SYNTHETIC ACTIVITY REPORT 2020

for the implementation of the Postdoctoral Project

PN-III-P1-1.1-PD-2016-0484

"*Assessing the influence of nonsteroidal anti-inflammatory drugs on physiological characteristics and secondary metabolites in autochthonous vegetables*"

Stage 3. Determination of the NSAIDs amount accumulated in the studied vegetables, and soil respectively

(January – April 2020)

Summary of Stage 3

Content of the scientific and technical report (RST)

4.1. Statistical and comparative interpretation of the obtained results

4.2. Results dissemination

5. Conclusion

Diclofenac, ibuprofen, and naproxen are easily available pharmaceuticals that belong to the nonsteroidal anti-inflammatory drug (NSAIDs) class. NSAIDs, as well as their metabolites, are introduced into the water cycle through the municipal wastewater treatment plant in all industrialized countries. Thus, these pharmaceutical products are found in the aquatic environment, globally, and traces of these drugs have been determined in different compartments of the environment, including surface waters. As a result, increasing quantities of these compounds reach water flows, lakes that often serve as drinking water reservoirs in industrialized countries. In this context, contamination of the environment with pharmaceuticals has become a significant concern for environmental policy, especially in the U.E.

To address these challenges, in the analysis of pharmaceuticals from environmental samples, solid-phase extraction (SPE) is widely used to isolate and pre-concentrate the analytes before separating and quantifying them using high-performance liquid chromatography (HPLC) with a diode array (DAD) or mass spectrometer (MS) detectors.

Thus, within this stage of the project (January – April 2020) is presented a SPE-HPLC/DAD procedure elaborated for the simultaneous determination of diclofenac, ibuprofen, and naproxen in vegetables *Atriplex patula* L., *Spinacia oleracea* L., and *Lactuca sativa* L., as well as in their soil.

The plant material was obtained by planting seeds of orache (*A. patula*, local sources, Cluj-Napoca, Romania), spinach (*S. oleracea*, Agrosel, Romania), and lettuce (*L. sativa*, Agrosel, Romania) to a depth of 1 cm, in plastic containers containing commercial garden soil. The vegetables were grown under controlled light conditions (for 12 h from 24 h) and day/night temperature of 25/18ºC.

The abiotic stress to which the vegetables were subjected consisted of watering them every two days with aqueous solutions of NSAIDs (diclofenac, ibuprofen, and naproxen) of 0.1 mg/l, 0.5 mg/l, and 1 mg/l concentrations. Also, control vegetables (untreated with NSAIDs) were grown and watered with the same volume, but with distilled water. The experimental measurements were made at eight weeks after the emergence of the vegetables. For a correct assessment of the effects

of NSAIDs on the selected vegetables, all experiments were performed three times with independent leaf samples that were grown under the same conditions.

The analysis of NSAIDs was performed using an HPLC-DAD (Shimadzu, Japan). Chromatographic separation was performed on a Nucleodur 100-3C18ec column (Macherey-Nagel, Germany) thermostated at 30 °C. The mobile phase used was methanol (A) and ultrapure water with 0.2% formic acid (B). The elution of the compounds was achieved isocratically (75% A and 25% B) with a flow rate of 0.43 ml/min. In order to obtain the calibration curves, standard working solutions were prepared by successive dilutions of the stock solution with methanol, in the concentration range between 0.5-100 μg/ml for diclofenac and ibuprofen, and 0.1-80 μg/ml for naproxen. A volume of 20 μL of each prepared solution was injected for analysis under the chromatographic conditions described above.

For simultaneous extraction of selected NSAIDs from vegetables and soil, the SPE Oasis HLB (hydrophilic-lipophilic balanced, 6 ml, 500 mg) polymer cartridge with hydrophilic and lipophilic characteristics, purchased from Waters (U.S.), was selected.

First, the SPE extraction method of NSAIDs from liquid matrices with the known concentrations of NSAIDs was established. Thus, the working protocol followed to extract NSAIDs of interest from liquid matrices consisted of several steps:

– conditioning the stationary phase with 10 ml methanol and 10 ml ultrapure water;
– passing a volume of 200 ml of a sample containing known NSAIDs (40 μg of each drug) through the extraction cartridge; the sample volume flow that was passed through the cartridge was 1.2 ml/min;
– the elution of the NSAIDs retained on the stationary phase was achieved with 6 ml methanol.

All of these steps of extracting NSAIDs from liquid matrices were performed using a Supelco Visiprep 24 DL manifold vacuum extraction device (Sigma-Aldrich, Germany). The extracts thus obtained were evaporated to dryness at 30 °C with a rotary evaporator (Laborota 4011-digital, Heidolph, Germany) and then taken up in 1 ml methanol. All extractions were performed three times and evaluated using the previously established HPLC-DAD method. Before analysis, the samples were filtered with a 13 x 0.45 μm nylon filter. Thus, for diclofenac a recovery degree of 100.73% was obtained, for ibuprofen 87.88%, and for naproxen 108.51%.

The extraction of selected drugs from vegetables and soil was performed by ultrasound-assisted extraction, and the extracts thus obtained were subjected to the previously established SPE method. Thus, in order to optimize the extraction of the three NSAIDs from the selected vegetables and their soil, 13 experimental variants were tested, which were generated using the Minitab 17 software. In these cases, the volume of extraction solvent and time extraction (ultrasound-assisted extraction) were selected as variables.

In the case of NSAIDs extraction from vegetables, over 0.5 g sample (fresh weigh – FW) with a known concentration (40 μg) of each selected NSAIDs was added a volume of extraction solvent (methanol) corresponding to the experimental variant established. The mixture thus obtained was subjected to ultrasound-assisted extraction, for a specific time, as well as the experimental variant. After the end of the sonication time, the extracts obtained were centrifuged for 10 min, at 7000 rpm, evaporated to dryness and taken up in 2 ml of methanol. The extracts were brought to the volume of 200 ml with ultrapure water and subjected to the SPE working protocol described above. The final extracts obtained were filtered, and the content of NSAIDs was determined using the established HPLC-DAD method. The best recoveries of the NSAIDs from the green leafy vegetables were obtained with the experimental variant no. 7, variant with a recovery degree of 96.23% for diclofenac, 81.27% for ibuprofen and 101.84% for naproxen (Fig. 1). These degrees of recovery were obtained using a volume of 20 ml of methanol and a sonication time of 30 min.

In the case of the optimization extraction of NSAIDs from the soil in which the treated vegetables were grown, to 1 g sample (soil) with a known concentration (40 μg) of each selected NSAIDs was added and equal amounts of methanol, ultrapure water and ethylenediaminetetraacetic acid (EDTA, 5%) was added, so the volume of final extraction solvent corresponds to the experimental variant established. The mixture thus obtained was subjected to ultrasonic-assisted extraction, for a specific time corresponding to the experimental variant. At the end of the sonication time, the extract obtained was centrifuged for 15 min, at 7000 rpm, evaporated to dryness and taken up in 2 ml methanol. This extract was brought to the volume of 200 ml with ultrapure water and subjected to the SPE working protocol described above. The final extracts obtained were filtered, and the content of NSAIDs was determined using the established HPLC-DAD method.

After testing the experimental variants to optimize the extraction of NSAIDs from the soil, it was concluded that the best recovery degrees of the selected NSAIDs were obtained with the experimental variant no. 5, in which a volume of 15 ml extraction solvent was used and a sonication time of 34.14 min. With this experimental variant, a recovery degree of 82.71% was obtained for diclofenac, 85.14% for ibuprofen, and 81.85% for naproxen (Fig. 1).

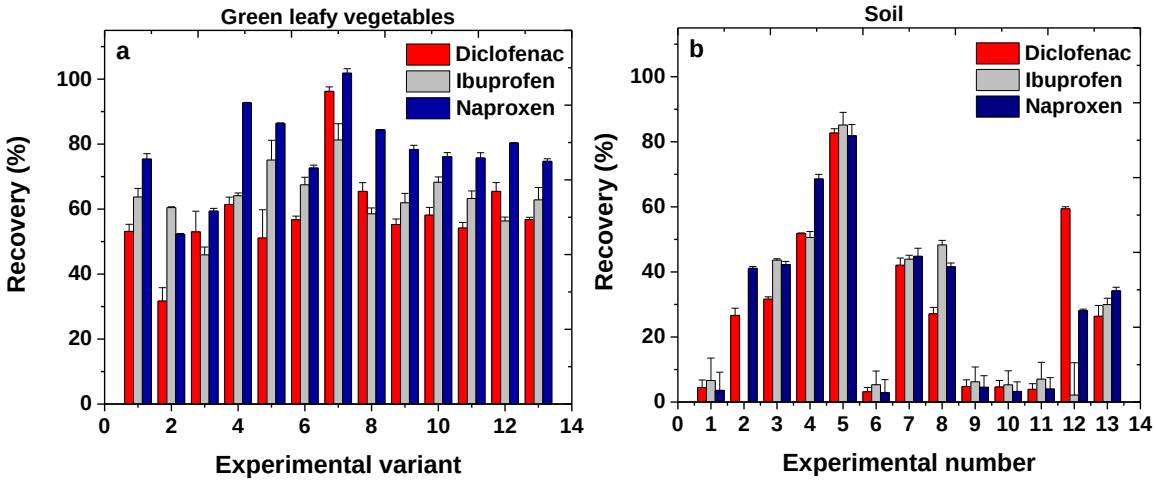

Fig. 1. The average recoveries (± RSD, %, three determinations) of the studied NSAIDs (diclofenac, ibuprofen, and naproxen) obtained by optimizing their extraction from the vegetables (a) and soil (b).

According to the previously established working protocols, the selected NSAIDs from the treated green leafy vegetables (*A. patula*, *S. oleracea* and *L. sativa*) and their soil were extracted and analyzed at eight weeks after the emergence of the vegetables. Thus, regarding the green leafy vegetables, diclofenac was not detected in any of the analyzed samples. In the case of vegetables, *A. patula* was determined naproxen in concentrations between 1.22 and 4.37 μg/g FW. Ibuprofen was detected (0.8 μg/g FW) in the leaves of *S. oleracea* vegetables treated with a concentration of 1 mg/l ibuprofen. Naproxen was also detected in the *S. oleracea* leaves treated with all three concentrations. Thus, in vegetables treated with 0.1 mg/l naproxen, a concentration of 5.43 μg/g FW was determined, for those treated with 0.5 mg/l a concentration of 6.39 μg/g FW, and for those treated with 1 mg/l a concentration of 6.70 μg/g FW. Also, in the case of *L. sativa* leaves were detected quantities of ibuprofen (3.37 μg/g FW in the treatments with 1 mg/l ibuprofen) and

naproxen (6.52 μg/g FW in case of the treatments with 0.1 mg/l naproxen; 10.08 μg/g FW in the case of 0.5 mg/l treatments; 8.98 μg/g FW in the case of the treatments 1 mg/l naproxen).

In soil samples in which the selected vegetables were grown and treated with the three NSAIDs, drug concentrations ranged from 0.38 μg/g soil to 9.00 μg/g soil. In the soil where *A. patula* vegetables were grown, ibuprofen (0.38 - 1.46 μg/g soil) and naproxen (2.51 - 3.15 μg/g soil) were determined. The soil in which *S. oleracea* vegetables were grown and treated with ibuprofen in the concentration of 0.5 and 1 mg/l had an ibuprofen content of 0.87 μg/g soil, respectively 0.65 μg/g soil. Treatments of *S. oleracea* vegetables with naproxen (all three concentrations used) led to naproxen concentrations in the soil of 4.35 μg/g soil, 8.46 μg/g soil, and 2.43 μg/g soil respectively. The soil where *L. sativa* vegetables were grown was the only soil where diclofenac was detected (6.45 μg/g soil, 1 mg/l diclofenac treatment). The soil where *L. sativa* vegetables were treated with ibuprofen had a content of ibuprofen between 1.22 μg/g soil and 4.27 μg/g soil. The highest amount of drug detected in soil samples was 9.00 μg/g soil, in the soil where *L. sativa* vegetables were grown and treated with 1 mg/l naproxen.

The results obtained during this stage of the project are:

a) **method** regarding the determination of NSAIDs accumulated in vegetables and soil;
b) **method** regarding the extraction of NSAIDs accumulated in vegetables and soil;
c) **study** regarding the accumulation degree of NSAIDs in the vegetables and soil, and its correlation with the experimental data obtained in the Stages I and II of the project;
d) **scientific and technical report** (RST);
e) dissemination of the results:
   - **update of the web page** (http://www.itim-cj.ro/PNCDI/ru12/index.html) of the project;
   - **scientific article** ("*Biotransformation of non-steroidal anti-inflammatory drugs induce ultrastructural modifications in green leafy vegetables*", autori: Ocsana OPRIȘ, Maria L. SORAN, Ildikó LUNG, Alexandra CIORÎȚĂ, Lucian COPOLOVICI) under review – Environemntal Research.

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