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OPTIMIZATION OF EXTRACTION FROM AMBROSIA ARTEMISIIFOLIA OF SOME SESQUITERPENE DERIVATIVES FOR UPLC ANALYSIS

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Abstract. *A two-step UPLC-MS method was developed for the separation and identification of five compounds — germacrene D, spathulenol, longipinanol, ambrosin, and cumanin — extracted from Ambrosia artemisiifolia, even in the absence of analytical standards. In the first step, the MassLynx software was employed to generate isotopic model spectra, allowing identification of the target compounds based on the recorded spectra during chromatographic separation. In the second step, the retention times of the eluted peaks were correlated with the logP values of the target compounds, providing an additional layer of identification. Another key objective of this study was to optimize the solid–liquid extraction process of the target compounds from different plant organs (root, stem, leaf). To achieve this, a full factorial experimental design was generated using Minitab software, minimizing the number of experiments while maximizing the information obtained. By evaluating factors such as the organ origin of the plant material, extraction time, and the hydrophobicity and volume of the extraction solvent, the number of experiments was effectively reduced to 36. The results highlighted that solvent hydrophobicity and solvent volume significantly influenced the extraction yield. This method provides an efficient and reliable strategy for both the identification and extraction optimization of compounds from A. artemisiifolia.*

Keywords*: design of experiments, factorial design, Ambrosia sp., germacrene, spathulenol, longipinanol, ambrosin, cumanin, sesquiterpene derivatives, MS spectra, solid – liquid extraction*

INTRODUCTION

In Europe, the genus *Ambrosia (Asteraceae)* comprise around 20 species(Slujic, 2008), but in Romania were identified only 3 species: A. *artemisiifolia*, *A. tifida* and *A. psilostachya* (Ianovici, 2009). Because pollen from *Ambrosia sp.* travels with a velocity up to 300 km/h these plants are considered to be very invasive (Genton, 2005). It was estimated that every 10 years the concentration of pollen of *Ambrosia sp*. increased 5 times (Slujic, 2008). The plant is extremely allergenic because produces large amounts of pollen (Simard, 2011). It invades cultivated fields and reduces crop productivity (Wang, 2006). It is well documented that this plant is responsible for major health and economic influence in the most infected areas of Europe, that include also Romania (Storkey, 2014).

In the same time, there are regions where *Ambrosia sp.* is cultivated and used as a tea in the treatment of pneumonia, fevers, nausea, intestinal cramps, diarrhea and mucous discharges and menstrual disorders (Wang 2006), due to the fact that plant contains several groups or chemical compounds with proved therapeutic activities (Sulsen, 2013). Among these groups of chemicals, the sesquiterpenes are very much studied in the last years (Villagomez, 2013) due to their beneficial effects in the treatment of some major diseases (Cotugno, 2012). For example, ambrosin is useful for treatment of renal pains, hypertension, diabetes (Jia, 2013); germacrene D has antimicrobial and insecticide properties (Adio, 2008); spathulenol is an inhibitor of proliferation of lymphocytes being an immunomodulator (Akram, 2010); cumanin

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facilitate the inhibition of synthesis of NO (Lastra, 2004) and longipinanol is a general insecticide, but also has antitumor effects (Cerda-Garcia-Rojas, 2010).

Due to the fact that the sesquiterpene derivatives are present in plant in very small quantities (around 1%) there are required sensitive analytical methods for identification and quantization of these compounds (Robinson, 2011). Most of the published papers regarding the analysis of sesquiterpenes refer to GC (Maggio, 2013) and HPLC methods (Liu, 2013).

In the present work, the optimization of the extraction from *Ambrosia artemisiifolia* of five sesquiterpene derivatives is presented, by the design of experiments approach, i.e. using some of the factors that may affect the extraction procedure to make a factorial design. The target compounds were identified by MS detection in a two steps procedure. First, based on the analysis of MS spectra of the eluted peaks, the compounds of interest were identified comparing the MS spectra of chromatographic peaks with software generated spectra of the target molecules and further, the retention times of identified peaks were correlated with the log P values of the target compounds, taking into account that these compounds were separated by reversed phase liquid chromatography.

MATERIAL AND METHODS

Sample collection. The entire mature plant of *Ambrosia artemisiifolia* was collected during October 2013, from a park in Timisoara, in a dry day. The vegetal material was air-dried at room temperature, in a shaded area. The dried material was separated by organ origin, i.e. root, stem and leaf, packaged in plastic bags and stored at 4°C in refrigerator.

Regents and materials. All the solvents were purchased from Sigma – Aldrich: methanol (Sigma-Aldrich, 14262 Fluka), hexane (Sigma-Aldrich, 34859), acetone (Sigma-Aldrich, 270725), formic acid (Sigma-Aldrich, 14265). The ultrapure water was prepared with SG UltraPure Water system (SG, Germany).

Extraction method. Around 30 mg of dried plant material was triturated with a pistil directly in a 2 mL Eppendorf tube. Different series of experiments were realized using root, stem and leaf material, respectively. Similarly, separate series of experiments using different solvents, i.e. methanol, hexane and acetone, were carried out. In each case, the plant material was mixed with 0.75 or 1.5 mL solvent, respectively. The mixture was homogenized 30 seconds by vortex movement (Velp Scientifica vortex) and the extraction was continued in an ultrasonic bath (Barnstased Lab-Line, Aqua Wave 9375) for 10 or 20 minutes, respectively. The tubes were centrifuged for 5 minutes at 12000 rpm (Mikro 22 R, Hettich Zentrifugen). From the tubes where 0.75 mL solvent was used, it was collected 0.5 mL supernatant and from the tubes where 1.5 mL solvent was utilized, it was recovered 1 mL supernatant. Then the solvent evaporated under a gentle stream of nitrogen at a temperature ranging from 40 to 70°C, depending on the solvent used. The residue was redissolved in 1 mL 50% methanol with 0.05% formic acid. After homogenization the solution was filtered using 0.22 µm syringe (cellulose) filter (Macherey Nagel, Germany) and injected in the UPLC system. In fact, using Minitab software program (Minitab 17.1.0, Minitab Inc.) a factorial design of experiments was created and further used to emphasize the influence of each factor that may affect the extraction yield of the target compounds and to reveal the possible interaction between these factors.

Analytical method. In the absence of standards, the target compounds were identified based on their molecular mass, fragmentation patterns and estimation of elution time based on logP values. The separation of the target compounds was carried out on a Waters Acquity UPLC-MS system (Binary Solvent Manager, Xevo TQD equipped with an electrospray ionization interface) (Waters, Milford, USA), using a Betasil Phenyl 5 µm column (2.1 x 50

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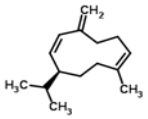
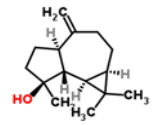
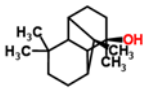
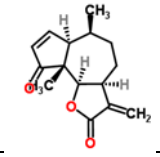
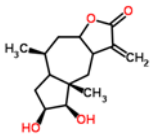
mm) (Thermo Scientific). The compounds were eluted using a 25 min gradient elution profile. Mobile phase A consisted in 0.05% formic acid in methanol and mobile phase B was 0.05% formic acid in 10% methanol. The equilibration of the column was realized in 100% B and then the following gradient profile was used: 0.0 – 25 min, 100% A; 25 – 26 min, 100% A; 26,0 – 26,1 min 100 % B. The column temperature was set at 30°C. The analyses were run at a flow rate of 0.5 mL·min-1, and the injected volume of the sample was 10 µL. The ESI parameters for Xevo TQD MS detector were fixed as follows: capillary voltage at 3,5 kV, cone voltage at 30 V, source temperature at 150 °C, desolvation temperature at 450 °C, and desolvation gas at 500 L·h-1. Nitrogen was used as the desolvation gas, and argon was employed as the collision gas. The eluted peaks were analyzed by a MS scan method created in MassLynx 4.1 (Waters, Milford, USA): cone voltage 30 V, collision energy 30 V, MS scan range 180 – 350 Da, ionization mode ES+, continuous data collection with a scan time of 0.3 secs (with minimum 12 points per chromatographic peak). For each target compound a chromatographic profile was extracted at the molecular mass mentioned in Table 1.

Table 1

Some of the properties of target compounds

Nr.	Compound Name	Molecular Formula	Chemical Structure	Molecular Mass (Da)	LogP value
1	Germacrene D	$C_{15}H_{24}$		204.3	6.57
2	Spathulenol	$C_{15}H_{24}O$		220.3	4.45
3	Longipinanol	$C_{15}H_{26}O$		222.3	4.84
4	Ambrosin	$C_{15}H_{18}O_3$		246.3	1.85
5	Cumanin	$C_{15}H_{22}O_4$		266.3	0.32

RESULTS AND DISCUSSIONS

The conventional way to perform a chromatographic analysis aiming to identify and quantify a chemical compound is to use an analytical standard, i.e. the pure target compound in the same analytical conditions as the unknown sample and further to compare the retention

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time and some specific properties of that compound, this depending on the detector used. In the absence of a standard, no calibration curve can be made, and no quantitation of analyte can be performed.

In the present study, in the absence of pure compounds, the target analytes were only identified based on their physico-chemical properties, i.e. the log P values and the molecular mass (as described in Table 1). For example, in Figure 1 there is presented the chromatogram of a leaf extract realized in 0.75 mL acetone. In the lowest panel there is presented the total ion chromatogram corresponding to the m/z range 180 – 350 Da. In the other panels there are presented the extracted chromatograms at m/z corresponding M+1 of the target analytes (see Table 1). Using this procedure, we can see only the peaks of the compounds that have the molecular mass equal with the selected m/z value. So, in panel b. of Figure 1 we may consider that the peak eluted at 7.3 min correspond to cumanin, that has a molecular mass M+1 = 267.3 Da. Additional information that can help to identify the eluted compound may be obtained from the spectral analysis of this peak. In Figure 2 there are presented 2 sets of spectra. In the panels a. and b. there are presented the continuous and the centroid spectra, respectively, of the compound eluted at 7.3 min (the example presented in Figure 1). In order to prove that this compound is cumanin, in the panels c and d of Figure 2 there are presented the models of the continuous and centroid spectra, respectively, of cumanin, i.e. the compound that has the elemental formula $C_{15}H_{22}O_4$. As can be seen from Figure 2 there is a great similarity between the spectra of compound eluated at 7.3 min and the spectra of the model, that may lead to the conclusion that the compound that has eluated at 7.3 min is indeed cumanin. A similar strategy was applied for the other target compounds, i.e. germacrene D, spathulenol, longipinanol and ambrosin, respectively. All the target compounds were identified by comparing their MS spectra with model spectra of compound having the same elemental formula. Another criterion for identification of the eluted compound was the correlation between the retention time and the LogP values of the compounds. As is can be concluded from the comparison of the LogP values presented in Table 1 and the elution order from Figure 1 the compound eluted at 7.3 min is cumanin with LogP = 0.32, the compound eluated at 10.4 min is ambrosin with LogP = 1.85, the compound having the retention time 12.7 and LogP = 4.45 is spathulenol, the compound eluted at 14.8 min and having LogP= 4.84 is longipinanol and, finally, germacrene D is the compound that eluted at 15.1 min and has a logP value of 6.57.

Another goal of this study was the optimization of the extraction procedure in order to obtain the highest quantity of the target compounds extracted from the raw materials. The quantity of a compound that was extracted may be appreciated by the area (or the height) of the eluted peak in the chromatographic separation applied to the sample extract. With other words, the area (or the height, or a combination of both) can be a measure of the extraction yield and can be considered the dependent variable in a mathematical function $y = f(x_i)$, where the independent variables x_i may be the factors that can affect the yield of extraction. In solid-liquid extraction procedures the independent variables can include type of solvent used for the extraction, the ratio between raw plant material mass and solvent volume, the temperature of extraction, the time of extraction, the procedure and the time of homogenization of the mixtures, and so on. In this work, it was studied the influence of the fallowing factors that affect the yield of extraction of the target compounds: the type of solvent (considering the logP values), the ratio between the quantity of plant material and the quantity of the solvent (by varying the solvent volume at the same amount of raw material) and the time of extraction. Regarding the solvent utilized for extraction there were used 3 solvents with different logP values: methanol (logP = -0,74) a solvent compatible with the mobiles phases used in liquid

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chromatography and which can extract well compounds with low and medium hydrophobicity; hexane (logP = 4) a relatively high hydrophobic solvent that will extract especially lipophilic compounds and acetone (logP = 0.3) frequently used for extraction of compounds from plant materials. Another factor that can be conducted to different amounts of extracted target compounds is the organ of the plant used as raw material. Here it was used roots, stems and leafs of *Ambrosia artemisiifolia*. That means the above equation can now be rewritten as follows: $y = f(x_4)$, were the four independent variables, or factors are the organ plant, the nature / hydrophobicity of the solvent, the solvent volume and the time of extraction.

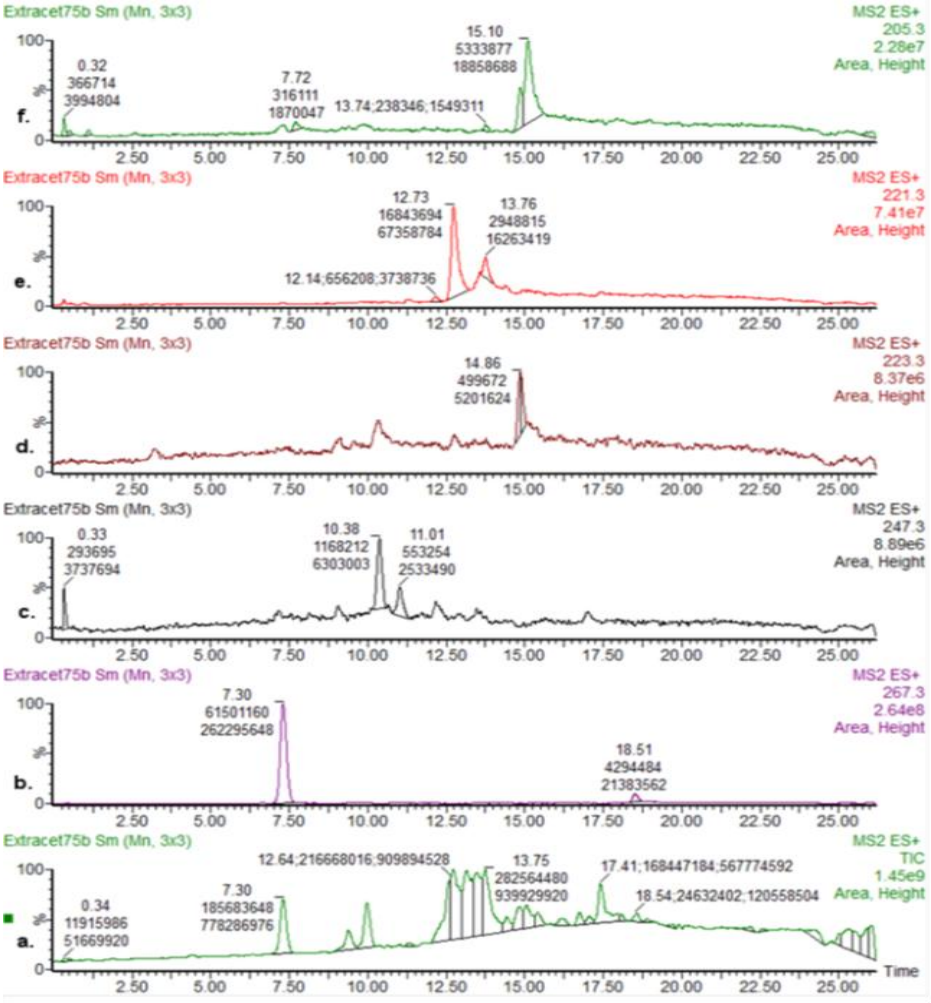

Figure 1. Chromatogram of an extract from leaf of *Ambrosia sp*. when 0.75 mL acetone was used. a. TIC MS range from 180 – 350 Da; b. Extracted chromatogram at 267.3 Da corresponding to cumanin; c. Extracted chromatogram at 247.3 Da corresponding to ambrosin; d. Extracted chromatogram at 223.3 Da corresponding to longipinanol; e. Extracted chromatogram at 221.3 Da corresponding to spathulenol and f. Extracted chromatogram at 205.3 Da corresponding to germacrene D

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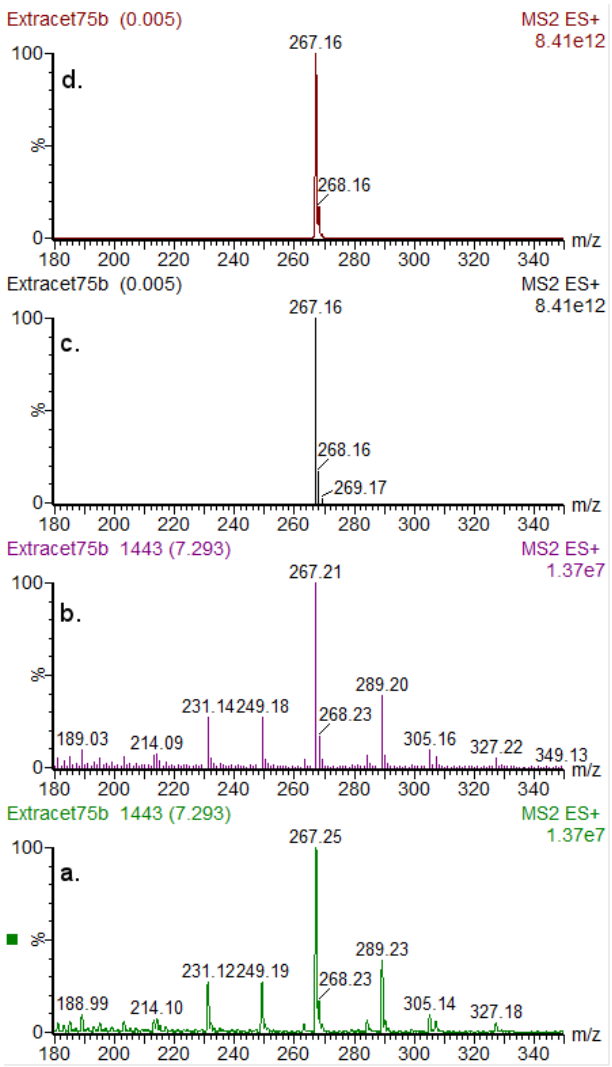

Figure 2. Spectral information for confirmation that the peak eluated at 7.3 min in chromatogram from Figure 1. b. belongs to cumanin. Panel a. The continuous MS spectrum of peak eluated at 7.3 min (Figure 1.a.); Panel b. The centroid MS spectrum of peak eluated at 7.3 min; Panel c. The isotopic elemental model of a compound with the molecular formula $C_{15}H_{22}O_4$; Panel d. A model of a continuous MS spectrum of a compound with the molecular formula $C_{15}H_{22}O_4$

The traditional methodology used for the optimization of analytical procedures is the one-factor-at-a-time approach, where every experimental factor is optimized independently of other factors. On the contrary, the factorial designs methodology supposes the simultaneous optimization of all factors at once. For the optimization of the extraction parameters the design of experiments (DOE) was realized using Minitab software. A general multilevel factorial design was considered, selecting 4 variables (factors) with 2 or 3 levels: A = the organ of the plant with 3 levels: root, stem and leaf; B = solvent type with 3 levels: methanol, acetone and

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hexane or their logP values; C = solvent volume with 2 levels: 0.75 mL and 1.5 mL (for 30 mg plant material) and D = extraction time with 2 levels: 10 minutes and 20 minutes. In some statistical analysis, for the variable B, i.e. the solvent type, instead of using the name of solvent it was used its logP value (this variable was changed from text type to numeric type). Considering 2 replicates, the above multilevel factorial design requested 72 experiments for optimization. The response variable was considered the peak area of each target compound and the sum of these areas, this being a measure of the yield of the extraction.

In order to graphically emphasize the influence of each factor, the experimental data were fitted to 2 level factorial design, considering only 3 factors. For example, from the 72 experiments there were selected those 16 experiments (considering 2 replicates) where the variables were LogP of solvent but considering only 2 values (i.e. for methanol logP = 0.74 and for hexane log P = 4), the volume of the solvent with 2 levels and the time of extraction, with 2 levels. The matrix for the 2^3 factorial design and the uncoded variables are presented in Table 2.

Table 2

Design matrix and level of variables for 2^3 factorial design

Exp.	Design matrix			Uncoded variables		
	B	C	D	B – logP value	C – solvent volume (mL)	D – extraction time (min)
1	-	-	-	-0.74	0.75	10
2	+	+	-	4	1.5	10
3	+	-	-	4	0.75	10
4	-	+	-	-0.74	1.5	10
5	+	+	+	4	1.5	20
6	-	+	+	-0.74	1.5	20
7	+	-	+	4	0.75	20
8	-	-	+	-0.74	0.75	20

Using as response variables the area of the chromatographic peaks attributed to germacrene D, spathulenol, longipinanol, ambrosin, cumanin, and the sum of these areas, an analysis of the factorial design was performed, at $\alpha < 0.05$. In Figure 3 there is presented an example of the way of the estimation of the magnitude and importance of the effect of each Using as response variables the area of the chromatographic peaks attributed to germacrene D, spathulenol, longipinanol, ambrosin, cumanin, and the sum of these areas, an analysis of the factorial design was performed, at $\alpha < 0.05$. In Figure 3 there is presented an example of the way of the estimation of the magnitude and importance of the effect of each factor considered on the yield of the extraction of a target compound. The biggest effect is attributed to the hydrophobicity of the solvent, while the volume used for extraction has a lesser influence, whereas time has no effect**.**

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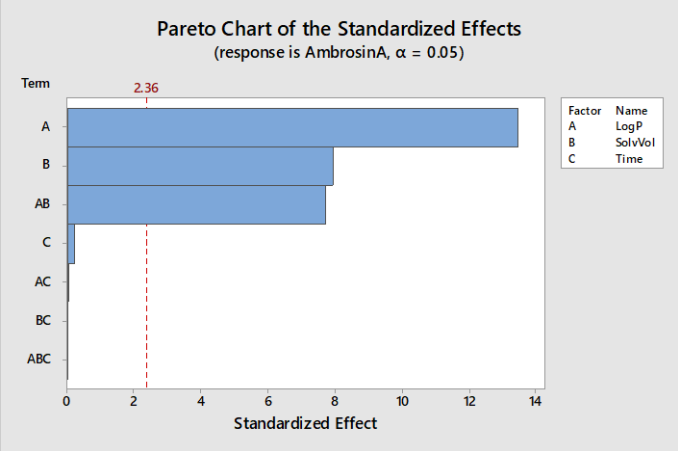

Figure 3. The Pareto chart showing the magnitude and the importance of the effect of factors taken into consideration to influence the yield of extraction of ambrosin

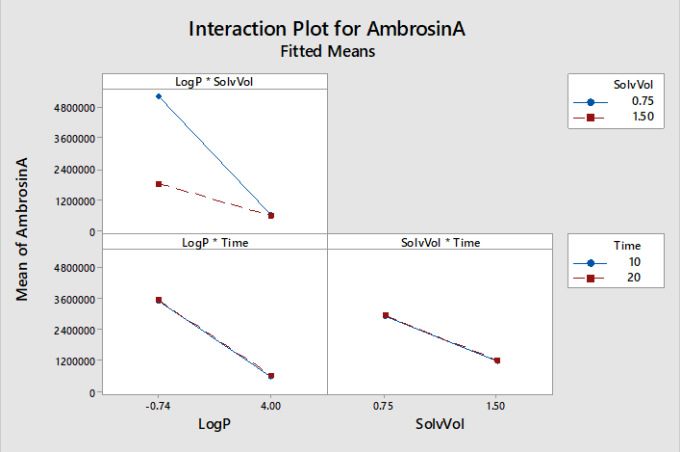

Figure 4. The interactions between the factors considered in DOE

These observations are supported by the analysis of variance, which for ambrosin gives p values smaller than 0.05 for logP and solvent volume factor and for their combination. The fact that a combination of 2 factors has an influence on the response, i.e. the yield of extraction is influenced by hydrophobicity * volume of solvent (as it can be seen from Pareto chart from **Error! Reference source not found.**) conducts to the supposition that between these factors may be an interaction. This can be evidenced by an interaction plot as shown in **Error! Reference source not found.**. Indeed, there are no interaction between time and hydrophobicity or time and volume of the solvent (as the lines are parallel) but there is an evident interaction between the hydrophobicity and the volume of solvent used for extraction (there is an intersection of the lines).

Similar analyses were conducted for each target compound and for the sum of the areas and in every case the conclusion was similar, i.e. the biggest influence on the yield of

http://doi.org/10.59463/RJAS.2025.1.30

extraction is given by the hydrophobicity of the solvent and in the second place is the volume of the solvent and these factors have an opposite influence

Analogous statistical analysis was performed considering the other two pairs of solvents methanol: acetone and hexane: acetone, respectively. In all cases the hydrophobicity of the solvent has the biggest effect on the yield of the extraction.

Factorial designs where the independent variables were organ of the pant (root, stem, leaf) and together with the solvent volume and extraction time, reveal that the origin of the raw material has no influence on the yield of the extraction. As proof, in Figure 5 there are presented the interactions plot for ambrosin. Again, there is an interaction between the hydrophobicity and volume of the solvent.

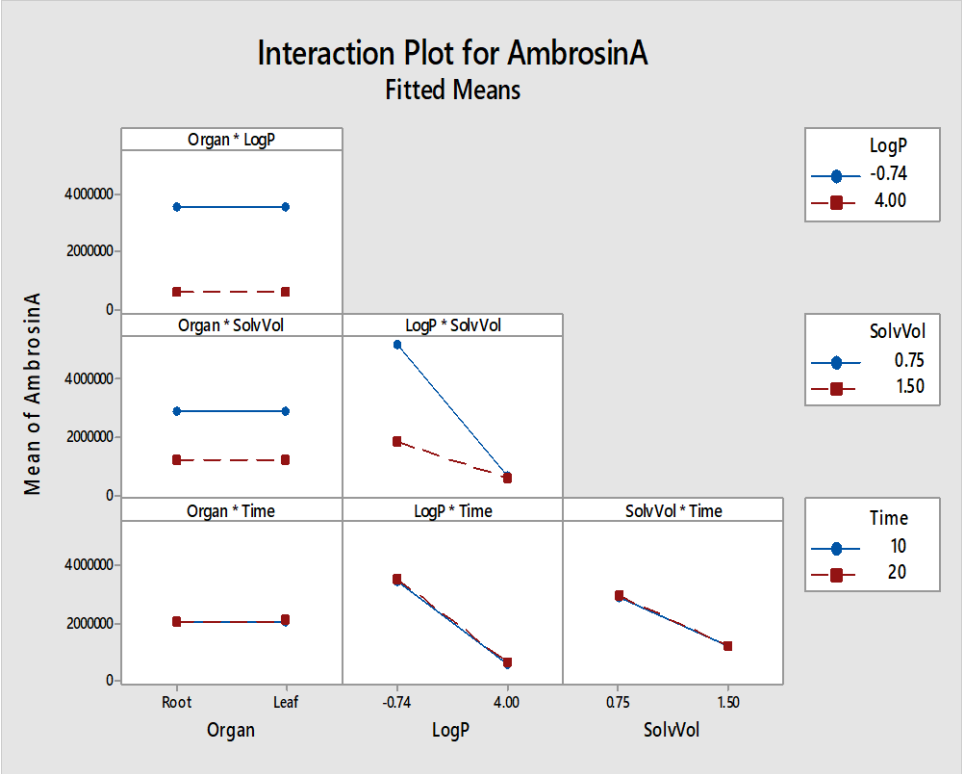

Figure 5. The interaction plot for a 23 factorial design with factors organ (levels root and leaf), logP (-0.74 and 4) and solvent volume (0.75 and 1.5 mL)

When all the 4 factors (2 of them with 2 levels and 2 with 3 levels) are considered for the construction of the factorial design, the analysis becomes complex as it can be seen from **Error! Reference source not found.** (where the example is for ambrosin).

Even so, the only factors that may influence the yield of extraction are the type of solvent, the volume of solvent and their combination. When the logP value of the solvent is high the volume of the solvent used can be smaller, i.e. when hexane is used as extraction solvent the ratio between the solvent volume and the amount of raw plant material can be smaller than when methanol must be used.

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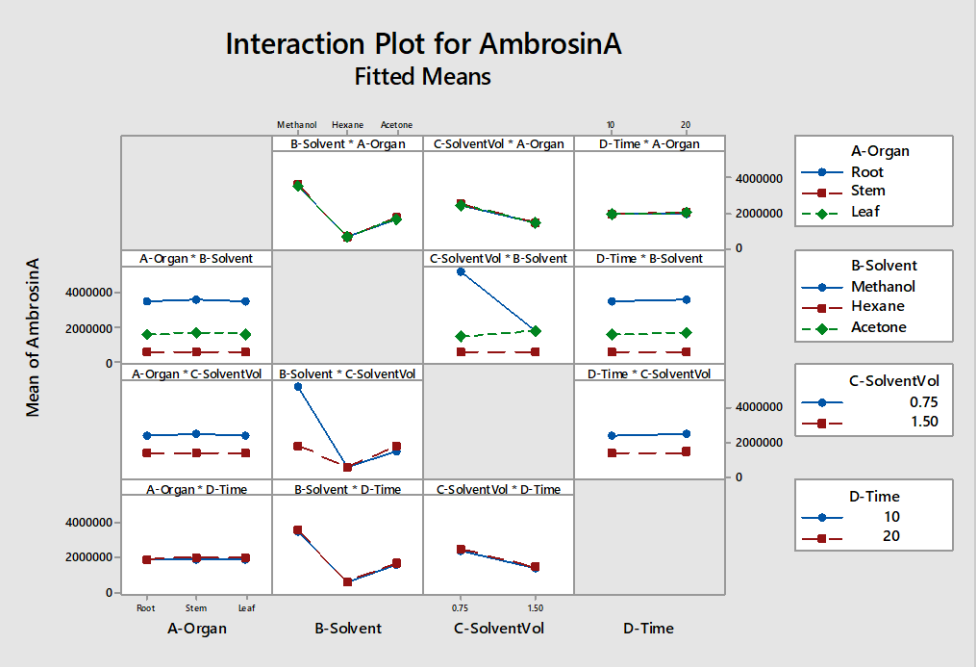

Figure 6. The interaction plot for ambrosin, in a factorial design with 4 factors with 2 and 3 levels. In this case the solvent type was settled up as a text variable

CONCLUSIONS

An uncomplicated UPLC-MS methods for separation and identification of five compounds (germacrene D, spathulenol, longipinanol, ambrosin and cumanin) from *Ambrosia artemisiifolia* was conducted in the absence of analytical standards. The identification of the eluted peaks was realized based on two criteria. First, the MS spectra of the chromatographic peaks were compared with isotopic model spectra generated by MasLynx software. Secondly, the order of elution of assigned peaks in the first step was compared with the logP values of the target compounds.

The process of solid – liquid extraction of the target compound was optimized regarding the hydrophobicity of the solvent used for extraction, the ratio between the plant mass and solvent volume and the extraction time. In order to decrease the number of experiments and to emphasize the influence of these factors upon the yield of the extraction of the target compounds, a factorial design technique was created with the help of Minitab software. It was evaluated also the influence of organs of the plant (i.e. root, stem and leaf, respectively) to the extraction yield and it was concluded that the origin of the plant material has no influence on final yield. The degree of hydrophobicity of the extraction solvent and the volume of the solvent used have an opposite influence upon the extraction.

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