OPTIMISATION OF HYPERICIN ULTRASOUND ASSISTED EXTRACTION: A MATHEMATICAL MODEL APPROACH

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This study focused on maximizing the ultrasound-assisted extraction (UAE) yield and purity of hypericin from Hypericum perforatum, as a function of ultrasound (US) power (60-120W), solvent:solid ratio (5-20 mL/g), and extraction time (10-60 min). The data were subjected to response surface methodology (RSM) and the results demonstrated the polynomial equations significance for all models. They did not show lack of fit proving their suitability for prediction purposes. Using desirability function, the optimum operating conditions to attain a higher extraction yield were found to be 120W US power, 10-minute extraction time, and 20:1 (mL/g) solvent (methanol-acetone 2:1 (v/v)):solid ratio.

Keywords: Design of Experiments, St. John's Wort, hypericin, polarity of solvent, ultrasound-assisted extraction.

1. Introduction

The botanical genus *Hypericum* comprises a number of approximately 450 species widely distributed around the world [1]. In Europe, there are 61 native species, out of which 12 can be found in Romania. Furthermore, these can be split into 11 spontaneous and one cultivated species [2]. However, despite the large variety and availability, only one species has truly perked the interest of the scientific world: *Hypericum perforatum*, more commonly known as St. John's Wort.

St. John's Wort (SJW) is a perennial plant and a freely branching shrubby herb, with a height of 40 to 80 cm on average. It typically starts flowering in the early summer, a time biblically associated with several feasts and celebrations dedicated to St. John, hence the more commonly used name [3]. When the plant reaches full maturity, it produces small yellow flowers, each of them having five petals [4]. Each has several small black dots on their edges and produces a reddish liquid when crushed. These have been associated with tissues containing high quantities of the naphtodianthrones hypericin and pseudohypericin [5]. Generally,

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towards late summer, the flowers mature, dry, and transform into capsules containing seeds.

SJW has been known and used as a medicinal plant for many years. The oldest written reference regarding the medical applications of this plant can be traced back to Ancient Greece and attributed to the pharmacologist and military medic Pedanius Dioscorides. In his book "*De Materia Medica*", SJW is described as having healing, diuretic, analgesic, and antimalaria properties, some of which are recognized and still in use today. But he first to truly get close to the modern applications of *Hypericum* was Swiss alchemist Paracelsus, who recommended it for ill temper and anxiety [3].

Nowadays, SJW has garnered attention by being a relatively safe and widely available nutraceutical and dietary supplement. It has been included in pharmacopoeias and has been thoroughly analyzed to reveal the phytochemicals responsible for all the therapeutic effects associated with its use.

One such phytochemical is hypericin, which has been proven to be one of the main constituents responsible for the antidepressant effect of SJW extracts. It has also been proved to be a good antiretroviral and an excellent cytotoxic. Moreover, its application in oncological phototherapy is being regarded as the future of curing skin cancer in a patient-friendly manner. The principle is rather simple but very effective: the tumor is injected with a hypericin-based preparation and the site is exposed to a beam of light with a specific wavelength at which hypericin starts generating reactive species within the cell. The presence of these radicals, along with the incapacity of the cells' own antioxidant systems to keep up with the demand, lead to apoptosis [6].

For this form of therapy to be feasible in a clinical setting, it is imperative to find a method to extract large quantities of hypericin from SJW. Also, it should be noted that while there are ways of laboratory synthesis, their yields had been found to be insufficient [7].

There are various methods used to extract hypericin, but the most popular ones are Soxhlet and UAE. Both entail using a solvent to remove the interest compound from the vegetal material. However, using a Soxhlet extractor is slow and time-consuming. It also requires large quantities of solvents, and the overall yield is unsatisfactory [8]. On the other hand, UAE has proven to be much faster, with a better overall yield. However, it is widely known that the compounds affinity towards the used solvent can have a significant impact on the extraction yield [9].

Therefore, the aims of this paper are identifying and testing a number of solvents that should allow for better extraction yields and purities to be obtained and optimizing the UAE of hypericin.

2. Materials and methods

2.1. Materials

Dried and powdered St. John's Wort was purchased from an authorised local medicinal plants and plant products supplier. HPLC grade methanol (MeOH) was purchased from Lab-Scan. AnalaR NORMAPUR® ACS acetone (Ac) and absolute ethanol were purchased from VWR. HPLC grade acetonitrile (AcN) was supplied by Merck.

2.2. Extraction of hypericin

Dried St. John's Wort was milled into a fine powder. It was then mixed with methylene chloride in a solid to liquid ratio of 1:10 for 2 hours in order to remove the high amounts of chlorophyll present. After this simple extraction, the suspension was filtered and the solids were dried at 50^0C for approximately one hour. The filtrate can be used to recover the extracted chlorophyll.

The dried SJW solids were further used for hypericin extraction, using the same experimental setup as described above. After the extraction, the mixtures were centrifuged, the supernatant was separated, and the precipitate discarded. The resulting solutions were red, varying from light to very dark and were stored in brown glass bottles in a cool environment, to prevent the light and temperature-induced degradation of the interest compound. [10].

To find the optimal extraction solvent, methanol, acetone, acetonitrile, and methanol-acetone mixtures were used. They were analyzed regarding their selectivity for hypericin when using a 1:10 solid to liquid ratio.

The extraction was performed using a UP200H Hielscher ultrasonic processor (200 W, 24 kHz). The sonotrode was lowered into a 100 mL cylindrical jacketed reactor, at 3 cm from the bottom of the vessel. A heating plate equipped with a magnetic stirrer was utilized. The temperature of the extraction mixture was maintained constant by circulating cooling water through the jacket. Furthermore, the photodegradation of hypericin was prevented by shielding the reactor from light with aluminum foil. The used parameters were: 80 W US power, 500 rpm stirring rate, and 30 minutes extraction time. After centrifugation, the solvent was completely removed from the filtrate by means of a rotary evaporator and the interest compound – hypericin – was redissolved in 20 mL of ethanol.

The ethanol-dissolved hypericin solutions were analyzed using the JASCO V-550 UV-VIS spectrophotometer, in glass cuvettes with a pathlength of 1 cm.

Hypericin was quantitatively determined using eq. (1) obtained from the calibration curve (see Fig. 1).

$$Conc_{HYPERICIN} = \frac{A_{592}}{0{,}2376} \quad (1),$$

where $Conc_{HYPERICIN}$ is the hypericin concentration expressed in mg/mL and A_{592} is the absorption of the sample at 592 nm.

The calibration curve was determined using column chromatography purified hypericin. A 200x20 (mm x mm) silica gel chromatography column (Merck) was used. The extract was eluted with chloroform, followed by chloroform:Ac 4:1 (v/v) and finally with MeOH:Ac:CH_2Cl_2 (75:10:15, v/v/v). The final fraction containing hypericin is evaporated to dryness using a rotary evaporator and used to prepare a stock solution in ethanol with a concentration of 20 mg/mL. The calibration curve is realized considering the absorbance determined at 592 nm.

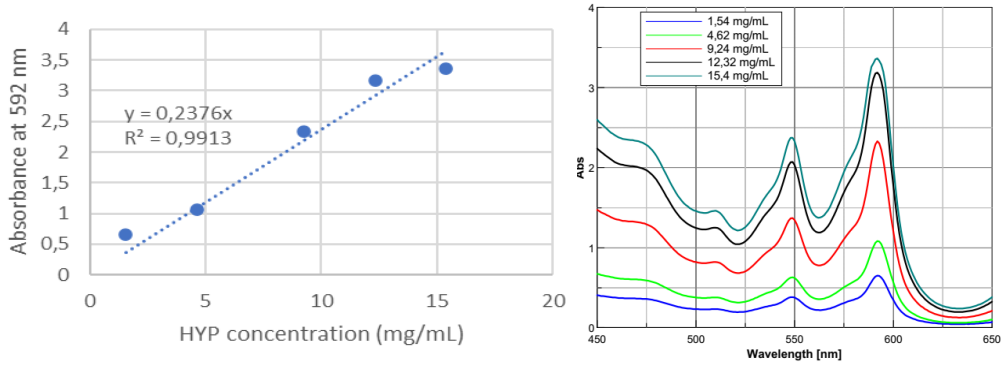

Fig. 1. Calibration curve for hypericin concentration determination

An important aspect to consider is the two peaks appearing on the absorbance spectra, at 592 and 539 nm. The first one is specific to hypericin, while the one at 549 nm can also indicate the presence of hyperforin. While both are valuable compounds, only hypericin displays the photodynamic effect necessary for the applications presented in the first part of this paper. As such, it is important to have higher yields of hypericin and a lower hyperforin content.

As a result, the difference in absorbance at 549 mm compared to the one at 592 nm can be considered as a purity estimate of the extracted hypericin. This estimation was calculated using equation (2).

$$Purity\ (\%) = \frac{A_{592} - A_{549}}{A_{592}} \cdot 100 \qquad (2),$$

where A_{592} and A_{549} are the absorbance values obtained at 592 and 549 nm, respectively.

The optimization of the ultrasound-assisted extraction of hypericin (UAE) is performed using a Design of Experiments (DOE) approach which allows the prediction of optimal values for experimental factors with the help of mathematical criteria included in the factorial design. The software of choice is Design Expert 11. The mathematical model built for testing is based on three numerical factors of continuous type: US power (60-120 W), solvent: solid ratio (5-20 mL/g) and

extraction time (10-60 min) with two responses: hypericin concentration (mg/g dry plant) and purity (%). The objective of the optimization is to maximize both responses. The chosen model was Optimal (Custom) Design with three replicates and two lack of fit. The search for design points was of the Coordinate Exchange type. The optimality of the model was of the I-optimal type (also called Integrated Variance) which is desirable for response surface methods (RSM) where prediction is important. The obtained experimental data were analyzed using Response Surface Methodology (RSM).

3. Results and discussions

3.1 Solvent selection based on its polarity

When discussing the best solvent choice for hypericin extraction the polarity is to be considered (see Table 1). This important parameter was determined for the two mixtures (MeOH:Ac 1:1 and 1:2) using equation (3).

$$P_{am} = \sum_{i=1}^{n} P_i \cdot \varphi_i \quad (3),$$

where P_{am} is the relative polarity of the mixture, P_i is the relative polarity of the individual components, and φ_i is the volumetric fraction of each individual component.

Table 1

Relative polarity of the used solvents

Solvent	Relative polarity
Methanol	0.355
Acetone	0.37
Acetonitrile	0.762

Analyzing the data in Table 2 can be observed that the quantity and the purity of extracted hypericin depend on the polarity of the solvent. Therefore, solvents with a medium polarity tend to extract more of our interest compound, compared with those of lower polarity (Fig. 2). Also, these tend to generate extracts of higher purity than the more polar counterparts (Fig. 3).

Table 2

Concentration and purity of hypericin extracts when using solvents of various polarities

Solvent	Relative polarity	A_{592}	A_{549}	Conc. HYP, mg/g dry plant	Purity, %
Methanol	0.355	0.385	0.189	16.20	50.90
Acetone	0.37	0.567	0.227	23.86	59.96
MeOH:Ac 2:1	0.46	0.745	0.467	31.36	37.31
MeOH:Ac 1:1	0.63	0.567	0.345	23.86	39.15
Acetonitrile	0.762	0.275	0.155	11.57	43.63

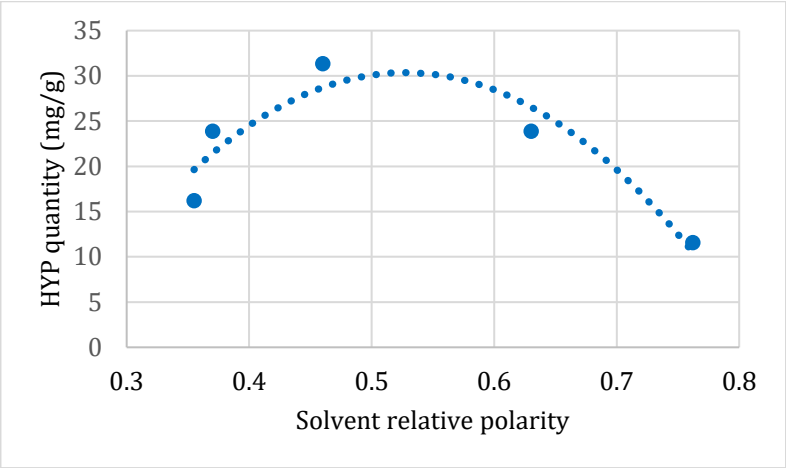

Fig. 2. Influence of the solvent relative polarity on the amount of hypericin extracted per gram of dry vegetal material

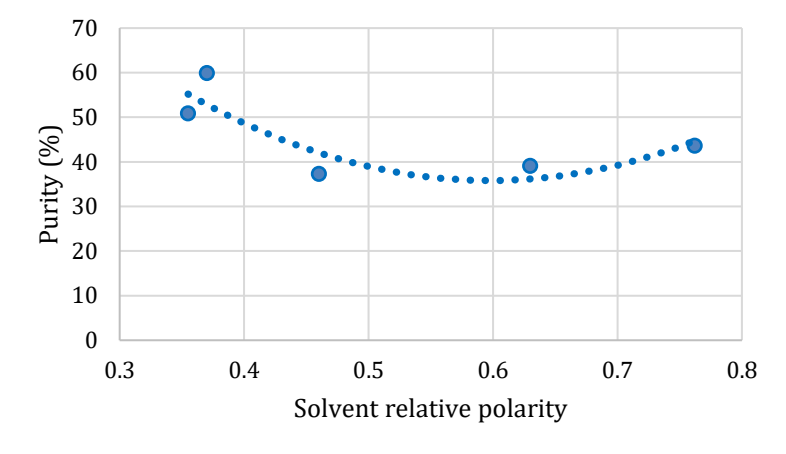

Fig. 3. Influence of the solvent relative polarity on the purity of the hypericin extract

From the obtained data was determined the optimal solvent, MeOH:Ac 2:1. When used, it accounted for a high extraction yield (~32 mg/g dry plant) and an acceptable hypericin purity (~38%).

3.2 The optimization of hypericin ultrasound assisted extraction (UAE)

In Table 3, the summary of the conversion of the experimental variables into code variables is given. The analyzed responses are hypericin concentration (mg/g dry plant) and purity(%), their maximization being one of the study objectives.

Table 3

Input parameters for hypericin UAE optimisation

Factor	Name	Units	Type	Min.	Max.	Coded low	Coded high	Mean	St. dev.
A	**US Power**	**W**	Numeric	60.00	120.00	-1 ↔ 60	+1 ↔ 120	85.33	24.46
B	**Solvent - solid ratio**	**mL/g**	Numeric	5.00	20.00	-1 ↔ 5	+1 ↔ 20	14.07	5.82
C	**Time**	**min**	Numeric	10.00	60.00	-1 ↔ 10	+1 ↔ 60	36.53	19.28

Based on these, the program generates 15 experimental runs which are further performed and analyzed, and the results are presented are presented in Table *4*.

Table 4

The generated experimental runs and responses obtained

		Factor 1	**Factor 2**	**Factor 3**	**Response 1 (predicted)**	**Response 1 (experimental)**	**Response 2 (predicted)**	**Response 2 (experimental)**
Run	**Space Type**	**A:US Power**	**B:Solvent-solid ratio**	**C:Time**	**Conc. hypericin**	**Conc. hypericin**	**Purity hypericin**	**Purity hypericin**
		W	mL/g	min	mg/g	mg/g	%	%
1	Vertex	60	5	60	162.36	163.07	35.91	36.26
2	Plane	60	12	34	173.12	170.78	39.38	38.91
3	Plane	60	12	34	173.12	175.08	39.38	38.84
4	Vertex	60	20	60	288.37	289.69	39.57	39.72
5	Plane	60	20	35	239.68	237.91	41.26	41.22
6	Vertex	60	20	10	191.35	190.24	42.95	43.61
7	Interior	80	5	12	163.22	161.07	39.03	39.5
8	Interior	80	14	13	210.20	216	41.16	40.21
9	Interior	100	12	58	207.91	207.03	37.49	37.26
10	Interior	100	12	58	207.91	205.25	37.49	37.76
11	Interior	100	20	32	264.16	267.69	41.21	41.27
12	Interior	100	20	32	264.16	260.51	41.21	41.74
13	Edge	120	5	40	196.07	197.97	36.87	36.97
14	Edge	120	14	10	276.94	275.19	41.10	41.04
15	Vertex	120	20	60	199.93	201	39.18	38.88

For response 1 (concentration of hypericin, mg/g dry plant) a Quadratic model was chosen based on the probability (*p*-value) of the various models taken under consideration (p-value<0.0001). Table 5 shows the ANOVA data for hypericin concentration (mg/g) quadratic model of UAE obtained extract. The coefficient was

accepted as significant for a *p*-value below 0.05. The Lack of Fit is shown to be not significant, meaning that the model accurately fits the data.

Table 5

ANOVA data for the hypericin concentration (mg/g) quadratic model of UAE obtained extract

Source	Sum of Squares	Δf	Mean Square	F-value	*p*-value	
Model	24888.19	9	2765.35	144.51	< 0.0001	**significant**
A-US Power	3929.32	1	3929.32	205.33	< 0.0001	**significant**
B-Solvent - solid ratio	11148.49	1	11148.49	582.58	< 0.0001	**significant**
C-Time	77.65	1	77.65	4.06	0.1001	**not significant**
AB	1947.24	1	1947.24	101.76	0.0002	**significant**
AC	10538.43	1	10538.43	550.70	< 0.0001	**significant**
BC	98.72	1	98.72	5.16	0.0723	**not significant**
A^2	1056.23	1	1056.23	55.20	0.0007	**significant**
B^2	23.08	1	23.08	1.21	0.3221	**not significant**
C^2	0.0899	1	0.0899	0.0047	0.9480	**not significant**
Residual	95.68	5	19.14			
Lack of Fit	59.08	2	29.54	2.42	0.2366	**not significant**
Pure Error	36.60	3	12.20			
Cor Total	24983.87	14				

Std. Dev.	4.37		**R^2**	0.9962
Mean	214.57		**Adjusted R^2**	0.9893
C.V. %	2.04		**Predicted R^2**	0.8003
			Adeq Precision	35.2778

Accordingly, the concentrations of the interest compound are higher when employing a higher solid to solvent ratio, lower US power, and shorter extraction times. These are illustrated by the graphs shown in Fig. 4.

If the US power increases, then there is a high possibility of degradation, which therefore negatively affects the overall yield and decreases the quantity of the extracted hypericin. Similarly, if the extraction times are longer, the resulted hypericin could degrade during the process and the final amount of our interest compound could be diminished.

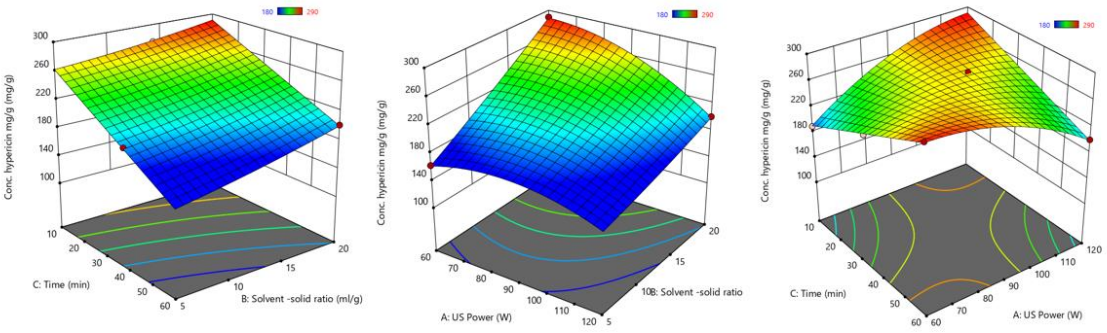

Fig. 4. 3D graphical representation of the quadratic model showing the parameters influence on the hypericin concentration, mg/g

The second response to be analyzed is the purity of the extracted HYP. The data in Table 6 show that the factors are correlated in a linear model. The F-values, p-values and the Lack of Fit retain the same meaning.

Table 6

ANOVA data for the hypericin purity (%) liniar model of UAE obtained extract

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	54.31	3	18.10	73.48	< 0.0001	**significant**
A-US Power	0.3535	1	0.3535	1.43	0.2562	**not significant**
B-Solvent - solid ratio	28.27	1	28.27	114.75	< 0.0001	**significant**
C-Time	23.69	1	23.69	96.13	< 0.0001	**significant**
Residual	2.71	11	0.2464			
Lack of Fit	2.47	8	0.3090	3.89	0.1455	**not significant**
Pure Error	0.2381	3	0.0794			
Cor Total	57.02	14				

Std. Dev.	0.4964		**R^2**	0.9525
Mean	39.55		**Adjusted R^2**	0.9395
C.V. %	1.26		**Predicted R^2**	0.9150
			Adeq Precision	27.4765

The purity of extracted hypericin is mainly influenced by the solid to solvent ratio: the higher it is, the more interest compound is extracted. Analogous to concentration, short extraction times favor better results, while longer ones lead to degradation. US power showed no significant influence on purity. These are presented in Fig. 5.

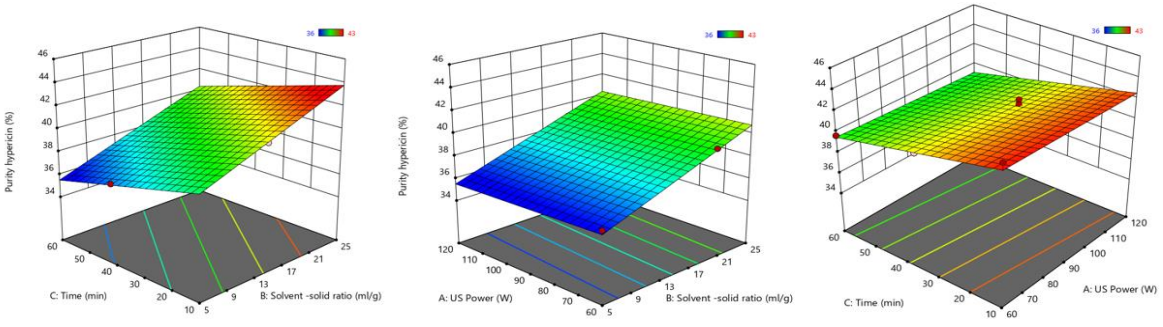

Fig. 5. 3D graphical representations of the *l*inear model showing the parameters influence on the hypericin purity, %

According to the data discussed up until this point, the program generated each model's equation and displayed it in the form of a table, where the first column shows the coefficient values and the second one shows the parameter. The concentration can be observed in Table 7 and the purity in Table 8.

Table 7

The mathematical model equation for hypericin concentration

Conc. Hypericin, mg/g	**=**
-373.71556	
+8.30838	**US Power**
+10.91066	**Solvent -solid ratio**
-0.087001	**US Power * Solvent -solid ratio**
-0.062705	**US Power * Time**
-0.023778	**US Power^2**

Table 8

The mathematical model equation for hypericin purity

Purity hypericin(%)	**=**
+39.13715	
+0.244182	**Solvent -solid ratio**
-0.067606	**Time**

Overall, the aim of the optimization of UAE hypericin using DOE approach is the maximization of both responses. The criteria for determining the optimal values of US power, solvent-solid ratio, and time were decided by selecting the "in range" option. A multiple response method called desirability makes use of an objective function called the desirability function. It reflects the desirable ranges for each response. The desirable ranges are from zero to one (least to most desirable, respectively). The simultaneous objective function is a geometric mean of all considered responses [11]. In the present case, the goal of the desirability function was to maximize both responses based on the ranges of values obtained

after performing the experimental variants imposed by the built model. The range of values for responses was 161.07 – 289.63 mg/g dry plant for hypericin concentration and 36.25 – 43.61 % for purity of hypericin. The optimal solution presented in Table 9 is the one with the highest desirability.

The verification experiments carried out according to the proposed optimal solution lead to responses that confirm the validity and adequacy of the built model. Moreover, these trials also proved that the predicted hypericin concentration and purity values for the model could be satisfactorily achieved within a 95% confidence interval of experimental values (Table 9).

Table 9

Optimum point solution and extraction verification of responses

	US Power	**Solvent - solid ratio**	**Time**	**Hypericin conc., mg/g**	**Hypericin purity, %**	**Desirability**
Predicted	118	20	10	290.484	42.576	0.927
Experimental	118	20	10	286±0.787	42.133±0.339	

4. Conclusions

Hypericin is a versatile phytochemical, with numerous applications, both in the biomedical field, as well as in the dyes and clean energy industries.

While there are many ways to extract hypericin from St. John's Wort, the one that has garnered most attention in recent years is the ultrasound-assisted extraction, which this paper sought to optimize. The technique of choice was Design of Experiments using a dedicated software, a fast and relatively cheap alternative to more traditional methods.

Overall, the goal of the optimization was to obtain higher extraction yields and purities. These were achieved by testing a built mathematical model that correlated the critical parameters (US power, solvent:solid ratio, and time) to concentration and purity, accordingly.

Considering these, the authors believe that future research pathways on this matter are the testing of an even larger variety of solvents, the optimization of other techniques used to extract hypericin and the industrial scaling of the optimized intensified processes.

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