Quality along the Dairy Chain for a Safe and Sustainable MILK
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Document information

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Executive Summary

Background	In previous tasks and deliverables of the project, *Thymus capitatus* essensial oil was selected for the further investigations considering its important biological activities. To preserve these activities, EO needs to be encapsulated in suitable delivery systems compatible with food applications. The delivery in nanoemulsions seems to ameliorate the efficacy of EO active compounds. Because of their nano-sized droplet that increases the active surface area, nanoemulsions are assumed to have a superior antimicrobial activity than conventional emulsions with significantly higher droplet size. The formulation process is a big problem as it requires many experiments that consume important EO amounts, time and resources. One of the best promising solutions to this problem is the experimental design methodology. Response surface methodology (RSM) represents a suitable alternative to reduce the experiment number perceptibly, while predicting the results with high accuracy. The RSM establishes better or innovative formulations meeting the requirements of the optimal requests and generates accurate conceptions about responses as well as interactions between independent factors. The present deliverable aims to stabilize the EO selected earlier in the project into an efficient and stable nanoemulsion.
Objectives	The encapsulation of thyme EO into an emulsion-based delivery system will be optimized using RSM targeting the smallest possible droplet size (nano-scale) and the better stability (low PDI). For this purpose, low-energy technologies of encapsulation will be adopted including the use of Ultrathorax and/or sonication apparatus. Different ingredients will be screened such as emulsifier, dispersed and aqueuses phase's caracteristics (ratio and compositions). The selected emulsion will be assessed for its physico-chemical proprities.
Methods	**Experimental design**. RSM was used in order to optimize the emulsification parameters. Four independent variables of stirring time (**X1**) ranging from 0-6 min, the sonication time (**X2**) (from 0-30 min], the percentage of the dispersed phase (**X3**) ranging from [5-15%] and percentage of the continuous phase (**X4**), vary in the range of [1-10%] were investigated. A response surface plane, spherical type, 1st degree hadamard was applied in order to study the effect of the independent variables (X1, X2, X3 and X4) on the response: diameter of the dispersed oil droplets ($d_{3.2}$). A total of 13 experimental runs were required to optimize the droplet size of the NE.

Results and implications	

Models fitting and statistical analysis

The 13 runs were randomly assessed in triplicate (Table 1). According to this table, it can be observed that obtained emulsions diameters varies greatly from the nano-scale (runs 1,2,3,5,7,8,9,10 and 11) to the milli-scale (runs 4 and 6) to even phase separation (runs 12 and 13). Taking into consideration the desired lowest droplet diameter, experiment 5 seems to present the better homogeneity and the highest stabilité ($d_{3.2}$ = 463 nm).

Table 1. Experimental Design of the four-factor three-level observed responses

	Coded value from independant variables				
Run order	**X1**	**X2**	**X3**	**X4**	**Response $Y_{d3.2}$ (nm)**
1	0	0	15	1	648,7
2	0	8	12	8	490,0
3	6	0	5	7	746,4
4	6	30	5	1	1035,7
5	0	30	15	7	463,0
6	6	0	15	1	1011,7
7	0	30	5	1	663,5
8	6	30(+1)	15	7	779,2
9	3	15	10	4	757,6
10	3	15	10	4	759,0
11	3	15	10	4	766,9
12	0	7	7	5	Phase separation
13	0	7	7	10	Phase separation

Variance analysis (ANOVA).

In order to check the significance of the different factors, the p values were calculated at 0.05. Results of regression analysis presented in Table 2 revealed that the coefficient was significant (*p* value is less than 0.05). In fact, all independent variables have significant linear effect (*p* value <0.05) (positive effect on diameter of droplet). The ANOVA results presented in Table 2, indicates that the model adequately fitted the experimental data as the lack of fit is insignificant (p>0.05). Moreover, the coefficients of determination R^2 = 0.981 indicate a good correlation between the independent variables and the response $Y_{d3.2}$.

The fitted model. The coefficients of the model make it possible to study the influence of factors and their interactions on the response. In this case study, it is the study of the influence of the percentages of the continuous and the dispersed phases, the sonication and agitation times as well as their interactions on the droplets diameter. According to the results reported in Table 2, the factors b_0, b_1, b_2 are considered significant while the factor b_3, b_4 are moderately significant, which confirms that the variation factors as well as their interactions influence the response.

Table 2. Regression coefficients and analysis of variance (ANOVA) of the predicted model for the droplet size diameter.

$Y_{d3.2}$ (R^2= 0.981)				
terms	**Regression coefficient**	**Standard deviation**	***t* value**	**Signification (%)**
b_0	724.10	1.51	478.94	***
b_1	-129.85	1.77	-73.24	***
b_2	183.27	1.77	103.38	***
b_3	25.30	1.77	14.27	**
b_4	15.60	1.77	8.80	**

Variation Source	Sum square	degree of freedom	mean square	*F*value	Signif
Regression	4.10674E+0005	4	1.02668E+0005	4083.3248	***
Residual	8.11427E+0003	6	1.35238E+0003		
Validity	8.06399E+0003	4	2.01600E+0003	80.1802	*
Error	5.02867E+0001	2	2.51433E+0001		
Total	4.18788E+0005	10			

(*, **;***) is the significance level at p<0.05 ; p<0.005 ; p<0.0005 respectively.

According to Table 2, and taking into consideration the statistically significant parameters (p value lower than .05), the predictive mathematical model representing the response is detailed in the following equation:

$$Y_{d3.2}= 724.10-129X1+183,27X2+25.30X3+15.60X4$$

with : X1 : stirring time

X2 : Sonication Time

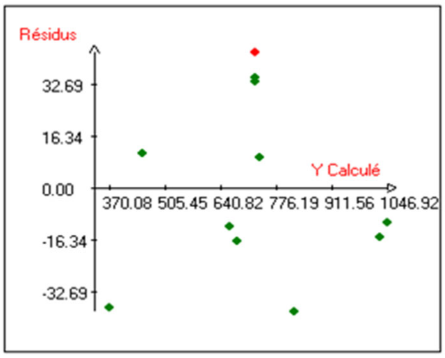
X3 : Percentage of the dispersed phase

X4 : Percentage of the continuous phase

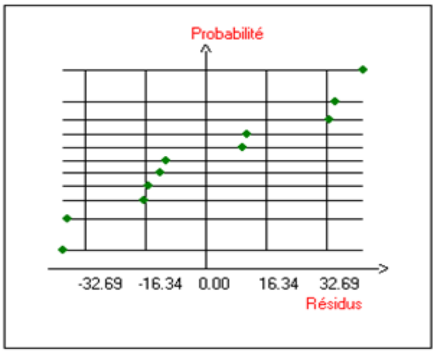
Model adequacy was confirmed through ANOVA which was checked by using Fisher-test. The Table 2 showed that the *F*-test value of regression coefficients is superior to the tabulated ($F_{regression}$ = >$F_{tabulated\ (6, 4, 0.05)}$). The corresponding p value was inferior to 0.0001 which indicates that independent factors have a significant effect on droplet diameter size. Furthermore, results from Table 2 indicated that the lack-of-fit is insignificant ($F_{lack\text{-}of\text{-}fit}$ <$F_{tabulated\ (4, 2, 0.05)}$; p value of 0.05) which means that the model is valid.. Thus, it can be conclude that the emulsion diameter and PDI is mainly influenced by the percentage of the aqueous phase

Statistical validation of the fitted model. The graphical study of the residuals of the Y response shows that the distribution of the points of the experimental and calculated values of the studied model are distributed at random and there is no particular structure that emerges from the representation of the residuals. This again confirms that the model is valid.

Graphic study of residuals



Normal Plot of residuals

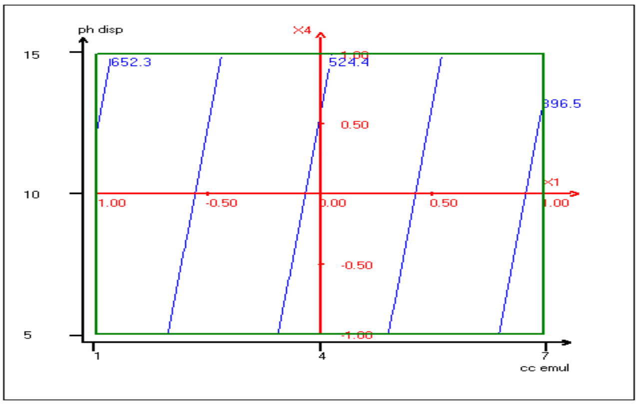


The superposition of the isoresponse curves shown in (figure 1) makes it possible to determine the response surface resulting from the interaction between the two variation factors X4 (percentage of the continuous phase) and X3 (percentage of the dispersed phase), thus allowing to specify the best zone of interaction between these two factors in order to have the optimum diameter of the NE:

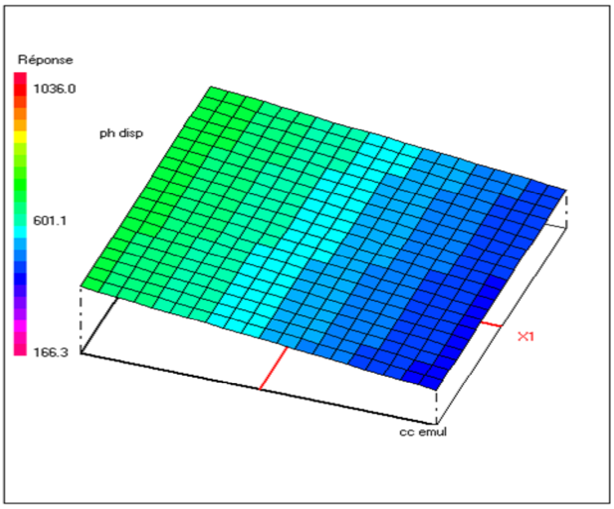
X4= 7 %

X3=5 %

From graphs 1 and 2, one can determine the estimated value of the desired parameter, at any point in the study area.



Graph 1: ***Response variation.*** Varied factors continous and dispersed phases percentages, fixed factors : tps agitation and sonication times = 0 min



Graph 2: ***Variation of the response in the plane.*** Varied factors continous and dispersed phases percentages

The study of desirability theoretically provided Experiment 14 as the optimum for encapsulation of thyme EO.

	N° Exp	tps agitat	tps sonicat	Dispersed phase percentage	Dispersed phase composition	Emulsifier percentage
Optimum	14	0	0	5	70%EO + 30% Oil	7%

Experimental verification of the optimal formulation. This experiment was confirmed experimentally and the values obtained are as follows

N° Exp	Agit/ son	Dispersed phase %	Dispersed phase composition	Emulsifier %	$D_{3.2}$ (nm)	PDI
14	-	5	70%EO + 30% Oil	7%	**260**	**0,221**

Nanoemulsion characterization. Once formulation was statistically validated, the optimized nanoemulsion was characterized for various parameters either physicochemical (droplet average diameter, pH, viscosity, density, turbidity, Zeta potential, conductivity, refractive index) or biological (antioxidant activity) as detailed in bellow:

Experiment 14	results	Units
Viscosity	0,10± 0.01	mPa/s
Refraction index	1,35± 0.08	-
pH	3,93± 0.23	-
Density	1,05± 0.07	$g.cm^{-3}$
Droplet diameter	280,93± 1.52	nm
PDI	0,26± 0.02	-
Turbidity	20.51± 0.95	-
Zeta Potential	-10,30 ± 0.78	mV
Conductivity	0,52± 0.06	ms/cm
Antiradical activity	44± 2.04	%