STAGE 4 SUMMARY

Different separation and purification procedures based on molecular imprinting technique may provide an alternative fast, simple and selective extraction of certain active components from complex matrices resulted from natural products standard extraction such as medicinal herbs). Yet, to get economically viable results, that can replace the cumbersome and expensive methods of hypericin (H) separation and purification from St. John's Wort extracts, new procedures should be addressed for the imprinting protocol. In this respect, the present project uses new H-molecularly imprinted polymers (MIP) as separation tools. This new approach aims at preparing MIPs with an concentrated and purified extract of naphtodianthrones as "template" in order to generate imprinted cavities selective for H only. This selectivity of MIPs for H is given by the difference of water solubility between the two naphtodianthrones of the extract with structural resemblance i.e. H insoluble in water and pseudohypericin (PH) 33 mg / L.

To validate the upper-stated obtaining technology of the molecularly imprinted pearls with hypericin by phase inversion, a series of studies, consisting of using as template for imprinting several sources of hypericin (a.k.a. the concentrated and purified hydro-alcoholic extract containing only hypericin and pseudohypericin and the commercial hypericin (high purity 98%) were made. In the previous stage, (stage III) an original protocol was developed to separate hypericin *in situ,* from hydro-alcoholic extracts of naphtodianthrones, directly into the imprinting stage. In this stage, as a preliminary and necessity step for establishing the preparation technology for the H-MIP by phase inversion, an interesting study was conducted to verify the previous proposed imprinting method that uses the pure commercial hypericin as template. The two methods are compared via structure, thermal behaviour, morphology and re-adsorption performance.

The original approach, with the natural naphtodianthrone extract of *Hypericum Perforatum* as a "phyto-template" used for the phase inversion (described in stage III), led to innovative ways for to produce macroporous adsorbents with advanced selective properties for hypericin. In the conventional imprinting approaches, template molecules must always be pure compounds as described in the literature -the first stage of the project. Our study has shown, however, that imprinting can work with natural extracts as well if they are concentrated and sufficiently purified. Therefore, using a basic principle related to compounds distribution (i.e. H and PH) between two solvents with different polarities, *in situ* separation (in parallel with the phase inversion of the copolymers) was possible. However, in order to validate the assumptions from earlier development stages of the laboratory technology (for the

preparation of molecular imprinted pearls with the phyto-template), it was necessary to apply the imprinting procedure using pure hypericin (commercial). Further on, the results with pure hypericin were less efficient than those obtained in the last round with the naphtodianthrone extract. Although pure compounds are usually used for imprinting, it seems that its activity after multiple purifications (used to obtain pure commercial substances) is diminished. This decrease in activity involved conformational distortions of the molecule, which no longer has the same active sites on which we can induce the specific complementary cavities, for hypericin only, in the polymer matrix. In conclusion it appears that these pearls are specific to similar structures from the naphtodianthrone class, similar to the results obtained usually with the "epitope" method, where only part of the template molecule is used to generate imprinted cavities capable of separating a class of structurally similar substances. Therefore, these results confirmed that imprinting can also be achieved with a purified and concentrated extract where the conformation of template compound is undamaged. As demonstrated in this stage, the activity of commercial hypericin is much lower than that of hypericin from the fresh extract.

Consequently, the production technology for molecularly imprinted polymer beads through the phase inversion was completed. The prospects of this innovative technology are important, both in terms of economic and productivity compared to current separation methods of naphtodianthrones. The study within this step offers another way to separate / concentrate / purify natural compounds, otherwise expensive. This technique takes into account the criteria of solubility and polarity, and thus, ensuring MIP selectivity for only one component of the initial extract.

In the next stage the preparation technology for the molecularly imprinted polymer beads and the process of suspension polymerization will be well-established. Further on, the specific tests on the resulted MIPs will be extended to solid phase extraction (SPE) experiments using MIPs, a technique called molecularly imprinted solid phase extraction (MISPE).

Structural analyzes of MIPs aimed at correlating their morphology with the adsorption properties. For this reason, imprinted polymers were analyzed at different stages of processing i.e. prior to extraction, after extraction or after final washing step.

In the case of imprinted pearls with pure hypericin, FTIR analysis revealed the effect of introducing a template through a phase inversion process in the preformed polymer structure. It was also underlined the absence of other newly established bands that could attest other covalent interactions between the polymer and the template. Thermal properties of the pearls were similar to those obtained with the naphtodianthrones extract in the previous step.

In addition, TGA-DTG analyzes confirmed structural changes due to the template, by shifting the cyclization peaks to higher temperatures for the pearls containing hypericin in their structure.

In this stage 5phase extraction tests were conducted in the laboratory, from dry plant material containing varying concentrations of hypericin (from 0.0431 to 0.0947% w / w p.v.), under different extraction parameters set by the kinetic extraction studies accomplished under phase III, in order to develop the obtaining technology for hypericin concentrates from St. John's Wort.

Extraction studies of total naphtodianthrones (expressed in hypericin) in the laboratory phase, under the specified extraction conditions, indicated that the type (aerial part, blossoms) and the quality of the vegetal product (total naphtodianthrones expressed in hypericin min. 0.08% g / g s.u., European Pharmacopoeia) are defining for obtaining a concentrate in hypericin of min. 0.3% w / w relative to the dry substance.

In the extraction tests, carried out in order to establish the extracting technology of total naphtodianthrones (expressed in hypericin units) in the laboratory stage, were taken into account:

- The quality of vegetal raw materials (min. 0.8% of total naphtodianthrones expressed in hypericin / p.v.);
- The preliminary processing of plant material, degreasing and dechlorophying (Soxhlet / reflux), for ensuring the optimum extraction of hypericin, by removing ballast substances;
-The purification of primary extracts (filter ballast substances type adsorbent material talc), necessary for processing the raw plant material in full or the one processed by reflux extraction of other ballast substances;
- The uniformity of plant material influences the extraction process of hypericin and establishing working intervals for the optimum extraction are required.

Test 3 extraction yielded a superior content of 0.5731% w / w (s.u.) in hypericin, providing the necessary data to establish the extraction technology in the laboratory stage. The technology was developed thereafter in the laboratory stage, to obtain a concentrated extract of hypericin from St. John's Wort (*Hypericum perforatum*). The applied process resulted in a phyto-complex concentrated in naphtodianthrones, in which the bioactive compound of interest was hypericin (min.0,3%).

Subsequently, the phyto-complexes were subjected to a series of analysis together with the supernants resulted from the adsorption tests applied to the molecularly imprinted polymers. All the carried-out tests showed that naphtodianthrones were separated efficiently.

A new accelerated solvent extraction in microwave field (ASE) was also tested and optimized to increase the efficiency of the primary extraction method. The evolution of tests was tracked on a DIONEX ASE 200 device (Dionex Corp., Sunnyvale, CA, USA) equipped with 33 ml stainless steel cells and with a solvent controller. The obtained results by this accelerated solvent extraction method indicated a more effective extraction than the standard processes regarding hypericin and pseudohypericin concentration.