STAGE 2 SUMMARY

Our country disposes of huge reserves of biomass, prominently including some of the most varied and least altered by pollution spontaneous flora in Europe. Among the spontaneous flora, the abundance of medicinal plants, which are an endless source of active ingredients, useful for diseases prevention or treatment, personal hygiene materials, cosmetics, dyes, etc., is remarkable.

The biggest problem in exploiting highly valuable compounds from medicinal plants belonging to the spontaneous flora is the fact that they are usually found in very small quantities in plants and are accompanied by a lot of other compounds, making their extraction and purification very difficult.

An example of such high valuable compound is hypericin, found in St John's wort, it's name deriving from the plant's Latin name: Hypericum perforatum. Hypericin is considered a substance with great future in photochemical therapy of skin cancer, but recent studies have demonstrated other pharmacological effects, such as antiviral and antidepressant action.

Extraction of this substance encounters the same difficulties as the extraction of other active ingredients, innovative technological solutions being required.

Molecular imprinting of polymers is a modern and explosively developing field, which has many advantages over conventional separation and purification techniques.

The purpose of this project is to develop a technology of obtaining polymeric materials molecularly imprinted with hypericin, suitable for its separation from primary extracts, and using these materials in selective separation of extracts of St John's wort, in order to obtain highly concentrated bioactive hypericin. The developed technology and the obtained polymers must also meet the requirements of industrial application.

The research work performed in the 2nd Stage of the project, for obtaining polymer materials molecularly imprinted with hypericin, started with a detailed characterization by HPLC of the compositions of primary St John's wort extracts, received from SC Plantavorel.

The molecular imprinting of polymers was achieved by two methods :

- Imprinting by phase inversion;
- Imprinting by suspension polymerization.

The rheological behavior of all imprinted copolymer solutions was pseudoplastic at low shear gradients, with a tendency to shift to Newtonian, when increasing shear gradient value. Pseudoplastic behavior is accentuated with increasing methacrylic acid content of the copolymer.

Imprinted copolymer solutions were converted into polymer beads by coagulation in 3 different compositions of inversion baths. The obtained pearls, imprinted with hypericin, show a similar appearance in all 3 experimental coagulation baths. However, it can be noticed that the polymer beads color becomes darker as the concentration of hypericin in solution increases.

Both imprinted polymers (MIP) and non-imprinted polymers (NIP) were subjected to thermal analysis, in order to identify differences that indicate the presence of target molecules in the polymer matrix, and to determine the thermal stability of the materials.

The characterization of imprinted polymer beads was performed both at ICECHIM and UPB, on different samples.

Thermal analysis, performed at ICECHIM, reveal the presence of hypericin in the molecular imprinted systems, based on four characteristic signals:

1. Concerning the mass derivative curves, a larger amount of hypericin is highlighted by the lower temperature of the first thermal decomposition stage and by the peak area;

2. On the same curves, a characteristic maximum at 310 ± 30 °C, matches for the total decomposition of the naphthodianthrone structure of hypericin;

3. Regarding the mass loss curves, significant differences in weight loss are observed in the temperature range of 80-480 °C, correlated with the content of hypericin as follows: the curves corresponding to systems with different amounts of hypericin show a greater mass loss the higher the percentage of hypericin used during imprinting;

4. Related to the heat flow curve, it can be noticed that hypericin appears to amplify the effect of the exothermic cyclization of polyacrylonitrile segments of the matrix, but to decrease the low exothermic effect of the dehydrogenation and aromatization reactions.

The characterization of the molecular imprinted and non-imprinted pearls, performed by UPB, revealed the following:

1. From the DSC curves it is noticed that the higher the content of hypericin of the sample, the greater the amount of water removed. In addition, by increasing the amount of methacrylic acid , the shoulder at 250 °C, specific to the cyclization phenomenon, no longer appears and exothermic peaks shift to higher temperature values;

2. TGA analysis show that the increase of the methacrylic acid content of the non-imprinted samples produces an increase in mass loss, ie a reduction in the amount of residue remaining at 600 ^{o}C. The increase of hypericin content of the imprinted samples also leads to increased mass loss;

3. DTG analysis reveals that all non-imprinted and imprinted samples show several stages of mass loss. In all samples the main decomposition occurs in two steps: one step of

decomposition at maximum temperatures between 315-390 ^oC and another at maximum temperatures between 390-420 ^oC;

4. In both imprinted and non-imprinted polymers FT-IR spectra, characteristic vibration bands of the polymer matrix can be easily distinguished. Besides the bands characteristic to the polymer and the intramolecular water, the appearance of an additional band, in the range 3600-3630 cm^{-1} is observed, due to ν_{-OH} (Ar-OH in hypericin) stretching vibration.

Investigations conducted on the second research direction (suspension polymerization) have shown the following:

1. Molecular imprinting by suspension polymerization method was based on a radical polymerization system using two types of initiators;

2. The extraction of Hypericin template from polymer particles was performed using two solvents;

3. It has been noticed that the appearance of the imprinted particles obtained by suspension polymerization is preserved after extraction, regardless of the medium used;

4. FT-IR spectra of the un-extracted imprinted polymers show, besides bands characteristic to the polymer and intra-molecular water, an additional band characteristic to hypericin. After applying the two methods of hypericin extraction, this band disappears, indicating a proper removal of hypericin from the polymer, thereby generating active imprinted cavities;

5. All SEM images reveal both spherical polymer particles and irregularly shaped particles. The first used solvent shows preservation of spherical particle shapes in a greater extent than when using the second solvent, but porous structure is evident in both types of extracted particles;

6. Calculation of Retention capacity and Imprinting factor, following HPLC analyzes, show that, of the four imprinted polymers, the best sample shows maximum values of Adsorption capacity and Imprinting factor, both for hypericin and for competitor named C1. Note that the extracted polymers showed very good adsorption and Imprinting factor values above 1 for hypericin. These results indicate both the formation of a significant number of imprinted cavities and a more efficient activation of these cavities, by using this extraction method to remove the template;

7. The effect of imprinting on selectivity was quantified using specific parameters, computed both for imprinted polymers and their non-imprinted counterparts. All imprinted polymers show values of selectivity coefficient, k ', above 1, indicating a preferential adsorption of hypericin, relative to competitor C1.

Studies performed for selective separation of hypericin from St. John's Wort phytoextracts demonstrated the following:

- Extracts obtained with ethanol, at room temperature and at reflux, are characterized by a high content of total naphthodianthrones, expressed as hypericin, thus representing an important source of active principles with bioactive potential. The hydroalcoholic solvent used to obtain the extracts above mentioned, has also allowed the extraction of:

> Chlorophyll pigments and fatty substances, these classes of compounds being considered ballast substances, that impede optimal extraction of hypericin from Hypericum perforatum (St. John's wort aerial part);

> Polyphenolic substances (polyphenolic carboxylic acids , flavonoid compounds, tannins) categories of bioactive principles, but which are not of interest in our current studies, and may impede optimal extraction of naphthodianthrone compounds expressed as hypericin;

- The chromatograms performed for identification of hypericin – after developing (at 254 and 366 nm) and spraying with the identification reagent (at 366 nm) - have shown the presence of the spot characteristic to hypericin in the analyzed samples. It was also observed that the dechlorophyillation process did not complete.

Due to the fact that hypericin is a photosensitive and thermolabile compound, successive stages of purification processes of crude extracts being able to lead to degradation of hypericin, other selective methods of obtaining extracts with a high content of hypericin were studied, namely subjecting the plant material to several dechlorophyillation and degreasing processes, advanced processing, as preliminary steps in the process of extracting naphthodianthrone compounds.

Of the two methods of extraction of ballast substances (refluxing and Soxhlet extraction), the Soxhlet method ensured complete extraction of chlorophylls and fatty substances. Selection of optimal conditions for plant raw material prior processing is the important starting point for further studying selective extraction of hypericin, establishing optimal parameters and phase extraction lab. technology, followed by extrapolating at micropilot and pilot scale, in order to obtain hypericin enriched extracts.

The hydro-alcoholic extract of St. John's Wort was subjected to procedures of separation and concentration in hypericin, using various traditional methods combined with preparative HPLC. The optimal method was a combination of extraction l-l combined with preparative HPLC, which showed 95 % yield and a final concentration of the extract of 130 mg / L.

The studies performed have demonstrated the possibility of obtaining polymer materials molecularly imprinted with hypericin and using them in selective separation of St. John's wort extracts. Research will be continued in order to develop technologies for obtaining molecular imprinted polymers and selective separation of hypericin from St. John's wort extracts.