XXVIII Congress of the Analytical Chemistry Division
Bari 22 – 26 September 2019

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DETERMINATION OF OXIDATIVE STRESS MARKERS IN BIOLOGICAL MATRICES BY MEANS OF UHPLC-MS/MS

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Oxidative stress (OS) is a physiological state leading to Reactive Oxygen Species (ROS) that are negative factor for human's health. OS is involved in aging processes and in many cardiovascular diseases, diabetes, and carcinogenicity processes. It is therefore evident that the need for reliable (bio-)markers for the identification of this physiological state is fundamental. Recently the class of isoprostanes of have been identified as gold standard for *in viv*o OS evaluation, thanks to their robust correlation in lipid peroxidation induced by the presence of ROS. Also other classes of molecules may be used as markers as they provide different information on the OS even in peripheral phenomena or by specific external threats towards the organism (oxysterols, resolvins).
The aim of this research was the development of tailored analytical methods for the evaluation of oxidative stress through the monitoring of specific markers, in order to evaluate the modifications in the physiological state by different point of view. The developed analytical tools may help to evaluate the effect on the oxidative state in humans or model organisms resulting from the exposure of xenobiotics or due to incorrect dietary regimes. The main goal was the development of a reliable analytical method for the determination of the levels of isoprostanes in the urine by means of dispersive Liquid-Liquid Micro Extraction (dLLME), subsequent micro Solid Phase Extraction (μSPE) clean-up and UHPLC-MS/MS analysis. The analytical targets were mainly high sensitivity and effective clean-up of the sample, for the quantification of 8-isoprotaglandine F2α (8-iso-PGF2α) and 5 isoprostane F2α (5-IPF2α), together with some metabolites and/or isomers.
Another goal was the identification and quantification of membrane oxysterols in different matrices, mainly swine and kelp spermatozoa. The presented method is focused mainly on the following oxidized forms: 20d-hydroxycholesterol, 25-hydroxycholesterol, 25-hydroxycholesterol, 22-hydroxycholesterol, 7α- and 7β-hydroxycholesterol and desmosterol. The samples deriving from spermatozoa were processed through a Liquid-Liquid Extraction (LLE) and subsequent clean-up by μSPE.
Some interesting results were also obtained for oxysterols in Zebrafish, as it was established as a model organism in studies of genetics, developmental biology, neurophysiology and biomedicine [1,2]. It is used as a biomarker of the effects of toxins and pollutants in the environment and in the study of the pathogenesis of some human diseases [3]. For Zebrafish embryos the samples were processed by LLE in an orbital shaker; after the withdrawing and drying of organic portion a μSPE clean-up step was performed.

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In addition also studies on resolvins in plasma were carried out as they are involved in inflammatory processes. This group of molecules arise from the n-3 fatty acid docosahexaenoic acid (DHA) or from the long-chain n-3 fatty acid eicosapentaenoic acid (EPA). These mediators, acting via G-coupled protein receptors, have potent anti-inflammatory and proresolving actions [5].
In the case of isoprostanes we must deal with compounds present in very small concentration in the urine; furthermore, they are a challenge for UHPLC-MS/MS analysis due to the relevant matrix effect [7]. Given this problem, we opted for a dLLME extraction which allows a significant enrichment factor of the analytes and a µSPE clean-up. For oxysterols, a different approach was applied, in fact they are not so prone to matrix effect as Isoprostanes and the requested LOQs are significantly higher. They, on the other hand, still face challenges: it was therefore difficult to completely and rapidly disrupt the membrane phospholipids, which was performed by an immersion probe followed by an LLE extraction and then a µSPE clean up to enrich the extract. The chromatographic separation was crucial as all analytes are positional isomers or epimers. Another difficulty of these compounds is their low sensitivity in mass spectrometry without a derivatization process [8]. This step was avoided by working on the extraction method and using an APCI source. For resolvins in plasma dLLME and µSPE were used for extraction and clean-up, while ESI negative ionization was applied for UHPLC-MS/MS analysis.
The obtained results for the different classes of compounds were satisfactory, in fact it was possible to obtain a good extraction with the dLLME (or LLE) technique, succeeded in concentrating the sample and then performing a µSPE clean-up which led to a lower matrix effect and good enrichment factors.

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