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RESEARCH ARTICLE

THE SCREENING OF PARAMETERS INFLUENCING THE HYDRODISTILLATION OF MOROCCAN *MYRTUS COMMUNIS* L. LEAVES BY EXPERIMENTS DESIGN METHODOLOGY

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ABSTRACT

Myrtle, *Myrtus communis* L., is an herb widely used throughout the world. It is one of the most popular plants in Morocco. For the purpose of examining the factors affecting extraction of this plant's essential oil by hydrodistillation, a screening study by Hadamard matrix type Placket and Burman was conducted. After an appropriate choice of seven variables, sixteen experiments lead to a mathematical model of first degree linking the response function (yield) to factors. After the experiment's realization and data analysis, we concluded that five factors have a significant effect on the hydrodistillation process, namely: the extracting time, the individuality effect, the harvest period, the material/water ratio and the heating temperature with the coefficients: 0.041, 0.025, - 0.021, 0.018, -0.015 respectively. Moreover, the plant material's drying and cutting present a statistically negligible effect.

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INTRODUCTION

The aromatic and medicinal plants represent a considerable value to the Moroccan economy (Farah *et al*., 2006). Among these plants, *Myrtus communis* L. is an evergreen shrub belonging to the family of Myrtaceae. It grows spontaneously in Morocco and is encountered in forest areas belonging to the thermo-Mediterranean series from the Atlantic coast to altitudes of 1100 m. Generally it develops on a siliceous substrate in semi-humid and humid bio-climates (Benabid, 1997). Myrtle oil has many reported benefits for skin especially acne and oily one. Research has also shown it to help the respiratory system with chronic coughs and tuberculosis. It is suitable to use for children's coughs and chest complaints and may help support the immune function in fighting colds, flu and infectious diseases. Myrtle oil is applied topically, diffused or used in a humidifier. In folk medicine, myrtle has been used as anti-inflammatory drug. It is also used as a culinary spice and flavoring agent for alcoholic beverages in the Mediterranean region (Charles, 2013). The essential oil of *Myrtus communis* reduced leukocyte migration to the damaged tissue and exhibited anti inflammatory activity. The oil also inhibited cotton pellet-induced granuloma and serum TNF-alpha and IL-6 in mice (Maxia *et al*., 2011) and it is a strong antioxidant (Gardeli *et al*., 2008). Thus, it is essential to understand the effects of factor acting on the hydrodistillation process, and see their close link with the essential oil yield's improvement. To achieve this objective, we proceeded by the application of statistical techniques such as the experimental designs to make this improvement increasingly accessible. These methods, which reduce the experimentation to minimum number of experiments (Zeboudj *et al*., 2005), give the

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opportunity to screen the factors from the most to the least important, and also optimize the operating conditions to achieve the best possible result.

The use of experiments designs in the analysis and the optimization of the essential oil's extraction process was reported by several authors. Some have used other types of designs such as complete factorial design (Ammar *et al*., 2010, Silou *et al*., 2004, Wognin *et al*., 2010) and others have performed directly the optimization by using response surface methodology (Tan *et al*., 2012, Mu'azu *et al*., 2012). In this paper, we made a screening of factors acting on the hydrodistillation operation of *Myrtus communis* L. We have used the screening designs which are best known for factors with 2 levels: the Hadamard matrices or Plackett and Burman design (Plackett and Burman., 1946). The experimentation highlights the effects of some factors on the studied response (Silou *et al*., 2004). The choice of screening design for our study instead of the complete factorial design is based on seven studied factors. This number, which is higher than the factor's number used in complete factorial design, will cause an increase in the number of experiments (2^7 = 128 experiments for seven factors). As for the response surface designs, they are generally used for optimization tests. In our case, a screening design of Plackett and Burman type is more advocated. The objective of this screening study is to determine the most important factors acting on hydrodistillation process of studied plant with a view to a more detailed study of parameters optimization. The perspective will be optimization study which will be concerned only with factors considered influential on the hydrodistillation process.

MATERIALS AND METHODS

Vegetable material

Myrtus communis L. plants were collected from the National Institute of Medicinal and Aromatic Plants garden in Taounate (Morocco).

Extraction material

The Clevenger type apparatus was used for hydrodistillation (Clevenger, 1928) according to the method recommended by the French Pharmacopoeia (Pharmacopée Française, 1983). The process operates at the atmospheric pressure and is equipped with a recycling system, permitting the mass plant/water ratio to be maintained at its initial level. During every experiment, plant material and water were placed, in determined proportions, in a one liter capacity glass flask. The mixture was heated to boiling temperature and the liberated steams crossed up the column and passed out of the condenser in a liquid state. At the end of the distillation, two phases were observed, an aqueous phase (aromatic water) and an organic phase (essential oil), less dense than water. The obtained essential oil was dried over anhydrous sodium sulphate and was stored in the refrigerator at 4°C in dark glass bottles until use.

GC and GC–MS analysis

The essential oil was analyzed using Gas chromatography (GC) coupled to mass spectrometry GC / MS (Polaris Q ion trap MS). A Hewlett-Packard (HP 6890) gas chromatograph (FID), equipped with a 5% phenyl methyl silicone HP-5 capillary column (30m x 0.25 mm x film thickness 0.25 µm) was used. The temperature was programmed from 50°C after 5 min initial hold to 200°C at 4°C/min. Gas chromatography conditions were as follows: N_2 as carrier gas (1.8 ml/min), split mode was used (Flow: 72.1 ml/min, ratio: 1/50), temperature of injector and detector was 250°C, Final hold time was 48 min. The machine was led by a computer system type "HP ChemStation", managing the functioning of the machine and allowing to follow the evolution of chromatographic analyses. Diluted samples (1/20 in methanol) of 1µl were injected manually.

Placket and Burman Design

Based on a process or phenomenon, the first problems which the experiments design can provide information about, are those of screening parameters. A screening study may be defined as a step for identifying rapidly, in a large number, factors that are actually influential on a process in a fixed experimental field. The most known matrices screening experiments are the matrices of Hadamard (Horadam, 2007) or matrices of Plackett and Burman, for which the number of experiments is close to the numbers of the studied factors (Claeys-Bruno *et al*., 2009). These designs are matrices with orthogonal columns composed only of values +1 or -1 (Tinsson, 2010). These deigns are generally saturated and the mathematical model is a model without interactions (Goupy, 2006). The Plackett and Burman design is a fractional factorial design and the main effect (the contrast coefficient) of such design may be simply calculated as the difference between the average of measurements made at the high level (+1) of the factor and the average of measurements made at the low level (-1). Contrast coefficients allow the determination of the effect of each factor. A large contrast coefficient either positive or negative indicates that a factor has a large impact on response; while a coefficient close to zero means that a factor has little or no effect (Levin *et al*., 2005)

Experimental domain of factors and responses

The studied factors

The levels of factors were selected by taking into account the operating experimental limits, the literature data on hydrodistillation conditions (Ganou, 1993), and the previous studies (Wognin *et al*., 2010, Mu'azu *et al*., 2012, Galadima *et al.,* 2012). Factors that could affect the essential oil yield can be divided into two categories:

Continuous or quantitative factors:

- The extracting time varies between 150 and 210 minutes.
- The ratio between the vegetable material and water in the distillation flacks: varies between 1/12 and 1/4 (x 100g/100ml).
- The flask's heating temperature which is directly related to the steam flow leaving the heated flask and hence the flow of condensation. To test this

parameter, two heating temperatures are used: 250 °C and 350 °C.

Qualitative factors:

- The harvest period of plant material which have two levels: the middle of May and the middle of October.
- The studied plant's drying level with two modalities: fresh plant and dried plant. The plant's drying is performed in the shade during eight days at a temperature room fixed at 25 °C.
- The individuality effect: we have two modalities "individual 1" and "individual 2" because we have studied two separate plants. The cutting of plants in small pieces before the distillation, which have two–modalities: entire plant and cut plant.

The seven factors which were simultaneously studied to quantify the each one's effect on the hydrodistillation operation are shown in Table 1.

Table 1. Parameter levels and coded values used in the experimental design

Factors	levels	Units	Coded variables	Coded levels
Harvest period	Middle of May Middle of October	-	X1	-1 1
Individuality effect	Individual 1 Individual 2	-	X2	-1 1
Cutting of leaves	Cut leaves Entire leaves	-	X3	-1 1
Material / Water Ratio	1/12 1/4	x 100g/100ml	X4	-1 1
Extracting time	150 210	Min	X5	-1 1
Heating temperature	250° 350°	°C	X6	-1 1
Drying	Fresh plant Dried plant	-	X7	-1 1

The studied response

The studied response is essential oil yield of *Myrtus Communis* L. expressed as:

$$Y = Y_{HE}(\%) = \frac{M_{HE}}{M_s} \times 100$$

Where Y_{HE} the essential oil yield (%), M_{HE} the essential oil mass (g) and Ms the dry vegetal matter mass (g)

Experimental matrix

Since we have seven factors, the experimental design was a matrix of eight experiments. For more precision, and in order to determine the pure error, we have duplicated the chosen design, which thus leads to a matrix of sixteen essays.

Mathematical model and statistical analysis

The resulting mathematical model is a polynomial of order one as:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_6X_6 + b_7X_7 + \varepsilon$$

With:

$Y= Y_{HE}$ (%) is the yield of essential oil (response).
b_0 represents the theoretical average value of responses.
b_1, b_2, b_3, b_4, b_5, b_6 and b_7 are the principal effects of factors X_1, X_2, X3, X_4, X_5, X_6 and X_7 respectively.

ε is an error term.

An analysis of variance consisting on F test at a 95% significance level was conducted. The mean squares (MS) were obtained as follows:

$$MS = {SS}/_{DF}$$

Where SS is the sum of squares of each variation source and DF is the respective degree of freedom.

The ratio between the mean square regression (MS_R) and the mean square residual (MS_r), $F_{ratio(R/r)}$, was used in order to establish whether the model was statistically significant (Ammar *et al*., 2010). The greater F value from unity adequately explains the variation of the data around its mean, in addition the estimated factor effects are real (Myer and Montgomery, 2002, Box *et al*., 1978).

The quality of fitting the first-order polynomial was also expressed by the coefficient of determination. The R^2 measures the proportion of total variation about the mean response explained by the regression, in fact it is the correlation between observed and predicted response and it is often expressed as a percentage (Draper and Smith, 1998).

The model coefficients were considered significant for values of *p-value* <0.05. The statistical significance of the model coefficients was determined by using the t-test (only significant coefficients with *p-value* < 0.05 are included). During this study, we have used the conception and the treatment software of experiments design Nemrodw (Mathieu, 2000).

RESULTS AND DISCUSSION

Chemical composition of the essential oils

The results obtained by GC and GC–MS analysis of the essential oils of two studied *Myrtus communis* individuals are presented in Table 2, GC–MS chromatograms are listed in Figure 1. Ten compounds were identified in the studied individual's essential oils, hence individual 1 contained α-pinene (47,62%) and 1,8-cineole (48,43%) as the major compounds. While, α-pinene (34.74%), 1,8-cineole (50.69%), α-Terpinolene (6.76%) and α-terpineol (5,16%) were the major compounds of individual 2. the difference between chemical composition of both individuals can be attributed to physiological factors as organ development or even to genetic factors (Figueiredo *et al*., 2008).

Table 2. Chemical composition analysis of the essential oils of two studied *Myrtus communis* individuals

Compounds		KI	Individual 1 (%)	Individual 2 (%)
1	α-pinene	941	**47,62**	**34,74**
2	3-carene	968	-	0,53
3	Sabinene	970	0,58	-
4	1,8-cineole	1020	**48,43**	**50,69**
5	α-Terpinolene	1091	-	**6,76**
6	α-terpineol	1176	1,48	**5,16**
7	Myrtenyl acetate	1333	0,15	0,25
8	α-Terpinyl acetate	1340	1,44	0,88
9	Caryophyllene	1399	0,16	0,27
10	Caryophyllene oxide	1565	-	0,19
Total			99,86	99,47

KI: Kovats indices; (-): not detected.

Experimental design

The observed response values with different combinations of seven studied variables are listed in Table 3.

Statistical validation of postulated model

According to the analysis of variance (Table 4), we can conclude that the regression explains the studied process since the significance of the risk *p-value* is lower than 0,05. Obviously, the calculation of $F_{Ratio(R/r)}$-(= 14.47) has shown that it is 4 times higher than the value of $F_{(0,05;7,8)}$ at 95% confidence level (=3.5). In general, a model has a statistical significance if the calculated F value is at least three to five times greater than the theoretical value (Kalil *et al*., 2000).

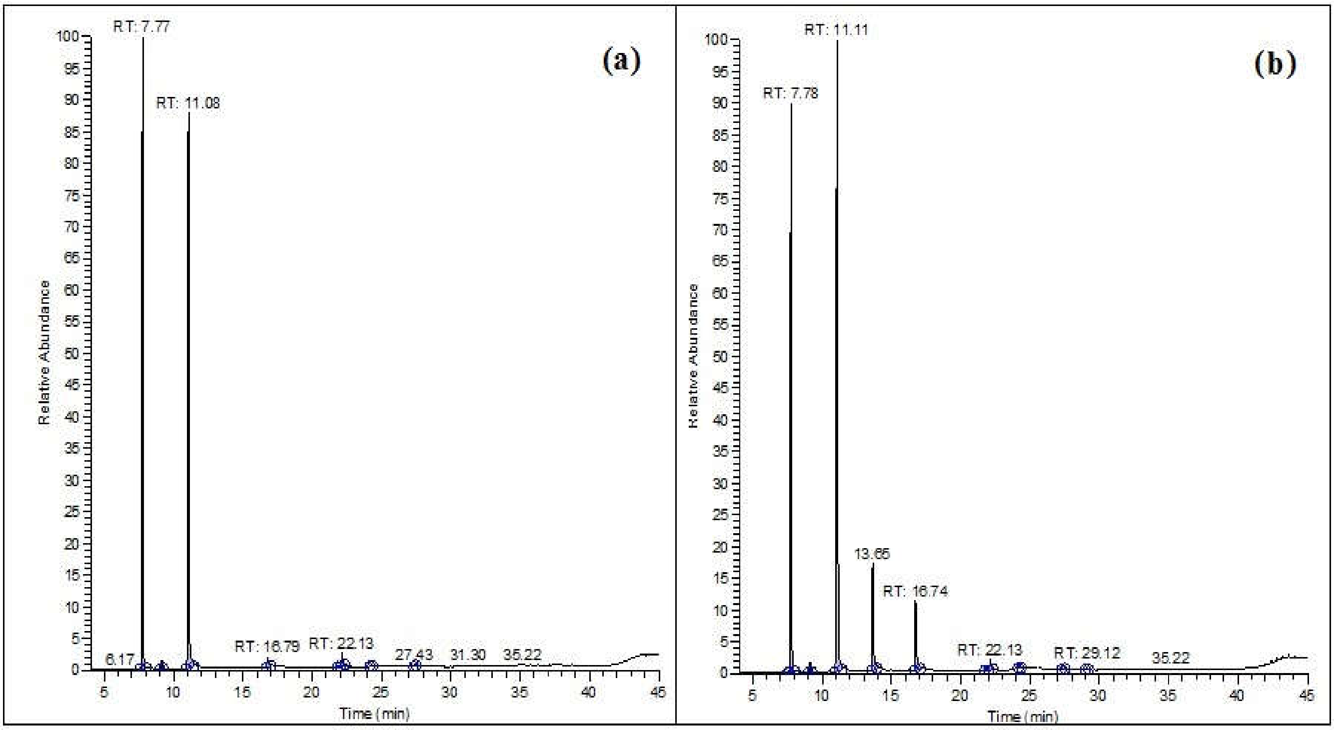

Figure 1. GC chromatograms of individual 1(a) and individual 2 (b) of Myrtus communis essential oil

Table 3. Experimental design of the hydrodistillation process of *Myrtus communis*L. with the observed answer for each experiment

N° of Experiment	Harvest period	Individuality	cutting of leaves	Ratio Material / Water	Extracting time	Heating temperature	Drying	Yield (%)
1	1	1	1	-1	1	-1	-1	0,75
2	1	1	1	-1	1	-1	-1	0,79
3	-1	1	1	1	-1	1	-1	0,58
4	-1	1	1	1	-1	1	-1	0,58
5	-1	-1	1	1	1	-1	1	0,65
6	-1	-1	1	1	1	-1	1	0,64
7	1	-1	-1	1	1	1	-1	0,63
8	1	-1	-1	1	1	1	-1	0,68
9	-1	1	-1	-1	1	1	1	0,72
10	-1	1	-1	-1	1	1	1	0,71
11	1	-1	1	-1	-1	1	1	0,59
12	1	-1	1	-1	-1	1	1	0,63
13	1	1	-1	1	-1	-1	1	0,64
14	1	1	-1	1	-1	-1	1	0,67
15	-1	-1	-1	-1	-1	-1	-1	0,63
16	-1	-1	-1	-1	-1	-1	-1	0,59

Table 4. Analysis of variance for the fitted model

Source of variance	DF	Sum of squares	Mean square	$F_{Ratio(R/r)}$	*p-value*
R	7	0,0532	0,0076	14,47	0,0008
r	8	0,0042	0,0005		
Total	15	0,0574			
R²	92,70%				

R: regression; r: residual; DF: Degrees of freedom; R^2 : Coefficient of determination

The coefficient of determination R^2=92,7% is sufficient. This value gives a good agreement between the experimental and predicted values of the adapted model.

These results are confirmed by those obtained in the graph (Fig. 2), showing a linear curve for the observed values in term of the predicted ones.

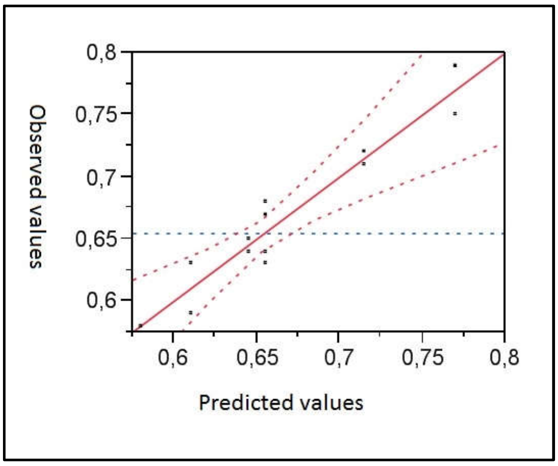

Figure 2. Curve of the observed values in terms of the predicted values

Study of the factors effects

The main effects of the seven studied variables are shown in Table 5. Each coefficient is associated with the values of *t-student* and *p-value*. The values of *t-student* are employed to determine the significance of the regression coefficients of each parameter and the values of *p* are defined as the lowest level of importance leading to the rejection of the null hypothesis (Ammar *et al*., 2010). In general, more the *t-student's* magnitude is larger, more the *p-value* value is smaller, and more the corresponding coefficient term is significant (Ravikumar *et al*., 2007). The value of the constant b_0 is equal to 0.65.

Table 5. Estimated regression coefficients for the Plackett and Burman design

Name of parameter	Coefficient	Effect	Standard error	*t-student*	*p-value*
Constant	b_0	0,655	0,006	114,35	< 0,0001 *
Harvest period	b_1	0,018	0,006	3,06	0,0154 *
Individuality effect	b_2	0,025	0,006	4,36	0,00251 *
Cutting of plan	b_3	-0,004	0,006	-0,65	0,537
Mass plant/water ratio	b_4	-0,021	0,006	-3,71	0,006 *
Extracting time	b_5	0,041	0,006	7,20	< 0,0001 *
Heating temperature	b_6	-0,015	0,006	-2,62	0,0299 *
Drying	b_7	0,001	0,006	0,22	0,827

*: Statistically significant factor for a confidence level of 95%.

The results show also that only the factors b_3 and b_7, which are related to the plant's cutting and drying respectively, doesn't have any influence on the hydrodistillation process, since their signification risk is superior than 0,05.

Fitted Model

The statistical mathematical model representing the response in terms of the most influential variables is:

$$Y = 0{,}655 + 0{,}018\,X_1 + 0{,}025\,X_2 - 0{,}021\,X_4 + 0{,}041\,X_5 - 0{,}015\,X_6 + \varepsilon$$

The calculated experimental error (ε) from the residual mean square was 0.023.

Statistically negligible parameters

The graph (Fig 3) shows that there is a small yield increase between fresh and dried plants and between the entire and cut leaves, but the test on coefficients b_3 and b_7 (Table 5) shows that these two factors don't have any influence on the hydrodistillation operation, since there signification risk is superior to 0.05 (0.537 and 0.827 respectively).

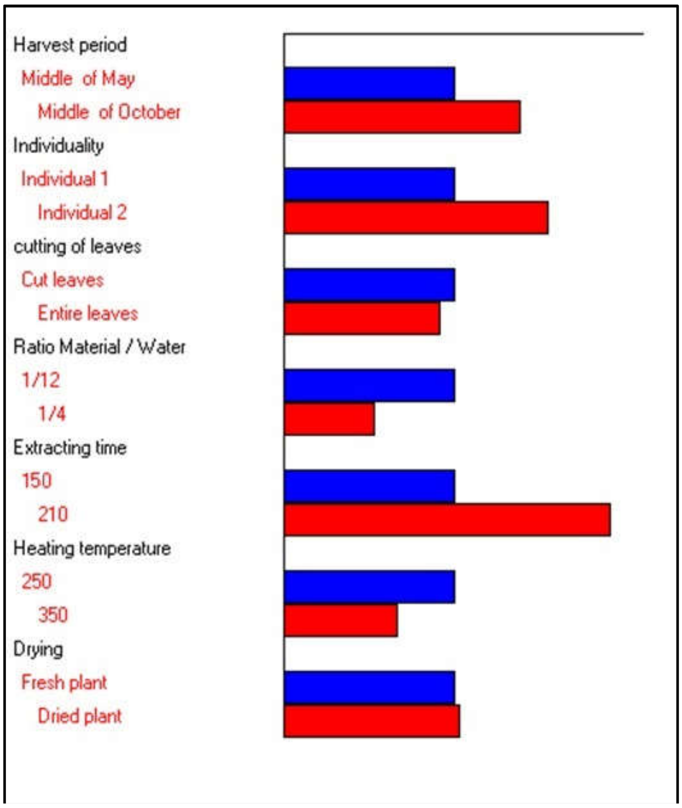

Figure 3. Variation of the response (Yield) in terms of the variation of each parameter

Cutting of leaves

The leave's cutting factor have a large influence on the yield because it induced the occurrence of an important surface contact between the plant material and water, facilitating the essential oil extraction process. However, the results obtained (Table 5) revealed a low effect of cutting.

This can be explained by the presence of myrtle essential oils in the surface and not in the heart of the plant material (Ammar *et al*., 2010).

Drying

A small yield increase was observed between fresh and dried plants (Fig 3). This result is similar to those found for other plants such as *Rosmarinus officinalis* L. (Benjilali, 2005) and *Tetraclinis articulata* (Bourkhiss *et al*., 2009) which shows that plant's drying for one week leads to remarkable increase in yield. However, in our case this increase was not statistically significant.

Statistically significant parameters

Extracting time

The two graphics (Fig 4) show that time (please precise here which time) (factor b_5) is the most influential factor on the hydrodistillation operation with a coefficient of 0.041. This lone factor contributed by 51.17% to the variability of the studied response. Obviously, the time influences directly the hydrodistillation operation and its impact on such operation has been demonstrated by several authors (Wognin *et al*., 2010, Tan *et al*., 2012, Mu'azu *et al*., 2012, Galadima *et al*., 2012).

Vegetable material / Water Ratio

The third factor that showed a significant influence on the yield is evidently the ratio between vegetable material and water in the distillation flask. This factor has a coefficient of --0,021 and a contribution of 13,58%. The minus sign indicates that the passage from the minimal level (the ratio 1/ 12) to the maximal level (the ratio 1/4) causes a fall in the essential oil yield. Several studies have shown that the increase in the ratio of vegetable material and water generates a decrease in yield (Ammar *et al*., 2010, Silou *et al*., 2004, Tan *et al*., 2012). This decrease can be explained by the fact that high amount of plant material in water prevents the water vapor formed in the bottom of the flask to rise up in the condensation tube which induce a yield reduction (Rabesiaka *et al*., 2012).

Harvest period

The harvest period comes in the fourth position with a coefficient of 0,018 and a contribution of 9,21%. This result indicates that yield becomes more important in the middle of October (Ripen fruits Period) compared to the middle of May (Pre-flowering Period). These results are in accordance with Pereira's and Amin's ones (Pereira *et al*., 2009, Amine, 2013) that indicated that October is the best period for better exploiting the Myrtle essences. Furthermore, Bradesi *et al*. (1997) recommend the period from June to November as the best harvest time for commercial production of the essential oil.

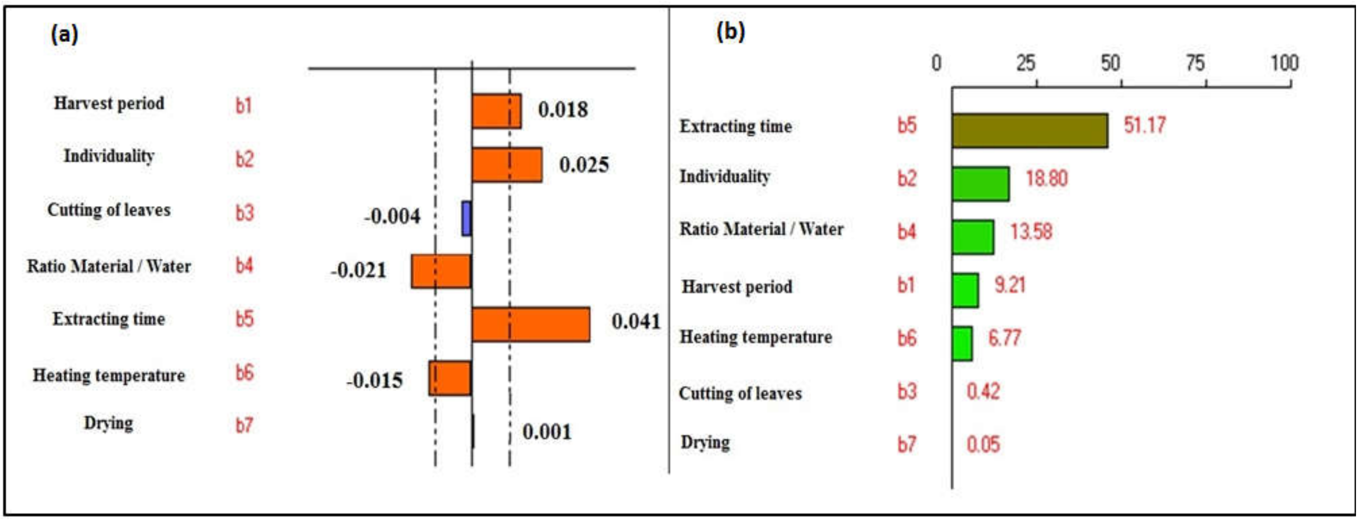

Figure 4. (a): The factors effect's graph, (b): the variation of the contribution percentage of each factor to the studied response

Individuality effect

The individuality effect is the second factor that affects the hydrodistillation process with a coefficient of 0,025 and 18,8% of contribution in the yield variability. The results show that the passage from the individual 1 to individual 2 in the realization of the experiments generates an increase in the essential oil yield. This change from one individual to another can be explained by the development stage of the plant organ (leaf, flower and fruit ontogeny) (Rowshan *et al*., 2012, Pereira *et al*., 2009), or by genetic (Figueiredo *et al*., 2008).

Heating temperature

The last factor having a significant effect on the yield is the heating temperature, with a coefficient of -0,015 and a contribution of 6,77 %. Its increase enhanced the steam condensation's flow. This increase has a negative effect on yield. Indeed, a big increase of the steam condensation's flow leads to a decrease of the condensate's residence time in the decanter, which does not leave time for essential oils to be separated from the liquid (Herzi, 2013). These results are concordant with those found by other authors (Ammar *et al*., 2010, Silou *et al*., 2004, Rabesiaka *et al*., 2012).

Conclusion

In this study, we were able to evaluate the effect of operating conditions on the essential oil yield of *Myrtus communis* L., by using the strategy of the experiments design to get the maximum results with a minimum of experiments. The results show clearly that this experimental methodology is an appropriate method for screening the factors affecting the hydrodistillation operation of the studied plant. The design of Placket and Burman which was applied led to a first-order model whose statistically significant coefficients are related to the most influential factors on the response. After the statistical validation of the obtained model, we performed the analysis of effects. All the studied factors influenced the hydrodistillation of *Myrtus communis* leaves. And hence its essential oil yield except the leave's drying and cutting, even though these two factors showed a negligible statistical effects. To complete this study, an optimization study must be conducted. It will seek the optimal operating conditions to have a better yield through using another type of experiments design developed for this type of study; namely, the response surface methodology by acting only on the continuous operating factors such as time processing, mass plant/water ratio, and heating temperature.

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