., Furlong, E.T., Kolpin, D.W., Werner, S.L., Schoenfuss, H.L., Barber, L.B., Blazer, V.S., Norris, D.O., Vajda, A.M., 2010. Antidepressant pharmaceuticals in two U.S. effluent-impacted streams: Occurrence and fate in water and sediment and selective uptake in fish neural tissue. Environmental Science and Technology 44, 1918-1925.
Sehonova, P., Svobodova, Z., Dolezelova, P., Vosmerova, P., Faggio, C., 2018. Effects of waterborne antidepressants on non-target animals living in the aquatic environment: A review. Science of the Total Environment 631-632, 789-794.
Tufi, S., Lamoree, M., de Boer, J., Leonards, P., 2015. Simultaneous analysis of multiple neurotransmitters by hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry. Journal of Chromatography A 1395, 79-87.
Vasudev, R., Rampal, U., Patel, H., Patel, K., Bikkina, M., Shamoon, F., 2016. Selective serotonin–norepinephrine reuptake inhibitors–induced Takotsubo cardiomyopathy. North American Journal of Medical Sciences 8, 312-315.